

WHO

operational
handbook on
tuberculosis

Module 3: Diagnosis

Rapid diagnostics for tuberculosis
detection

Third edition

Web Annex C. Technical manual for
culture-based drug susceptibility
testing of anti-tuberculosis drugs
used in the treatment of tuberculosis



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Contents

Acknowledgements.....	v
External Review Group	v
Acknowledgement of financial support	v
Glossary of terms.....	viii
1 Introduction.....	1
1.1 Background.....	1
1.2 Scope	2
1.3 Biosafety	3
1.4 Evidence basis for determining critical concentrations for DST	3
1.5 Recommendations for DST.....	3
2 Susceptibility testing for anti-TB agents using solid media.....	12
2.1 Recommended drug powders.....	12
2.2 Proportion method using Löwenstein–Jensen medium	14
2.3 Proportion method using Middlebrook 7H10 or 7H11 agar media	20
3 Susceptibility testing for anti-TB agents using liquid media.....	29
3.1 Principles	29
3.2 Preparing the medium	30
3.3 Anti-TB agents and critical concentrations for testing.....	30
3.4 Preparing solutions of anti-TB agents	31
3.5 Calculating the dilution factor for the MGIT medium.....	34
3.6 Adding an anti-TB agent to the medium.....	34
3.7 Preparing the mycobacterial inoculum.....	34
3.8 DST set carriers.....	37
3.9 Interpreting and reporting results	38
3.10 BD EpiCenter™ TB eXiST module.....	39
3.11 Important considerations.....	39
3.12 Quality control.....	40
4 References	41
Annexes.....	43
Annex A. Storing stock cultures.....	43
Annex B. McFarland Turbidity Standards	44
Annex C. Quality control testing	45
Annex D. Preparing and storing solutions for anti-TB drugs	51

List of Tables

Table 1. Critical concentrations (CC) for first-line drugs recommended for the treatment of drug-susceptible TB.	5
Table 2. Critical concentrations (CC) and clinical breakpoints (CB) for drugs recommended for the treatment of RR-TB and MDR-TB.	6
Table 3. WHO-recommended diagnostic approaches, reference methods and clinical interpretation for anti-TB drugs.....	9
Table 4. Availability of pure powders from GDF and other manufacturers	12
Table 5. Concentrations and solutions needed to prepare first- and second-line anti-tuberculosis agents for use with 500 mL of Löwenstein–Jensen medium	15
Table 6. Final test concentrations of first- and second-line anti-tuberculosis (anti-TB) agents for use with 7H10 or 7H11 medium.....	21
Table 7. Concentrations and solutions needed to prepare first- and second-line anti-tuberculosis (anti-TB) agents for use with 500 mL of 7H10 medium.....	22
Table 8. Concentrations and solutions needed to prepare first- and second-line anti-tuberculosis (anti-TB) agents for use with 500 mL of 7H11 medium.....	23
Table 9. Quantifying and reporting the growth of bacteria on culture plates	27
Table 10. Reconstitution volumes and final concentrations for first-line anti-TB agents	31
Table 11. Reconstitution volumes and final concentrations for lyophilised second-line anti-TB agents available from BD	32
Table 12. Concentrations and solutions needed to prepare second-line anti-TB agents for use with the BACTEC MGIT 960 system	32
Annex Tables	
Annex Table 1. Amounts of barium chloride and sulphuric acid needed to achieve different McFarland Turbidity Standards, and their correlation to the number of Escherichia coli per millilitre of suspension	44
Annex Table 2. Recommended quality control strains.....	46

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Abbreviations

7H10 = Middlebrook 7H10 medium
7H11 = Middlebrook 7H11 medium
AMK = amikacin
BDQ = bedaquiline
CAP = capreomycin
CB = clinical breakpoint
CC = critical concentration
CFZ = clofazimine
CLSI = Clinical & Laboratory Standards Institute
DCS = cycloserine
DLM = delamanid
DMSO = dimethyl sulfoxide
DR-TB = drug-resistant tuberculosis
DST = drug susceptibility testing
ECOFF = epidemiological cut-off value
FQ = fluoroquinolone (e.g., levofloxacin, moxifloxacin)
GDF = Global Drug Facility
GFX = gatifloxacin
gNWT = genotypically non-wild-type
GTB = Global TB Programme
gWT = genotypically wild-type
HPLC = high performance liquid chromatography
KAN = kanamycin
LFX = levofloxacin
LPA = line probe assay
LJ = Löwenstein-Jensen medium
LZD = linezolid
MDR = multidrug-resistant
MFX = moxifloxacin
MGIT = Mycobacterial Growth Indicator Tube
MIC = minimum inhibitory concentration
MTBC = *Mycobacterium tuberculosis complex*
mWRD = molecular WHO-recommended rapid diagnostics
NAAT = nucleic acid amplification test
OACD = Oleic acid, albumin, dextrose, catalase
OFX = ofloxacin
OD = optical density
Pa = pretomanid
PK/PD = pharmacokinetic/pharmacodynamic
pNWT = phenotypically non-wild-type
PZA = pyrazinamide
pWT = phenotypically wild-type
QC = quality control
R = resistance/resistant
RR = rifampicin resistant
S = susceptible/susceptibility
SDW = sterile distilled water
SIRE = streptomycin, isoniazid, rifampicin, ethambutol
TB = tuberculosis

TRD = terizidone
WHO = World Health Organization
WRD = WHO-recommended rapid diagnostic
WT = wild-type
XDR = extensively drug-resistant

Glossary of terms

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

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

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

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

Cross-resistance is resistance to multiple anti-TB agents caused by a single genetic change (or multiple changes, in case the given resistance mechanisms requires several genetic alterations), although in practice, such genetic mechanisms may not be known.

Epidemiological cut-off value (ECOFF), phenotypically wild type (pWT) and non-wild type (pNWT) strains

- Typically, when MICs that are tested using a standardised method are aggregated for one species, a single Gaussian-shaped MIC distribution is formed, which corresponds to the **pWT** distribution for that species (i.e. the distribution for organisms that lack natural phenotypically detectable resistance mechanisms). Additional distributions with higher overall MICs are sometimes identified, even prior to the clinical use of the particular drug in question (or prior to the clinical use of another, related drug that shares the same resistance mechanism), that correspond to intrinsically or naturally resistant organisms. In this case, the distribution with the lowest MICs corresponds to the pWT distribution and the other distributions correspond to one or more **pNWT** distributions.
- The **ECOFF** corresponds to the upper end of the pWT distribution (i.e. it typically encompasses 99% of pWT strains).
- Excluding the scenario where it is difficult to distinguish pWT and pNWT strains because of methodological variation in MIC testing (i.e. where both distributions overlap), pWT strains are, by definition, genotypically WT (gWT). However, this does not mean that gWT strains are identical genotypically since they may harbour mutations in genes associated with resistance that do not change the MIC (e.g. the *gyrA* S95T mutation does not affect the MICs of fluoroquinolones (FQ)).
- Conversely, organisms with MICs above the ECOFF are by definition pNWT. Again, excluding the possibility of methodological testing variation close to the ECOFF, there should be a genetic basis for this phenotype (i.e. the strains should be genotypically NWT (gNWT)). Yet in practice, these gNWT strains may appear to be gWT if:
 - The gene conferring the phenotype was not interrogated.
 - The gene conferring the phenotype was only partially interrogated (e.g. only 81 base pair region of *rpoB* gene for rifampicin)
 - The gene was interrogated, but the genetic change conferring the phenotype was not detected, as it occurred at a frequency below the level of detection of the molecular test (i.e. heteroresistance).
 - The genetic change was detected but could not be interpreted because of an incomplete understanding of the genotype-phenotype relationship.

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

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

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

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

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

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

1 Introduction

1.1 Background

In 2022 more than 10 million people fell ill with tuberculosis (TB), including almost half a million with multi-drug or rifampicin-resistant TB (MDR/RR-TB) (1). Only one third of people with MDR/RR-TB were known to have been diagnosed and enrolled on treatment. The number of individuals falling ill with TB that is susceptible to rifampicin but resistant to isoniazid is estimated to be more than two times higher than RR-TB and remains largely undetected and inadequately treated (2). Globally, the prevalence of fluoroquinolone (FQ) resistance among MDR/RR-TB is approximately 20%. Recently the World Health Organization (WHO) endorsed the use of a novel all-oral 6-month regimen composed of bedaquiline, pretomanid, linezolid and moxifloxacin (BPaLM) in people suffering from MDR/RR-TB, including those with additional resistance to fluoroquinolones (pre-XDR-TB, BPaL without moxifloxacin). The newly recommended BPaLM regimen offers better outcomes and shortens the duration of treatment considerably, thus significantly improves quality of life for people with MDR/RR-TB (3). An emerging concern is bedaquiline and linezolid resistance, as these two drugs are the backbone of the shorter MDR/RR-TB regimens, and it is anticipated that these drugs will remain essential for treatment of drug-resistant TB in future regimens.

To end the global TB epidemic by 2030, we must drastically expand access to drug-resistance testing, including to the drugs that constitute the best available regimens recommended for drug-susceptible and drug-resistant TB. This requires ensuring access to WHO-recommended rapid diagnostics and universal access to drug susceptibility testing (DST) for all persons with signs and symptoms of TB and no longer only prioritised for persons at greater risk of MDR-TB and/or HIV-associated TB. WHO defines universal access to DST as rapid DST for at least rifampicin and further DST for at least fluoroquinolones among all TB patients with rifampicin resistance.

Currently there are no WHO-recommended rapid diagnostics (WRDs) that can detect resistance to all drugs in a single test to inform treatment decisions, nor are there any WRDs that detect resistance to new and repurposed drugs such as bedaquiline, clofazimine, linezolid, delamanid and pretomanid (4). These drugs can however, be tested by culture-based phenotypic DST which is still the reference standard for most drug resistance detection. This methodology is time-consuming and requires specific laboratory infrastructure, skilled staff and quality control.

Traditionally, phenotypic DST (pDST) for *Mycobacterium tuberculosis* complex (MTBC) has relied on the testing of a single, critical concentration (CC), which is used to differentiate resistant from susceptible isolates of MTBC and is specific for each anti-TB agent and test method. In order to perform pDST, mycobacteria are often initially grown in a variety of solid or liquid culture media. Most frequently used media are Löwenstein–Jensen (LJ), Middlebrook 7H10 enriched agar (7H10), Middlebrook 7H11 enriched agar (7H11) and Middlebrook 7H9 enriched broth (7H9). The latter is used as a medium for the BACTEC mycobacterial growth indicator tube (MGIT) automated *M. tuberculosis* culture system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Bacterial growth on solid medium can be identified visually (i.e. by identifying characteristic growth) or in MGIT liquid medium by automated detection of fluorescence, which indicates a reduction in oxygen tension due to

bacterial growth. All positive cultures must be tested to confirm the detection of MTBC and to exclude the presence of any nontuberculous mycobacteria or other bacteria prior to performing DST.

The indirect proportion method is used for testing the phenotypic susceptibility of MTBC isolates. In this method, a defined inoculum is used to inoculate the drug containing media and a two 10-fold serial dilutions (10^{-2} dilution) of the inoculum is used to inoculate the drug-free control medium. The growth (i.e. the number of colonies corrected for the dilution factor) on a control medium without an anti-TB agent is compared with the growth present on a medium containing the critical concentration of the anti-TB agent being tested. Resistance is defined when at least 1% of growth is observed at the critical concentration of drug in the culture medium.

Other methods, such as the absolute concentration and resistance ratio methods require additional controls and the use of a standardized inoculum (5). As these methods have not been adequately validated for all anti-TB agents, they are currently not recommended for use. Commercial liquid culture systems for DST reduce the time to result to as little as 10 days, compared with the 28–42 days needed for DST using solid media. Hence, can improve patient management. DST using the BACTEC MGIT system is the preferred method for performing DST for many anti-TB agents given the standardization of the MGIT media and instrument.

There are a growing number of molecular WHO-recommended rapid diagnostics (mWRDs) for the detection of resistance to rifampicin (RIF) and isoniazid (INH) as well as molecular tools to detect resistance to other drugs. In addition to RIF and INH, these tests are capable of detecting resistance to fluoroquinolones (FQs), amikacin (AMK), pyrazinamide (PZA) and ethionamide (ETO) either individually or combined formats.

WHO now recommends the use of targeted next generation sequencing (tNGS) for the detection of resistance to RIF, INH, PZA, ethambutol (EMB), FQ, bedaquiline (BDQ), linezolid (LNZ), clofazimine (CFZ), amikacin (AMK) and streptomycin (STR), which is an attractive alternative to culture-based methods (6).

Importantly, the transition to rapid molecular testing does not eliminate the need for pDST as they are still needed for conducting DST for drugs for which a molecular tool /well characterised resistance mutation is not available, conducting DST to guide drug dosing determinations, monitoring the response to TB treatment and investigating discordant results from diagnostic testing. In particular, pDST is needed for testing the new and repurposed Group A and other drugs used for treatment.

1.2 Scope

This technical manual focuses on the available pDST methods for both first- second-line, Newer and repurposed anti-TB agents. Culture-based DST methods for certain anti-TB drugs are reliable and reproducible, but these methods are time-consuming, require specific laboratory infrastructure, skilled staff and adherence to quality control. Only indirect pDST procedures for anti-TB drugs are described in this document, which include LJ, 7H10 agar, 7H11 agar and 7H9 broth (BACTEC MGIT instrument).

WHO recommends the use of rapid molecular DST tests as the initial tests to detect drug resistance prior to the initiation of appropriate therapy for all TB patients, including new patients and patients that require retreatment. If rifampicin resistance is detected, rapid molecular tests for resistance to

relevant drugs (based on WHO-recommended treatment guidelines) should be performed promptly to inform which second-line drugs should be used for the treatment of MDR/RR-TB.

1.3 Biosafety

Activities involving the propagation and manipulation of cultures of *M. tuberculosis* complex bacteria, especially those suspected or known to be multidrug-resistant (MDR) or extensively drug resistant (XDR), should be carried out in an appropriate TB containment laboratory meeting or exceeding the minimum requirements for high-risk laboratory facility for TB. Further details to establish and to properly implement a bio-risk management system can be found in the 2020 WHO TB laboratory biosafety guidelines (7).

1.4 Evidence basis for determining critical concentrations for DST

In 2018, WHO Global TB Programme commissioned FIND to perform a systematic review of available minimum inhibitory concentration (MIC) data for phenotypically wild type (pWT) as well as phenotypically non-wild type (pNWT) strains, including associated sequencing data for relevant resistance genes for the critical first-line anti-TB drugs, rifampicin and isoniazid. Table 1 presents the critical concentrations and clinical breakpoints for first-line drugs recommended for the treatment of drug-susceptible TB as updated in the 2021 Technical Report (8) on critical concentrations for drug susceptibility testing of isoniazid and the rifamycins. Table 3 provides information on diagnostic interpretations.

The 2018 review complements the previous systematic review of available MIC data and associated sequencing data for relevant resistance genes for second-line drugs performed by FIND in 2017. The drugs included in the latter review were the second-line injectable agents (kanamycin, amikacin and capreomycin), clofazimine and bedaquiline, cycloserine and terizidone, linezolid, delamanid, and the fluoroquinolones (ofloxacin, levofloxacin, gatifloxacin and moxifloxacin). The following media were considered: Löwenstein Jensen, Middlebrook 7H10/7H11 and BACTEC™ Mycobacterial Growth Indicator Tube™ (MGIT) (9). Two of the injectable agents (kanamycin and capreomycin) are no longer recommended to treat DR-TB. For the class of fluoroquinolones, only the later generation drugs (levofloxacin and moxifloxacin) are currently recommended for use in the treatment of DR-TB, the specific agent used in a treatment regimen should be the one tested (i.e. test moxifloxacin if moxifloxacin is used) (9). Table 2 presents the critical concentrations and clinical breakpoints for second-line drugs recommended for the treatment of and MDR/RR-TB.

1.5 Recommendations for DST

1.5.1 First-line anti-TB agents

The End TB Strategy calls for early diagnosis and prompt treatment for persons of all ages presumed to have any form of TB. This requires ensuring access to WHO-recommended rapid diagnostics and universal access to DST for all patients with signs and symptoms of TB (10). WHO defines universal access to DST as rapid DST for at least rifampicin among all patients with bacteriologically confirmed TB, and further DST for at least fluoroquinolones among all TB patients with rifampicin resistance (10).

Patients with presumed drug-susceptible TB and TB patients who have not been treated previously with anti-TB agents and do not have other risk factors for drug resistance should receive a WHO-recommended first-line treatment regimen using quality assured anti-TB agents. The standard 6-month regimen for drug-susceptible TB (2 months of isoniazid, rifampicin, pyrazinamide and ethambutol followed by 4 months of isoniazid and rifampicin, denoted as 2HRZE/4HR) is one of the recommended regimens (10). WHO recently approved the shorter DS-TB regimen for people aged 12 years or older with drug-susceptible pulmonary TB, who may receive a 4-month regimen of isoniazid, rifapentine, moxifloxacin and pyrazinamide (11).

Table 1. Critical concentrations (CC) for drugs recommended for the treatment of drug-susceptible TB.

Drug	Abbreviation	Critical concentrations (µg/mL) for DST by medium			
		Löwenstein–Jensen ^a	Middlebrook 7H10 ^a	Middlebrook 7H11 ^a	BACTEC MGIT liquid culture ^a
Rifampicin ^b	RIF	40.0	0.5	1.0	0.5
Isoniazid ^c	INH	0.2	0.2	0.2	0.1
Ethambutol ^d	EMB	2.0	5.0	7.5	5.0
Pyrazinamide ^e	PZA	-	-	-	100
Moxifloxacin	MFX (CC)	1.0	0.5	0.5	0.25

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

^b As per the revised rifampicin critical concentrations report."gDST and, where applicable, pDST for rifampicin should serve as surrogate for rifapentine DST. Additional data are needed to clarify whether the RIF CC for Löwenstein–Jensen is set correctly. The RIF CC for 7H11 was based on limited data and might be too high in light of the fact that the RIF CC for 7H10 had to be lowered to 0.5 µg/mL."

^c Patients with MTBC isolates that are resistant at the critical concentration may be effectively treated with high dose isoniazid. Formerly, a higher concentration of INH (0.4 µg/mL in MGIT) has been used to identify strains that may be effectively treated with a higher drug dose. However, molecular patterns of INH resistance may be more reliable for predicting patient outcomes than pDST (12). To date no clinical breakpoint concentration has been established for INH.

^d All pDST methods for ethambutol produce inconsistent results. pDST is not recommended.

^e BACTEC MGIT 960 liquid culture method for PZA susceptibility testing, is reportedly associated with a high rate of false-positive resistance results. Careful inoculum preparation is essential for performing PZA testing reliably.

The GenoScholar PZA-TB is the only line probe assay approved for PZA (12). The detection of resistance conferring mutations in the *pncA* gene using DNA sequencing may be the most reliable method for the detection of pyrazinamide resistance although there is emerging evidence of non-*pncA* mutational resistance to PZA (13).

1.5.2 Isoniazid

Isoniazid is one of the most important first-line drugs for the treatment of active TB and TB infection, with high bactericidal activity and a good safety profile. The emergence of TB strains resistant to isoniazid threaten to reduce the effectiveness of first-line TB treatment. In 2019, globally 11% of all incident TB cases had rifampicin-susceptible, isoniazid-resistant TB (Hr-TB). Hr-TB globally, is more prevalent than MDR-TB. WHO recommends that in patients with confirmed Hr-TB, treatment with rifampicin, ethambutol, pyrazinamide and levofloxacin [(H)REZ-Lfx] for a duration of 6 months (14).

Efforts need to be made by all countries to move towards testing of both isoniazid and rifampicin at the start of TB treatment and to ensure the careful selection of patients eligible for the recommended Hr-TB regimen [(H)REZ-Lfx] regimen (15, 16). The minimum diagnostic capacity to appropriately implement the Hr-TB recommendations requires rapid molecular testing for rifampicin and isoniazid prior to the start of treatment with the Hr-TB regimen. Further, fluoroquinolone resistance should be ruled-out by either phenotypic or genotypic DST as soon as possible. Rapid molecular tests such as WHO-recommended TB-nucleic acid amplification tests (NAAT), line probe assays (LPA) and tNGS for

isoniazid, rifampicin and fluoroquinolones are preferred to guide patient selection for the (H)REZ-Lfx regimen.

In vitro evidence seems to suggest that when specific *inhA* promoter mutations are detected (and in the absence of any specific *katG* resistance-associated mutations), increasing the dose of isoniazid may be effective. In the case of *katG* mutations, which more commonly confer higher level resistance, the use of isoniazid even at higher-dose is less likely to be effective. The presence of combined mutations in the *inhA* promoter and coding region and/or *katG* gene result in considerable increase in the MICs (17). Table 3 presents an overview of the phenotypic and genotypic methods for performing DST against first-line anti-TB drugs and provides a clinical interpretation of the results.

1.5.3 Second-line anti-TB agents

Treatment for RR/MDR-TB is increasingly becoming more individualized as a result of innovations in diagnostics and growing scientific understanding of the molecular basis for drug resistance and the pharmacokinetics and pharmacodynamics of TB drugs (18). Three signals are clear from the current scientific evidence assessment:

- The feasibility of effective and fully oral treatment regimens for most patients;
- The need to ensure that drug resistance is excluded (at least to the fluoroquinolones and amikacin) before starting patients on treatment, especially for the shorter MDR-TB regimen;
- The need for close monitoring of patient safety and treatment response and a low threshold for switching regimens for non-responding patients or those experiencing drug intolerance

In 2018, critical concentrations were revised or established for performing DST for the Group A drugs that are strongly recommended to be used in the treatment of DR-TB. These include the later generation FQs (levofloxacin and moxifloxacin), bedaquiline, and linezolid. A critical concentration for the oral core agent clofazimine was established for MGIT medium only. Critical concentrations for the Group C (add on) agents were established or validated for delamanid, amikacin, and pyrazinamide. In 2023, the critical concentrations for pretomanid and cycloserine were established (19). Table 2 summarizes the established critical concentrations for drugs used to treat MDR/RR-TB, however, a recommendation has not been made for select agents and media as the reliability and reproducibility of testing is uncertain. The use of the indirect proportion method is recommended. Other methods using solid media (such as the resistance ratio or absolute concentration) have not been adequately validated for anti-TB agents.

Table 2. Critical concentrations (CCs) and clinical breakpoints (CBs) for drugs recommended for the treatment of RR-TB and MDR-TB.

Group	Drug	Abbreviation	Critical concentrations (µg/mL) for DST by medium			
			Löwenstein–Jensen	Middlebrook 7H10 ^a	Middlebrook 7H11 ^a	BACTEC MGIT liquid culture ^a
Group A	Levofloxacin (CC)	LFX ^b	2.0	1.0	-	1.0
	Moxifloxacin (CC)	MFX ^b	1.0	0.5	0.5	0.25
	Moxifloxacin (CB) ^c		-	2.0	-	1.0
	Bedaquiline	BDQ	-	-	0.25	1.0

	Linezolid	LZD	-	1.0	1.0	1.0
Group B	Clofazimine	CFZ	-	-	-	1.0
	Cycloserine	CS	-	-	-	16
	Terizidone	TZD	-	-	-	-
Group C	Ethambutol ^d	EMB	2.0	5.0	7.5	5.0
	Delamanid ^e	DLM	-	-	0.016	0.06
	Pyrazinamide ^f	PZA	-	-	-	100.0
	Imipenem-cilastatin	IMP/CLN	-	-	-	-
	Meropenem	MPM	-	-	-	-
	Amikacin ^g (Or Streptomycin)	AMK (S)	30.0 4.0	2.0 2.0	- 2.0	1.0 1.0
	Ethionamide Prothionamide	ETO PTO	40.0 40.0	5.0 -	10.0 -	5.0 2.5
	<i>Para</i> -aminosalicylic acid	PAS	-	-	-	-
Other	Pretomanid ^h	Pa	-	-	-	0.5
			-	-	-	2

^a Other methods using solid media (such as the resistance ratio or absolute concentration) have not been adequately validated for anti-TB agents.

^b Levofloxacin and moxifloxacin CCs for LJ established despite very limited data.

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

^d DST not reliable and reproducible. DST is not recommended.

^e Delamanid should be stored away from light and heat as per the manufacturers materials safety data sheet (MSDS)

^f Pyrazinamide is only counted as an effective agent when DST results confirm susceptibility in a quality-assured laboratory.

^g Amikacin and streptomycin are only to be considered in case of rescue regimens/individualised treatment and only if DST results confirm susceptibility.

^h Testing should be performed at both test concentrations, growth at 0.5 mg/L and no growth at 2.0 mg/L = susceptible, with a comment added to the laboratory report stating there is an interpretive uncertainty of this result and close patient follow-up is required; growth at 2.0 = resistant. This has been defined and interpretation guidance is provided in the updated WHO policy statement on critical concentrations (20) for pretomanid and cycloserine.

Testing of ofloxacin is not recommended as it is no longer used to treat DR-TB and laboratories should transition to testing the specific later generation FQs used in treatment regimens.

Knowledge of PZA susceptibility can inform decisions on the choice and design of effective DR-TB regimens. Culture-based PZA pDST requires additional consideration during preparation to reduce unreliable results (21, 22). Currently, BACTEC MGIT 960 liquid culture method, the GenoScholar PZA-TB line probe assay and targeted next generation sequencing (tNGS) are WHO-recommended methods for PZA susceptibility testing. Even though a high rate of false-positive resistance results has been reported in some laboratories for the BACTEC MGIT 960 liquid culture method, in a quality assured laboratory, PZA DST in MGIT can be performed reliably and reproducibly (23).

The thioamides (prothionamide and ethionamide) have been tested extensively in solid media and in liquid media in a few studies. It is difficult to determine thioamide resistance accurately because the change in MIC associated with resistance is small and the drugs are thermolabile. Hence, the distributions of probable susceptible and probable resistant strains are not well separated leading to discrepancy between predicted clinical outcome and *in vitro* susceptibility patterns (24). Resistance conferring mutations in the promoter region of the *inhA* gene that are normally associated with low-level resistance to isoniazid also confer cross-resistance to the class of thioamides, which may be a more reliable method for the detection of resistance to these drugs than performing pDST (25, 26).

Mutations within the *ethA* and *inhA* structural genes have also been associated with relatively high levels of ethionamide resistance (27). Although the Xpert MTB/XDR provides rapid results for ethionamide, sensitivity is limited since it is detected by a single target shared with isoniazid (promoter region of the *inhA* gene) (28).

Primarily, the reproducibility of DST for cycloserine is poor for both solid media and liquid media, and it was not recommended to be tested (29), however, on review of the current literature available this decision has been revised and a critical concentration established for cycloserine. Routine DST is not recommended for other Group C anti-TB drugs (*p*-aminosalicylic acid, imipenem-cilastatin, meropenem) as there are no reliable and reproducible DST methods available. Ideally, DST should be performed at the time of treatment initiation against the drugs for which there is a reliable method available. If baseline DST is not possible, DST should be performed on the first positive culture isolated from the patient during treatment monitoring. Positive cultures isolated from patients during treatment monitoring should be stored frozen. If drug resistance or treatment failure is suspected, pDST and next generation sequencing, if available, should be performed to detect mutations that are associated or determine association with TB drug resistance, especially for the newer drugs.

DST for second-line anti-TB agents should be built on a foundation of reliable, quality assured DST for first-line anti-TB agents. Therefore, it is necessary to establish and validate high-quality DST procedures. For laboratories in resource-limited countries this is usually done in collaboration with a member of the WHO TB Supranational Reference Laboratory Network. When introducing DST for second-line agents, it is necessary to follow the protocols for test validation and to establish a reproducible procedure (i.e. ensure that inter-test agreement is 90% or greater) before beginning to test clinical isolates and report those results (see Annex C for information on quality control testing). Table 3 presents an overview of the phenotypic and genotypic methods for performing DST against second-line anti-TB drugs and provides a clinical interpretation of the results.

Table 3. WHO-recommended diagnostic approaches, reference methods and clinical interpretation for anti-TB drugs

Drug	Initial diagnostic test	Phenotypic DST	Reference Method	Comment
First-line anti-TB drugs	Rifampicin	WRDs LPAs tNGS	MGIT may not be reliable for isolates with borderline resistance mutations	DNA sequencing of the entire <i>rpoB</i> gene Any mutation (excluding silent mutations) observed in the 81bp RRDR ^a hotspot region of the <i>rpoB</i> gene are known or assumed to be associated with rifampicin resistance. In a few cases, mutations in the <i>rpoB</i> gene outside the RRDR region are associated with rifampicin resistance, these would require DR-TB treatment Consider conducting sequencing if the suspicion of resistance is high.
	Isoniazid	Moderate complexity automated NAATs Low complexity automated NAATs LPAs tNGS	Reliable and reproducible when testing the CC in all media.	MGIT If specific inhA promoter mutations are detected (and in the absence of any katG mutations), increasing the dose of isoniazid is likely to be effective. Low and moderate complexity NAATs and LPA for RIF and INH detection are preferred to guide patient selection for the (H)RZE-Lfx regimen. Rifampicin resistance should be excluded before starting the Hr-TB regimen and FQ resistance should be excluded as soon as possible. Consider conducting pDST if the suspicion of resistance is high.
Group A second-line anti-TB drugs	Levofloxacin	Moderate complexity automated NAATs Low complexity automated NAATs LPAs tNGS	Reliable and reproducible when testing the CC in LJ, 7H10 and MGIT media	MGIT Strains with known or assumed resistance mutations should be considered to be resistant. Most strains without mutations should be susceptible. However, a strain with no mutations detected by NAATs/LPAs may still be resistant. Consider conducting pDST if the suspicion of resistance is high.
	Moxifloxacin (Critical concentration)	Low complexity automated NAATs LPAs tNGS	Reliable and reproducible when testing the CC in LJ, 7H10, 7H11 and MGIT media.	MGIT A strain of TB with no mutations detected by LPA or Xpert MTB/XDR may still be resistant. Confirm with pDST both the CC and CB concentrations.
	Moxifloxacin (Clinical breakpoint concentration)	Low complexity automated NAATs LPAs tNGS Certain mutations detected by NAATs,	Clinical breakpoint concentration (CB) for 7H10 and MGIT apply to high-dose moxifloxacin (i.e. 800 mg daily).	MGIT Moxifloxacin- even at high dose- is unlikely to be effective if resistant at the CB concentration or if certain high confidence mutations associated with high MICs are detected

	tNGS or LPA result in very high MICs for which even high-dose moxifloxacin is not effective.				
	Bedaquiline	tNGS	CCs established for testing in 7H11 and MGIT media.	MGIT	Ideally, perform pDST at the time of treatment initiation. If baseline DST is not performed, perform DST with the first strain isolated from patients sample during treatment monitoring. ^a
	Linezolid	tNGS	CCs established for testing in 7H10, 7H11 and MGIT media.	MGIT	Ideally, perform pDST at the time of treatment initiation. If baseline DST is not performed, perform DST with the first strain isolated from patients sample during treatment monitoring. ^a
Group B second-line anti-	Clofazimine	tNGS	CC established for testing MGIT media only.	MGIT	Ideally, perform pDST at the time of treatment initiation. If baseline DST is not performed, perform DST with the first strain isolated from patients sample during treatment monitoring. ^a
	Cycloserine Terizidone	No rapid method currently exists for the detection resistance	CCs have been established for cycloserine on MGIT media only	MGIT	Ideally, perform pDST at the time of treatment initiation. If baseline DST is not performed, perform DST with the first strain isolated from patients sample during treatment monitoring. ^a
C second-line anti-TB drugs	Ethambutol	No rapid method currently exists for the detection resistance	DST not reliable and reproducible	N/A	Both gDST and pDST are not reliable. Ethambutol, if used, cannot be counted as an effective drug in an MDR/RR-TB treatment regimen
	Delamanid	No rapid method currently exists for the detection of resistance	CCs established for testing in 7H11 and MGIT media.	MGIT	Ideally, perform pDST at the time of treatment initiation. If baseline DST is not performed, perform DST with the first strain isolated from patients during treatment monitoring. ^a
	Pyrazinamide	tNGS or LPAs	DST method standardised in the MGIT. False resistant results can be detected if DST inoculum not properly prepared	DNA sequencing of the <i>pncA</i> gene	In a quality assured laboratory, a susceptible DST result for PZA can be used to guide the inclusion of PZA in a DR-TB treatment regimen. Do not include PZA if resistance is detected or if used do not count as an effective agent
	Amikacin (or Streptomycin)	Low complexity automated NAATs tNGS (for Amikacin and Streptomycin) -LPAs ^b (for Amikacin)	CCs established for testing in LJ, Middlebrook and MGIT media.	MGIT	Injectable agents are no longer part of the routine DR-TB regimen. However, testing should be completed if planned to be included in the DR-TB regimen A strain with no mutations in the <i>rrs</i> and <i>eis</i> genes detected by genotypic assays may still be resistant to AMK. Confirm with pDST. If streptomycin is used, perform pDST at the time of treatment initiation if possible.
	Imipenem-cilastin Meropenem	No rapid method currently exists for the detection resistance	CCs have not been established for any DST media	N/A	DST is not recommended as both imipenem and meropenem are highly unstable in liquid media
	Ethionamide Prothionamide	Mutations in the promotor region of the <i>inhA</i> gene: LPAs, and Moderate	DST not reliable and reproducible	DNA sequencing of the <i>inhA</i> promotor	Do not include thioamides if resistance associated mutations are detected

		complexity automated NAATs and Low complexity automated NAATs		region and <i>ethA</i> and <i>ethR</i> genes.	
	<i>Para-aminosalicylic acid</i>	No rapid method currently exists for the detection of resistance	CCs have not been established for any DST media	N/A	DST is not recommended
Other	<i>Pretomanid</i>	No rapid method currently exists for the detection of resistance	CCs have been established for MGIT of media only	MGIT	Ideally, perform pDST at the time of treatment initiation. If baseline DST is not performed, perform DST with the first strain isolated from patients sample during treatment monitoring. ^a

^a Perform phenotypic DST for strains isolated from patients during treatment monitoring. If resistance is detected, store strains and if possible, perform WGS to collect data on mutations associated with resistance

^b SL-LPAs do not cover the relevant region of the *rrs* gene or other genes associated with resistance to streptomycin

2 Susceptibility testing for anti-TB agents using solid media

2.1 Recommended drug powders

Recommended drug powders for pDST based on review of literature related to the establishment of the critical concentration.

Table 4. Availability of pure powders from Global Drug Facility (GDF) and other manufacturers

Drug	Description and ingredients	Manufacturer (Catalogue No.)	Quantity	GDF Catalogue No	GDF Quantity	Storage
Levofloxacin	>98% HPLC	Sigma- Aldrich (28266-IG-F)	1 g	106560	1 g	RT
Moxifloxacin	Moxifloxacin Hydrochloride 249ug/vial	BD REF: 215404	6 vials of 249 µg each	Please refer to GDF catalogue or contact GDF or manufacturer for more information	6 vials of 249 µg each	2-8°C
Bedaquiline	Bedaquiline fumarate 12 mg BDQ fumarate salt equivalent to 10 mg BDQ base	Available (free of charge) ^a through: BEI Resources https://www.beiresources.org	20 mg	Not applicable	Not applicable	RT
Bedaquiline	Bedaquiline fumarate 170 µg (active) Ficoll (inactive). Contents of vial soluble in 2 mL DMSO)	BD REF: 215449	170 µg (2 vials)	Please refer to GDF catalogue or contact GDF or manufacturer for more information	2 vials of 170 µg	2-8°C
Ethionamide	830 µg (active); Ficoll (inactive) Contents of vial soluble in 4 mL sterile, distilled water	BD REF: 215355	830 µg (6 vials)	Please refer to GDF catalogue or contact GDF or manufacturer for more information	Contact GDF or BD	2-8°C
	Pure substance	Sigma Aldrich (E6005)	5 g	106316	5g	2-8°C
Linezolid	Pure substance ≥ 98% activity	(1) Sigma (PZ0014-5MG); (PZ0014-25MG) (2) Cayman Chemical (CAS 165800-03-3)	5 mg / 25 mg 25mg	106653	25 mg	RT
Clofazimine	Pure substance	Sigma-Aldrich (C8895-1G)	1 g	106654	1 g	RT
Delamanid	Pure substance	Available through: BEI Resources ^b (https://www.beiresources.org/Catalog/BEIAntimicrobialCompoundsandOtherChemicals/NR-51636.aspx)	10 mg	Not applicable	Not applicable	RT (protect from light and heat)

Amikacin	Amikacin disulfate salt potency: 674-786 µg per mg (as amikacin base)	Sigma-Aldrich (A1774-250MG)	250 mg	106318	5 g	2-8°C
		BD REF 215350	6 vials of 332 µg each	106586	6 vials of 332 µg each	2-8°C
Streptomycin	Streptomycin sulphate. Potency ≥720 µg per mg (as streptomycin base)	Sigma-Aldrich (D7253-5G)	5 g	106311	5 g	2-8°C
Pretomanid	Pure substance	TB Alliance	10 or 50 mg	Contact TB Alliance	Contact TB Alliance	RT
		Sigma-Aldrich (SML1290)	10 or 50 mg	Please refer to GDF catalogue or contact GDF or manufacturer for more information	Please refer to GDF catalogue or contact GDF or manufacturer for more information	-20°C
Cycloserine	Pure-substance ≥ 98%	Sigma-Aldrich (C6880)	1 mg or 5 mg	Not available	Not available	See footnote ^c

^a Free of charge shipment when specifying carrier as JNJ

^b Collection from the closest airport and customs clearance of the drug shipment is the responsibility of the receiving laboratory

^c Given the known heat instability of DCS, DCS powder should be stored as instructed by the manufacturer and stocks solutions in sterile distilled/deionised water should be stored at -70° C ± 10° C for no longer than six months (i.e. lower temperatures should not be used and vials should never be re-frozen).

RT – room temperature

2.2 Proportion method using Löwenstein–Jensen medium

2.2.1 Principles

The proportion method can be used with Löwenstein–Jensen (LJ) medium to determine whether isolates of *M. tuberculosis* are susceptible to anti-TB agents. Media containing the critical concentration of the anti-TB agent is inoculated with a dilution of a culture suspension (usually a 10^{-2} dilution of a McFarland 1 suspension) and control media without the anti-TB agent is inoculated with a 1:100 dilution of the this (usually a 10^{-4} dilution of a McFarland 1 suspension). Growth (i.e. number of colonies) on the agent-containing media is compared to the growth on the agent-free control media. The ratio of the number of colonies on the medium containing the anti-TB agent to the number of colonies (corrected for the dilution factor) on the medium without the anti-TB agent is calculated, and the proportion is expressed as a percentage. Provisional results for susceptible isolates may be read after 3–4 weeks of incubation; definitive results may be read after 6 weeks of incubation. Resistance may be reported within 3–4 weeks. DST for second-line agents is similar to DST for first-line agents in terms of preparing the media, bacterial suspension and dilutions; inoculating the media; incubating the cultures; and the schedules for reading and reporting (30, 31). Some of these procedures are described in the following sections and appendices.

Preparing the medium

LJ medium is prepared without potato starch following the procedure described below.

- Use fresh eggs that are not more than 1 week old from chickens that have not been fed antibiotics.
- Scrub the eggs with a soap solution and leave them in the solution for 30 minutes.
- Rinse the eggs thoroughly under running water, and then soak them in 70% ethyl alcohol for 15 minutes.
- Place eggs on a sheet of clean paper towel and allow to air dry
- Break the eggs into a sterile flask, and then shake the flask by hand to homogenize the eggs.
- Filter the egg suspension through four layers of sterile gauze and collect the filtered suspension in a sterile measuring cylinder so that it can be measured.
- Prepare 600 mL of the recommended salt solution as described below and autoclave at 121°C for 30 minutes. Cool to room temperature.

LJ media salt solution can be prepared from a commercial base following the manufacturer's instructions. Alternatively prepare the salt solution dissolve the ingredients in the following order.

Monopotassium phosphate (anhydrous)	2.4 g
Magnesium sulphate 7H ₂ O	0.24 g
Magnesium citrate	0.6 g
Asparagine	3.6 g
Glycerol (reagent grade)	12.0 mL
Distilled or deionized water	600.0 mL

- Add 20.0 mL malachite green solution to the cooled salt solution; use freshly prepared 2% malachite green solution in water.

- Add 1000 mL of the homogenized eggs.
- Mix thoroughly and dispense 5.0 mL into each screw-capped sterile tube.
- Place the tubes in an inspissator on an angle.
- Inspissate the tubes for 40–50 minutes once the temperature has stabilised at 85 C ± 1 C.
- To prepare the medium containing the agent to be tested, the agents are incorporated into the liquid mixture before it is dispensed into tubes and inspissated. All tubes should be labelled with the name of the agent and the concentration.

Inspissated Löwenstein–Jensen medium with and without anti-TB agents incorporated can be stored at 6 °C ± 2 °C for 1 month.

After inspissation, randomly select about 5% of the tubes for a sterility test. The number of tubes selected depends on the number of tubes prepared in each batch. If a smaller number of tubes has been prepared, select at least 10 tubes for sterility testing. These tubes should be incubated at 35–37 °C for 48 hours as an initial sterility check, and then at least 5 tubes should be incubated at 35–37 °C for 4 weeks to test for slow-growing bacteria and fungi. Tubes incubated for 48 hours and found to be sterile may be used for routine culture work.

- A quality control test should be run on each batch of freshly prepared medium (see Annex C).

2.2.2 Anti-TB agents and critical concentrations for testing

The anti-TB agents recommended for DST using LJ media and the established critical concentrations for testing are listed below (29, 32). Newer agents, such as bedaquiline, linezolid and clofazimine have not been validated for testing using LJ media. Bedaquiline is known to bind significantly to protein.

Isoniazid	0.2 µg/mL
Rifampicin	40.0 µg/mL
Ethambutol	2.0 µg/mL
Levofloxacin	2.0 µg/mL
Moxifloxacin	1.0 µg/mL
Amikacin	30.0 µg/mL
Streptomycin	4.0 µg/mL

Note: Pure formulations of the agents being tested should be used to prepare the medium. The agents to be tested should be stored in a desiccator or according to the manufacturers' instructions. The weight of the agent in powder form must be calculated for each preparation based on the potency (activity) provided by the manufacturer (see Annex D for more detailed information). Details of the concentrations for different anti-TB agents and the preparation of solutions are given in Table 5.

Table 5. Concentrations and solutions needed to prepare first- and second-line anti-TB agents for use with 500 mL of Löwenstein–Jensen medium

	Final concentration of anti-TB agent (µg/mL) in LJ medium	Stock solution and solvent	Subsequent dilutions in LJ medium

First-line anti-TB drugs	Isoniazid (0.2)	Dissolve 20.0 mg INH in 40 mL sterile distilled water (Solution A) -500 µg/mL Dilute 2.0 mL solution A to a final volume of 50 mL of sterile distilled water – 20 µg/mL (Solution B)	Add 5 mL of solution B to 500 mL LJ medium
	Rifampicin (40.0)	Dissolve 80.0 mg/potency ^a in 5.0ml of absolute methanol - 1600 µg/mL Dilute further to a final volume with 5 mL of 95% ethanol - 800 µg/mL (Solution B) (self-sterilizing)	Add 2.5 mL of solution B to 500 mL LJ medium
	Ethambutol (2.0)	Dissolve 10.0 mg EMB in 50 mL sterile distilled water (Solution A) -200 µg/mL	Add 5 mL of solution A to 500 mL LJ medium
Second-line anti-TB drugs	Levofloxacin (2.0)	Dissolve 10 mg LFX in 5 mL sterile 0.1M NaOH ^b . (Solution A) -2000 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10 mL – 200 µg/mL (Solution B)	Add 5 mL of solution C to 500 mL LJ medium.
	Moxifloxacin (1.0) Critical concentration	Dissolve 10 mg MFX in 5 mL sterile 0.1M NaOH ^b . (Solution A) -2000 µg/mL Dilute 1 mL solution A with sterile water to final volume of 20 mL – 100 µg/mL (Solution B)	Add 5 mL of solution C to 500 mL LJ medium
	Amikacin (30)	Dissolve 30 mg ^a AMK in 10 mL sterile distilled water (Solution A) -3000 µg/mL	Add 5 mL of solution A to 500 mL LJ medium
	Streptomycin (4.0)	Dissolve 20 mg/potency ^a STR in 50 mL sterile distilled water. (Solution A) - 4000µg/mL Dilute 5 mL solution A with sterile water to final volume of 50ml – 400 µg/mL (Solution B)	Add 5 mL of solution C to 500 mL LJ medium

NaOH, sodium hydroxide.

^a Adjust for potency. See Annex D

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

Note: the preparation scheme shown in Table 5 may be changed as needed. For details of sourcing, weighing, calculating potency, and freezing anti-TB agents, see Annex D.

2.2.3 Preparing the mycobacterial suspension

Indirect DST is carried out using a primary isolate or a sub-culture on LJ medium. A fresh primary isolate is preferred because population characteristics (e.g. proportion of resistant bacteria) may change after sub-culturing, especially after repeated sub-culturing. Regardless of whether an original isolate or a subculture is used, an actively growing culture should be used within 1–2 weeks after the growth appears. If it is more than 2 weeks from the day that positive growth on the medium was observed, sub-culture again, and use the freshly grown subculture.

Precautions: The handling of cultures, and additions and manipulations should be done inside a BSC Class 2 (As recommended by 2020 WHO TB laboratory biosafety guidelines). WHO's recommendations for these procedures or established national guidelines should be followed. Use properly sterilized and quality-controlled reagents, and aseptic techniques throughout the DST procedure.

Procedure

The following points outline the procedure for preparing a culture suspension.

- Obtain a representative portion of bacterial growth by sampling as many colonies as possible. Be careful not to scrape off any medium because residual medium will give false turbidity readings.
- Transfer the colonies to a glass tube containing 5–6 mL normal sterile saline (0.85% sodium chloride). (Note: A smaller volume or 0.5–1.0 mL can be used to facilitate better homogenisation and minimise clumping).
- The suspension can be homogenized by using 3 mm glass beads (use about 5–10 beads) and then vortexing for 2–3 minutes (1 minute if small volume of water is used). Before vortexing, ensure that the cap is tightly closed.
- Leave the tube undisturbed for 30 minutes to allow large clumps of bacteria to settle. Without disturbing the sediment, withdraw 2–4 mL of the supernatant, and transfer to a sterile tube.
- Adjust the turbidity of the suspension to that of the McFarland number 1 standard by comparing to a known standard or by measuring optical density (OD). This will be the working suspension for pDST. It should be homogeneous, without any visible clumps. The bacterial suspension corresponds to approximately 1 mg of wet bacterial mass/mL (about one full loop with an inner diameter of 3 mm). The turbidity of the suspension is visually adjusted by comparing it to the McFarland number 1 reference standard (see Annex B for information on preparing McFarland standards).

2.2.4 Diluting the suspension and inoculating the Löwenstein–Jensen medium

Ensure that all tubes are properly labelled upfront. Make serial 10-fold dilutions of the standard suspension by diluting sequentially 1.0 mL of the culture suspension in tubes containing 9 mL of sterile distilled water or normal saline (0.85% sodium chloride). Make sure to mix each dilution thoroughly. Dilutions of 10^{-2} (for Control 1) and 10^{-4} (for Control 2) should be inoculated as the growth controls (that is, LJ medium without any anti-TB agents). Some laboratories inoculate duplicate LJ tubes for both Control 1 and Control 2. One tube with medium containing each anti-TB agent is inoculated with only the 10^{-2} dilution. The volume of the inoculum is 0.1 mL. The inoculum should be placed onto the middle two thirds of the slant avoiding the edges.

2.2.5 Incubation

After inoculation, the tubes are placed at an angle and are incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Some laboratories incubate the tubes with the screw caps slightly loosened to allow for evaporation of the inoculum. Then, after 24–48 hours, the caps are tightened and the tubes are incubated for about 6 weeks in an upright position. Extreme caution in accordance to biosafety level 3 should be taken if the caps are left loose.

2.2.6 Examining the cultures

Examine the inoculated tubes after 7 days of incubation to assess contamination. For interpretation, the results are read 21, 28 and 40 days after inoculation. The results can be read in two steps:

- Count the total number of colonies growing on the different tubes. Control 1 usually has confluent growth; Control 2 should have countable colonies. If two tubes have been inoculated as controls, then an average of the colony count for the two is used for calculations.
- The proportion of resistant bacilli is calculated by comparing the counts on Control 1 as follows:
 1. Check the acceptability criteria of minimal/max growth on Control 1 and Control 2
 2. If acceptable, then check whether growth on the drug-containing tube is equal to or greater than Control 2.
- A percentage is calculated using the following formula.

$$\frac{\text{Number of colonies on the medium containing the anti-TB agent}}{\text{Number of colonies on Control 1 (10}^{-2}\text{ dilution)}} \times 100$$

Example 1

1. Growth control (10^{-2} dilution) has 80 colonies;
2. The tube with the anti-TB agent has 6 colonies;
3. $6/80 \times 100 = 7.5\%$, therefore, the strain is resistant.

If Control 2 (10^{-4} dilution) has countable colonies, then multiply the number of colonies by 100 and compare this number with the number of colonies on Control 1 (the tube that has been inoculated with the 10^{-2} dilution); Control 1 should have confluent growth.

Example 2

1. Control 1 has confluent growth
2. Control 2 has 80 colonies
3. The tube with the anti-TB agent has 6 colonies (10^{-2} dilution); Control 2 (10^{-4} dilution) has 80 colonies. To estimate the number of colonies in Control 1 (10^{-2} dilution), multiply the number of colonies in Control 2 by 100. This will give 8000 colonies (this conversion gives the number of colonies expected on the 10^{-2} dilution so it can be used in the formula given above)
4. $6/8000 \times 100 = 0.075\%$, therefore the strain is susceptible

Note: Do not count colonies that are growing only on the upper part of the slant because this indicates that the anti-TB agent has been inactivated in that portion. **The number of colonies grown on Control 1 must be approximately 100 times the number of colonies on Control 2; this indicates that the 1:100 dilution has been prepared properly.**

2.2.7 Interpreting and reporting results

- Results are interpreted using the 1% proportion method. This means that if 1% or more of the populations are resistant then the culture is reported as resistant. If the ratio of the number of colonies on the medium containing an anti-TB agent to the number of colonies on the control medium is less than 1%, then the strain is reported as susceptible.
- If on day 21 or 28 the ratio of the number of colonies on the medium containing the anti-TB agent to the number of colonies on the control medium is 1% or greater, then the culture is resistant. If there are no colonies on the medium containing the anti-TB agent and Control 1 has confluent growth, the strain can be reported as susceptible without further reading. Except for these two circumstances, all other results should be reported after day 40.
- On day 28 if Control 1 or Control 2 has 20 colonies or fewer, this indicates that the medium has been insufficiently inoculated. If there is growth on the medium containing the anti-TB agent it can be reported as resistant, but if there is no growth on the medium containing the anti-TB agent, the test should be repeated using fresh inoculum.
- If Control 2 has confluent growth, this indicates that the medium has been over-inoculated, and the test should be repeated. In this situation, growth on the medium containing the anti-TB agent could be due to over inoculation, and the culture should not be interpreted as resistant. However, if there is no growth on the medium containing the anti-TB agent, the test can be reported as susceptible.
- Results of the tests should be reported only as “susceptible” or “resistant” to facilitate use by the clinician. Results should be reported as soon as they are available. Results can be reported after 3 weeks if there is growth on the medium containing the anti-TB agent and all other parameters are satisfactory.

2.2.8 Quality control

It is important to perform quality control of DST. The best practice is to run a quality control strain with each batch being tested. *M. tuberculosis* H37Rv can be used for quality control because it is susceptible to all anti-TB agents. It is further recommended to include testing a well characterised resistant strain in each batch even that may allow for the detection of major errors in the preparation of drug stock solutions. See Annex C for details of quality control testing and validation.

2.3 Proportion method using Middlebrook 7H10 or 7H11 agar media

2.3.1 Principles

The proportion method can be used with Middlebrook 7H10 (7H10) or Middlebrook 7H11 (7H11) agar media to determine whether isolates of *M. tuberculosis* are susceptible to first and second-line anti-TB agents. A standardized suspension of the test organism is prepared, and specified dilutions are made; these are then inoculated onto media containing antimicrobial agents and onto control media that do not contain an antimicrobial agent. After incubating the cultures for 3–4 weeks, the growth (that is, the number of colonies) on the medium containing the anti-TB agent is compared with the growth on the control medium. The ratio of the number of colonies on the medium that contains the anti-TB agent to the number of colonies on the medium without the anti-TB agent is calculated (corrected for dilution), and the proportion is expressed as a percentage. An isolate is considered resistant if the proportion of the number of colonies on the medium containing the anti-TB agent to the number of colonies on the control medium is 1% or greater (33).

2.3.2 Preparing the medium

The recommended media are 7H10 and 7H11 agar. Both 7H10 and 7H11 media can be used for DST although 7H11 is more suitable for the growth of isoniazid-resistant strains. DST using Middlebrook media is often performed using plastic quadrant petri dishes (**Note: for bedaquiline, polystyrene petri dishes must be used**) (34) in which quadrants contain either medium without an anti-TB agent or medium with one of the anti-TB agents being tested. Nevertheless, tubes can be used as well. Middlebrook 7H10 or 7H11 medium is prepared from a dehydrated base following the manufacturer's instructions. After the medium has been prepared, a supplement containing oleic acid, albumin, dextrose and catalase (OADC) is added, which is essential for the growth of *M. tuberculosis* complex bacteria.

Each quadrant of a petri dish contains 5 mL of medium: 500 mL of medium is needed to fill 1 quadrant on each of approximately 100 plates. The procedure for mixing the medium is briefly described below. Label the plates and quadrants ensuring that all plates and quadrants have been labelled with the correct anti-TB agent and concentration.

- Dispense 450 mL of distilled water into a 1000 mL Erlenmeyer flask.
- Weigh 7H10 or 7H11 agar base and adjust the weight of the agar base according to the manufacturer's instructions in the package insert. Add the agar base to the distilled water.
- This quantity is sufficient for each assigned quadrant of 100 plates, or alternatively, for 250 tubes with 5ml each
- Add 2.5 mL glycerol to each flask.
- Mix well.
- Stopper each flask with cotton or a screw cap, and autoclave at 121 °C for 15 minutes.
- While the media flasks are being autoclaved, remove the stock solutions of anti-TB agents and the OADC supplement from the refrigerator and bring to room temperature.
- After autoclaving, allow the flasks with the medium to cool to 50–56 °C.
- Add 50 mL of OADC supplement to 450 mL of the liquid agar base and mix well.
- For the medium containing the anti-TB agent, add the appropriate amount of the agent (see *Tables 6 and 7*), mix well, and immediately dispense 5.0 mL into each quadrant using aseptic

techniques. Ensure that all quadrants/tubes have been labelled with the name of the anti-TB agent and the concentration.

- Allow the agar to solidify at room temperature.
- Plates/tubes should be stored at 2-8 °C in an inverted position in ziplock bags and protected from light.
- The sterility of each batch of plates should be tested by incubating 1–5% of the total number at 35–37 °C for 48 hours. The number chosen for the sterility test depends upon the number of plates prepared in each batch.

2.3.3 Anti-TB agents and test concentrations

Both 7H10 and 7H11 agar media can be used for DST for selected first and second-line anti-TB agents. The concentrations of antimicrobial agents used in testing are the established critical concentrations for these media used for testing *M. tuberculosis* (See Table 6). The critical concentrations determined for certain second-line agents in Middlebrook media and the procedures used to prepare media containing these antimicrobials are given in Table 7 and Table 8. Only those second-line agents with established critical concentrations should be tested.

Note: the critical concentrations of some anti-TB agents are not available for both 7H11 and 7H10 media.

Table 6. Final test concentrations of first- and second-line anti-tuberculosis (anti-TB) agents for use with 7H10 or 7H11 medium

DRUG	7H10	7H11
Isoniazid	0.2 µg/mL	0.2 µg/mL
Rifampicin	0.5 µg/mL	1.0 µg/mL
Ethambutol	5.0 µg/mL	7.5 µg/mL
Levofloxacin	1.0 µg/mL	-
Moxifloxacin (CC)	0.5 µg/mL	0.5 µg/mL
Moxifloxacin (CB)	2.0 µg/mL	-
Bedaquiline ^a	-	0.25 µg/mL
Linezolid	1.0 µg/mL	1.0 µg/mL
Delamanid	-	0.016 µg/mL
Amikacin	2.0 µg/mL	-
Streptomycin	2.0 µg/mL	2.0 µg/mL

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

Table 7. Concentrations and solutions needed to prepare first- and second-line anti-tuberculosis (anti-TB) agents for use with 500 mL of 7H10 medium

	Final concentration of anti-TB agent (µg/mL)	Stock solution and solvent	Subsequent dilutions in Middlebrook 7H10 media
First-line anti-TB drugs	Isoniazid (0.2)	Dissolve 20.0 mg INH in 40 mL sterile distilled water (Solution A) -500 µg/mL Dilute 2.0ml solution A to a final volume of 50 mL of sterile distilled water – 20 µg/mL (Solution B)	Add 5 mL of solution B to 500 mL 7H10 medium.
	Rifampicin (1.0)	Dissolve 10.0 RIF mg/potency ^a in 5.0 mL of absolute methanol (or DMSO) - 2000 µg/mL Dilute further to a final volume with 5 mL DMSO of 95% ethanol - 100 µg/mL (Solution B) (self-sterilizing)	Add 5 mL of solution A to 500 mL 7H10 medium.
	Ethambutol (5.0)	Dissolve 10.0 mg EMB in 20 mL sterile distilled water (Solution A) -500 µg/mL	Add 5 mL of solution A to 500 mL 7H10 medium.
Second-line anti-TB drugs	Levofloxacin (1.0)	Dissolve 10 mg LFX in 10 mL sterile 0.1M NaOH ^b . (Solution A) -1000 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10ml – 100 µg/mL (Solution B)	Add 5.0ml of solution B to 500 mL 7H10 medium.
	Moxifloxacin (0.5) Critical concentration	Dissolve 10 mg MFX in 20 mL sterile 0.1M NaOH ^b . (Solution A) -500 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10 mL – 50 µg/mL (Solution B)	Add 5.0 mL of solution B to 500 mL 7H10 medium.
	Moxifloxacin (2.0) Clinical breakpoint	Dissolve 10 mg MFX in 5 mL sterile 0.1M NaOH ^b . (Solution A) -2000 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10 mL – 200 µg/mL (Solution B)	Add 5.0ml of solution B to 500 mL 7H10 medium.
	Linezolid (1.0)	Dissolve 10 mg LZD in 10 mL sterile distilled water. (Solution A) -1000 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10ml – 100 µg/mL (Solution B)	Add 5.0 mL of solution B to 500 mL 7H10 medium.
	Amikacin (2.0)	Dissolve 10 mg AMK in 5 mL sterile distilled water (Solution A) -2000 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10 mL – 200 µg/mL (Solution B)	Add 5.0 mL of solution B to 500 mL 7H10 medium.

NaOH, sodium hydroxide. Dilute to 1:10 to achieve 0.1M strength.

^aAdjust for potency. See Annex D

^b For 1M NaOH: dissolve 40 g NaOH in 1 litre of distilled or deionized water (or dissolve 4.0 g in 10 mL)

Note: the preparation scheme shown in Table 7 may be changed as needed. For details of sourcing, weighing, calculating potency, and freezing anti-TB agents, see Annex D.

Table 8. Concentrations and solutions needed to prepare first- and second-line anti-tuberculosis (anti-TB) agents for use with 500 mL of 7H11 medium

	Final concentration of anti-TB agent (µg/mL)	Stock solution and solvent	Subsequent dilutions in 7H11 media
First-line anti-TB drugs	Isoniazid (0.2)	Dissolve 20.0 mg isoniazid in 40 mL sterile distilled water (Solution A) -500 µg/mL Dilute 2.0 mL solution A to a final volume of 50 mL of sterile distilled water – 20 µg/mL (Solution B)	Add 5 mL of solution B to 500 mL 7H11 medium.
	Rifampicin (1.0)	Dissolve 10.0 mg/potency RIF in 5.0 mL of absolute methanol (or DMSO ^b) - 2000 µg/mL Dilute further to a final volume with 5 mL of 95% ethanol - 100 µg/mL (Solution B) (self-sterilizing)	Add 5 mL of solution A to 500 mL 7H11 medium.
	Ethambutol (7.5)	Dissolve 15.0 mg EMB in 20 mL sterile distilled water (Solution A) -750 µg/mL	Add 5 mL of solution A to 500 mL 7H10 medium.
Second-line anti-TB drugs	Moxifloxacin (0.5) Critical concentration	Dissolve 10 mg MFX in 20 mL sterile 0.1M NaOH ^a . (Solution A) -500 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10 mL – 50 µg/mL (Solution B)	Add 5.0 mL of solution B to 500 mL 7H11 medium.
	Bedaquiline ^c (0.25)	Dissolve 12 mg BDQ fumarate salts (equivalent to 10 mg BDQ base) in 20 mL sterile DMSO ^b . (Solution A) -500 µg/mL Dilute 1 mL solution A with DMSO to final volume of 20ml – 25 µg/mL (Solution B)	Add 5.0 mL of solution B to 500 mL 7H11 medium.
	Linezolid (1.0)	Dissolve 10 mg LZD in 10 mL sterile distilled water. (Solution A) -1000 µg/mL Dilute 1 mL solution A with sterile water to final volume of 10 mL – 100 µg/mL (Solution B)	Add 5.0 mL of solution B to 500 mL 7H11 medium.
	Delamanid (0.016)	Dissolve 10 mg DLM in 2.5 mL sterile DMSO ^b (Solution A) - 4000 µg/mL Dilute 0.5 mL solution A with DMSO to final volume of 12.5 mL – 160 µg/mL (Solution B) Dilute 1.0 mL solution B with DMSO water to final volume of 10 mL – 16.0 µg/mL (Solution C) Dilute 1.0 mL solution C with DMSO to final volume of 10 mL – 1.6 µg/mL (Solution D)	Add 5.0 mL of solution D to 500 mL 7H10 medium.

NaOH, sodium hydroxide. Dilute to 1:10 to achieve 0.1M strength.

a For 1M NaOH: dissolve 40 g NaOH in 1 litre of distilled or deionized water (or dissolve 4.0 g in 10 mL);

b DMSO – Dimethyl sulphoxide. The drug should dissolve completely and there should be no sediment or turbidity in the drug solution

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

d Delamanid is light sensitive, exposure to direct light must be minimized

Note: the preparation scheme shown in Table 8 may be changed as needed. For details of sourcing, weighing, calculating potency, and freezing anti-TB agents, see Annex D.

2.3.4 Preparing the mycobacterial suspension

A fresh primary isolate is preferred because population characteristics (e.g. proportion of resistant bacteria) may change after sub-culturing, especially after repeated sub-culturing. Regardless of whether an original isolate or a subculture is used, an actively growing culture should be used within 1–2 weeks after the growth appears. If it is more than 2 weeks from the day that positive growth on the medium was observed, sub-culture again, and use the freshly grown subculture. The culture should be thoroughly checked for purity by performing an acid-fast bacilli (AFB) smear and then by streaking the suspension on a blood agar plate; growth should be checked after the plate has been incubated at 37 °C for 48 hours.

The turbidity of the suspension should be equal to the McFarland number 1 standard. Inoculating the medium with 0.1 mL of a 10^{-2} dilution or of a 10^{-4} dilution, or both, of this standardized suspension should produce a countable number of separate colonies on the medium that does not contain the anti-TB agent.

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

Procedure

Using growth on solid medium

Scrape colonies from the surface of the solid medium. Sample growth from the entire surface of the culture medium. Take care not to scrape off the medium because residual medium will give false turbidity readings for the suspension.

Transfer the bacterial mass to an appropriately labelled tube containing 5-6 mL of 7H9 broth with albumin-dextrose-catalase (ADC) supplement, 0.05% Tween 80 and 3 mm glass beads (use about 6–10 beads). (Note: Alternatively, a smaller volume of 0.5-1.0 mL can be used to facilitate better homogenisation and minimise clumping). Cap the tube.

Vortex vigorously for approximately 1 minute to homogenize the sample.

Allow the tube to stand for 30 minutes to let large clumps of bacteria settle.

Withdraw 1–2 mL from the upper portion of the suspension, and transfer this to a sterile tube. Care must be taken not to disturb the sediment.

Adjust the turbidity of the suspension to that of a McFarland number 1 standard. (See Annex B for information on the McFarland turbidity standards.)

Using growth in liquid medium

Middlebrook 7H9 medium may also be used. For sub-culturing, 7H9 medium is usually used with OADC and 0.05% Tween 80.

- Transfer 5 mL of a culture that has been freshly grown in a liquid medium to an appropriately labelled tube containing 3 mm glass beads (use about 6–10 beads). (Note: Alternatively, a smaller volume of 0.5-1.0 mL can be used to facilitate better homogenisation and minimise clumping). Cap the tube.
- Vortex vigorously for approximately 1 minute to homogenize the sample.
- Allow the tube to stand for 30 minutes to let large clumps of bacteria settle.
- Withdraw 1–2 mL from the upper portion of the suspension, and transfer this to a sterile tube.
- Adjust the turbidity of the suspension to that of a McFarland number 1 standard. (See Annex B for information on the McFarland turbidity standards.)

2.3.5 Diluting the suspension and inoculating the medium

Make two dilutions of the suspension in sterile water or saline: one of 10^{-2} and one of 10^{-4} as described in the points below. The inclusion of 0.01% Tween 80 (final concentration in the medium) may reduce clumping.

Transfer 0.1 mL of the test organism to a tube with 9.9 mL of diluent, and mix it thoroughly. The resulting suspension will be a 10^{-2} dilution of the original sample.

Transfer 0.1 mL of the 10^{-2} suspension to another tube with 9.9 mL of diluent, and mix it thoroughly. The resulting suspension will be a 10^{-4} dilution of the original sample.

Appropriately label duplicate sets of plates/tubes containing control and anti-TB test media to indicate concentration of the test inoculum.

Inoculate 0.1 mL of the 10^{-2} dilution onto the control quadrant of each plate and onto each of the quadrants containing the anti-TB agent. This can be done using a sterile glass or plastic Pasteur pipette to inoculate each quadrant of each plate with three clean drops of the suspension. Take care not to touch the agar surface with the pipette tip or splash drops in adjacent quadrants.

In a similar manner, inoculate 0.1 mL of the 10^{-4} dilution onto the control quadrant and onto each of the quadrants containing the anti-TB agent. Allow the plates to stand at room temperature with the agar surface facing up until the liquid has been absorbed into the agar.

Seal each plate with a CO₂-permeable heat-shrink ring, or place in a CO₂-permeable, sealable bag.

2.3.6 Incubation

Incubate plates with the agar side facing down) at $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Make sure that the temperature of the area in the incubator where the plates are placed maintains the recommended temperature. Protect from light. The plates can optionally be incubated in an incubator with an atmosphere of 5–10% CO_2 .

2.3.7 Examining the cultures

Examine the plates after 3–7 days of incubation to assess contamination. Discard contaminated plates. If contamination has occurred, repeat the susceptibility test.

After 21 days of incubation, examine each quadrant using a dissecting microscope. For isolates that are known to grow slowly, or if there is insufficient growth on the control medium, the incubation period may be extended to 28 days.

Count or estimate the total number of colonies (including micro-colonies) in each quadrant for both dilutions and record those values.

Note: Micro-colonies may represent true resistance (for example, a slowly growing resistant strain), partial resistance (such as a strain with an MIC close to the tested concentration) or false resistance (for example, due to the breakdown of an anti-TB agent during the 3–4 weeks of incubation). Staff must interpret the presence of micro-colonies in light of past experiences at the laboratory. For example, if a laboratory usually finds that the bacteria from a micro-colony on a plate containing an anti-TB agent can grow when plated on fresh medium containing the agent, the micro-colonies most likely arose from resistant bacteria. On the other hand, if such bacteria usually fail to grow when plated on fresh medium containing the anti-TB agent, the micro-colonies most likely arose from susceptible bacteria. Medium that is stored for a long time tend to produce more micro-colonies. Quantify and report growth as shown in Table 9.

Table 9. Quantifying and reporting the growth of bacteria on culture plates

Number of colonies	Report
0–50	Actual count
50–100	1+ (use the actual count)
100–200	2+ (use the approximate count)
200–500	3+
Confluent growth	4+

2.3.8 Interpreting and reporting results

Examine the growth on the control medium.

- If fewer than 50 colonies are present on the control medium inoculated with the 10^{-2} dilution, the test must be repeated owing to insufficient inoculation. This is because the number of colonies on a plate forms a Poisson distribution reflecting the number of colony-forming units per millilitre in the dilution. As such, isolates may appear to be falsely susceptible due to a lack of reliability in detecting low proportions of resistant bacteria in a sample of fewer than 50 bacteria. However, if colonies are present on one or more of the quadrants containing the anti-TB agent, the results should be reported to the clinician while the test is being repeated because false resistance is unlikely to occur due to a sampling error.
- If growth on the control medium inoculated with the 10^{-4} dilution is 3–4+, it represents over inoculation of the medium; care must be taken in interpreting this result because it may represent the appearance of naturally resistant colonies on the medium containing the anti-TB agent (particularly on the medium inoculated with the 10^{-2} dilution). The presence of a large number of bacteria can reduce the effective concentration of an anti-TB agent through the mechanisms of absorption or degradation to a point where susceptible bacteria can grow, and this may result in false resistance. In such cases DST should be repeated with more carefully prepared fresh inoculum. On the other hand, if there is no growth on the medium containing the anti-TB agent, then the isolate is considered susceptible and the result can be reported.
- If necessary, the number of colonies on the control medium inoculated with the 10^{-4} dilution can be used to calculate the number of colonies on the medium without the anti-TB agent that was inoculated with the 10^{-2} dilution (Table 9).

- Calculate the proportion of resistant colonies and express this as a percentage following the procedure below.

$$\frac{\text{Number of colonies on the medium containing the anti – TB agent}}{\text{Number of colonies on the control medium}} \times 100$$

Note: the calculations shown above are for the number of colonies on the control medium and the medium containing the anti-TB agent that have been inoculated with the same dilution. If different dilutions are used to inoculate the control medium and the medium containing the anti-TB agent then apply the dilution factor to the calculation. For example, if there are 80 colonies on the medium containing the anti-TB agent that was inoculated with the 10⁻² dilution and if there are 75 colonies on the control medium that was inoculated with the 10⁻⁴ dilution, then multiply 75 by 100 (this gives the number of colonies expected for the 10⁻² dilution).

A result should be interpreted as resistant when the proportion of the growth on the medium containing the anti-TB agent to the growth on the control medium is 1% or greater. If resistance is less than 1%, then the strain of the organism is considered susceptible. Results should be reported as resistant or susceptible. Reports may include the proportion of resistant colonies.

Precautions: The lids should be secured to the plates, and the plates should be sealed in CO₂-permeable bags. Make sure that there is no water condensate because it may leak through the plastic bag and become a biosafety hazard.

2.3.9 Quality control

Quality control is an essential part of DST. The best practice is to run a quality control strain with each batch being tested. *M. tuberculosis* H37Rv can be used for quality control because it is susceptible to all anti-TB agents. It is further recommended to include testing a well characterised resistant strain in each batch even that may allow for the detection of major errors in the preparation of drug stock solutions. See Annex C for details of quality control testing and validation.

3 Susceptibility testing for anti-TB agents using liquid media

The procedures described in this manual are for use with the automated BACTEC MGIT 960 system. This manual complements the MGIT manual published by the Foundation for Innovative New Diagnostics which is available at:

https://www.finddx.org/wp-content/uploads/2023/02/20061101_rep_mgit_manual_FV_EN.pdf

3.1 Principles

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

In addition to the Middlebrook 7H9 liquid medium, the MGIT tube contains an oxygen-quenched fluorochrome – tris (4,7-diphenyl-1,10-phenanthroline) ruthenium chloride pentahydrate – embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced by CO₂. The depletion of free oxygen results in fluorescence of the sensor within the MGIT tube when visualized under ultraviolet light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion. The BACTEC MGIT 960 instrument automatically detects this fluorescence.

Susceptibility testing for second-line agents can be performed based on the same principle as that of first-line agents. Two MGIT tubes are inoculated with the test culture: one with a known concentration of the anti-TB agent added and one MGIT without the anti-TB agent (growth control tube). To assess the 1% criteria, the inoculum of the control tube is diluted 100-fold compared to the drug containing tube. After incubation growth in the MGIT containing the anti-TB drug is compared to the control MGIT. If the test agent is active against the mycobacteria, it will inhibit the growth and, thus, there will be suppression of fluorescence while growth in the control tube will be uninhibited and fluorescence will increase.

Growth is monitored each hour by the MGIT 960 instrument, and is recorded in values known as Growth Units (GU). To assess the 1% criteria, the inoculum of the control is diluted 100-fold compared with the inoculum for the tube containing the anti-TB agent. In the case of first-line agents, the instrument automatically interprets the difference in GU values between the control tubes and the tubes containing the agent; results are reported as susceptible or resistant (provided the carrier system is used). If the EpiCenter TB eXiST module is used interpretation will be manual. GU values are available for second-line or newer agents but the user must interpret the values manually as no carrier system is available yet for these agents.

For manual interpretation growth in the tubes containing the anti-TB agent is compared when growth in the control tube reaches a predetermined threshold of 400 GU. Susceptibility is defined if the drug-containing tube is less than 100 GU, and resistance if at least 100 GU.

The protocol for the BACTEC MGIT 960 susceptibility test usually takes 4–13 days for both first-line and second-line agents. In some instances, it may be necessary to extend the incubation period for some drug-resistant strains that may take longer than 14 days to grow. PZA uses a 21 days protocol and a 1:10 growth control (GC) is used for this drug.

3.2 Preparing the medium

3.2.1 Medium

The medium used with the BACTEC MGIT 960 system is modified Middlebrook 7H9 broth supplemented, prior to inoculation, with a growth supplement known as OADC (consisting of oleic acid, albumin, dextrose and catalase) available from the manufacturer.

The MGIT medium is complete only after the growth supplement has been added to the medium. If the Becton Dickson SIRE supplement is not available, then the BBL OADC growth supplement may be used (catalogue number: 245116) in the same volume as the SIRE supplement (i.e. 500 µl). For pyrazinamide DST, use pyrazinamide (PZA) DST supplement.

For DST, one tube is needed as the control (without anti-TB agent); for each agent and concentration to be tested, an additional tube is needed. Specific DST carriers are available for first-line drugs to place the tubes in the instrument. These are available in different sizes and use is based on the number of drugs that are being tested for DST. Alternatively, the TB eXiST module can be used and negates the need for the carrier system, with tubes pre-labelled for the drug to be tested (number of drugs tested is not limited) and placed individually in the BACTEC MGIT 960 instrument.

3.3 Anti-TB agents and critical concentrations for testing

The concentrations of antimicrobial agents used in MGIT DST have been reviewed and validated (See Table 1 and 2) (8, 35). In addition, revised critical concentrations for rifampicin is presented.

3.3.1 First-line DST

Materials for first-line DST

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

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

Reconstitute each BACTEC SIRE kit drug with 4 mL of sterile distilled water, or 8 mL DMSO for rifampicin, to achieve the desired stock concentration (Table 9).

BACTEC MGIT 960 PZA kit (cat No. 245128BD Cat No. GDF 106033) contains two lyophilised vials of pyrazinamide and six vials of PZA supplement. Reconstitute each BACTEC MGIT PZA kit drug with 2.5 mL of sterile distilled water to achieve the desired stock concentration (Table 10). The stock solutions

can be aliquoted, depending on the number of tests as per the laboratory's workflow, and stored at -20°C for up to six months or -70°C for up to one year.

Table 10. Reconstitution volumes and final concentrations for first-line anti-TB agents

Drug lyophilised	Volume added	Concentration of reconstituted agent (µg/mL)	Volume added to each MGIT tube	Final concentration in MGIT tubes
Isoniazid	4 mL	8.3	100 µl	0.1 µg/mL
Rifampicin	8 mL	4.15	100 µl	0.5 µg/mL
Ethambutol	4 mL	415	100 µl	5.0 µg/mL
Pyrazinamide	2.5 mL	8000	100 µl	100 µg/mL

3.3.2 Second-line DST

The critical concentrations determined for certain second-line agents in MGIT media and the procedures used to prepare media containing these antimicrobials are given in Table 11. Only those second-line agents with established critical concentrations with reliable and reproducible results should be tested.

Critical concentrations for the anti-TB agents bedaquiline, cycloserine, delamanid, linezolid, clofazimine, pretomanid are included.

3.4 Preparing solutions of anti-TB agents

3.4.1 Using lyophilised agents

A limited number of lyophilised anti-TB agents are available from the manufacturer. However, when these become available, the procedures given by the manufacturer should be followed, but it is important to ensure that the final test concentrations are those recommended by WHO (Table 11). The stock solutions can be aliquoted, depending on the number of tests as per the laboratory's workflow, and stored at -20°C for up to six months or -70°C for up to one year.

Table 11. Reconstitution volumes and final concentrations for lyophilised second-line anti-TB agents available from BD

Drug lyophilised	BD catalogue No.	GDF catalogue No.	µg/vial	Volume added	Concentration of reconstituted agent µg/mL	Volume added to each MGIT tube	Final concentration in MGIT tubes
Moxifloxacin	215404	106585	249	3 mL Dilute 1:4	83 20.75	100 100	1.0 µg/mL (CB) 0.25 µg/mL (CC)
Bedaquiline	215406	TBA	170	2 mL	83	100	1.0 µg/mL
Amikacin	215350	106586	332	4ml	83	100	1.0 µg/mL
Streptomycin ^a	245123	106028	332	4ml	83	100	1.0 µg/mL

^a Streptomycin catalogue numbers refer to the full SIRE kit
TBA to be announced.

3.4.2 Using pure drug powders

Table 11 provides an overview of manufacturers providing the pure powders. In order to prepare and sterilize anti-TB agent solutions make the needed dilutions in such a way that when 100 µl is added to 7 mL of MGIT medium, the desired test concentration is achieved based on the volume of the medium. Guidelines on preparing the recommended critical concentrations are shown in Table 12.

Table 12. Concentrations and solutions needed to prepare second-line anti-TB agents for use with the BACTEC MGIT 960 system

Final concentration of anti-TB agent (µg/mL)	Stock solution and solvent	Subsequent dilutions in sterile distilled water (SDW) or DMSO as specified
Levofloxacin (1.0)	Dissolve 10 mg LFX in 2.5 mL sterile 0.1M NaOH ^a . – 4000 µg/mL (Solution A) Dilute 1 mL solution A to a final volume of 5mL with SDW (Solution B) 800 µg/mL	Add 1.05 mL of solution C to 8.95 mL of SDW ^b . 84 µg/mL (Working solution)
Moxifloxacin (0.25) Critical concentration	Dissolve 10 mg MFX in 5 mL sterile 0.1 MM NaOH ^a . (2000 µg /mL (Solution A) Dilute 1 mL solution A to a final volume of 10 mL with SDW (Solution B) 200 µg/mL	Add 1.05 mL of solution B to 8.95 mL of SDW ^b . 21 µg/mL (Working solution)
Moxifloxacin (1.0) Clinical breakpoint	Dissolve 10 mg MFX in 2.5 mL sterile 0.1M NaOH ^a . 4000 µg /mL (Solution A) Dilute 1 mL solution A to a final volume of 5 mL with SDW (Solution B) 800 µg/mL	Add 1.05 mL of solution B to 8.95 mL of SDW ^b . 84 µg/mL (Working solution)
Bedaquiline ^c (1.0)	Dissolve 12 mg BDQ fumarate salts (equivalent to 10 mg BDQ base) in 2.5 mL sterile DMSO. 4000 µg /mL (Solution A) Dilute 1 mL solution A to final volume of 5 mL with DMSO (Solution B) 800 µg/mL	Add 1.05 mL of solution B to 8.95 mL of DMSO ^d . 84 µg/mL (Working solution)
Linezolid (1.0)	Dissolve 10 mg LZD in 10 mL DMSO. 1000 µg /mL (Solution A) Dilute 4 mL of Solution A to a final volume of 5 mL with DMSO. 800 µg/mL (Solution B)	Add 1.05 mL of solution B to 8.95 mL of DMSO. 84 µg/mL (Working solution)

Clofazimine (1.0) ^e	Dissolve 10 mg clofazimine in 2.5 mL sterile DMSO (Solution A - 4000 µg/mL) Dilute 1 mL Solution A to a final volume of 5 mL with DMSO – 800 µg/mL (Solution B)	Add 1.05 mL of solution B to 8.95 mL of DMSO^d 84 µg/mL (Working solution)
Delamanid (0.06)	Dissolve 10 mg DLM in 2.5 mL sterile DMSO. Dilute with DMSO to final volume of 10ml – 1,000 µg/mL (Solution A) Dilute 1 mL solution A with 9 mL of DMSO (Solution B) 100 µg /mL	Add 0.5 mL of solution B to 9.5 mL of DMSO 5 µg/mL (Working solution)
Amikacin (1.0)	Assume potency of 0.71 (varies between lots) Dissolve 140 mg amikacin in 10 mL sterile distilled water - 10,000 µg/mL (Solution A). Dilute 1 mL solution A with 9 mL of sterile distilled water (Solution B) 1,000 µg/mL Dilute 4 mL Solution B to a final volume of 5 mL with SDW (Solution C) 800 µg/mL	Add 1.05 mL of solution B to 8.95 mL of SDW. 84 µg/mL (Working solution)
Streptomycin (1.0)	Assume potency of 0.71 (varies between lots) Dissolve 140 mg streptomycin in 10ml sterile distilled water -10,000 µg/mL (Solution A). Dilute 1 mL solution A with 9 mL of sterile distilled water (Solution B) 1,000 µg/mL Dilute 4 mL Solution B to a final volume of 5 mL with SDW (Solution C) 800 µg/mL	Add 1.05 mL of solution B to 8.95 mL of SDW. 84 µg/mL (Working solution)
Cycloserine (16.0)	Dissolve 20 mg DCS in 10 mL sterile water (Solution A)	Add 6.7 mL of Solution A to 3.3 mL of SDW 1344 µg/mL (Working solution)

NaOH, sodium hydroxide.

^a For 1M NaOH: dissolve 40 g NaOH in 1 litre of distilled or deionized water (or dissolve 4.0 g in 10 mL); dilute 1:10 to achieve 0.1M strength.

^b Use sterile distilled water (SDW).

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

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

^e Further research and evidence is warranted to investigate the use of polystyrene or glass material for preparation.

Note: The preparation scheme shown in Table 12 may be modified as needed. However, the final concentration of each anti-TB agent should not be altered. The stock solutions can be aliquoted, depending on the number of tests as per the laboratory's workflow, and stored at -20°C for up to six months or -70°C for up to one year.

For details about weighing anti-TB agents, calculating their potency and storing the solutions, see Annex D.

3.5 Calculating the dilution factor for the MGIT medium

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

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

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

3.6 Adding an anti-TB agent to the medium

To prepare a set of MGIT tubes for DST, take the required number of MGIT tubes and label each tube with the relevant information that will identify the name and concentration of the antimicrobial agent being tested, and the patient. Do not add the anti-TB agent solution to the growth control tube. Aseptically add 0.1 mL (100 µl) of the properly dissolved and diluted solution of the agent being tested to each labelled MGIT tube. It is important to always add the test solution 0.1 mL (100 µl) at a time, and to add the correct amount of each test solution to each tube. Any slight variation in the quantity added to the tubes may affect the results.

Use a calibrated micropipette to make each addition to the tubes. The same micropipette tip may be used to add the same test solution to several MGIT tubes, but a separate pipette or micropipette tip should be used for each anti-TB agent being tested. After adding the solution, homogenise the sample by gently inverting several times.

3.7 Preparing the mycobacterial inoculum

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

Rapid tests, both molecular and antigen-based, can be used for definitive differentiation of *M. tuberculosis* from nontuberculous mycobacteria (36). It is important to establish the purity of the culture before performing DST, particularly if contamination is suspected.

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

3.7.1 Using inoculum from the MGIT tube

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

The day that an MGIT tube is positive as determined by the instrument is considered Day 0. Growth is not yet ready for DST.

The tube should be incubated for at least 1 more day before being used for DST (it may be incubated in a different incubator at $37\text{ °C} \pm 1\text{ °C}$). Day 1 starts after the tube has been incubated for 1 day.

A positive tube may be used for DST up to and including the fifth day after incubation (that is, on day 1, 2, 3, 4 or 5). A tube that has been incubated for more than 5 days should be sub-cultured in a fresh MGIT tube that has been supplemented with MGIT 960 growth supplement; it should then be incubated in the instrument until it is positive. This tube may be used from day 1 until day 5 as described above.

If growth occurs in a tube on day 1 or day 2, mix the tube well by shaking vigorously or vortexing for 2–3 minutes to break up any clumps. Leave the tube undisturbed for about 5–10 minutes to let larger clumps settle to the bottom. Carefully collect the supernatant (using a sterile transfer pipette placed just below the liquid surface) in a clean sterile container and use undiluted to inoculate the tubes with medium containing the anti-TB agent.

If growth occurs on day 3, 4 or 5, mix the tube well to break up the clumps, either by hand or with a vortex mixer.

Leave the tube undisturbed for 5–10 minutes to let the larger clumps settle to the bottom.

NOTE: For pyrazinamide leave the tube undisturbed for 15-20 minutes.

Transfer (using a sterile transfer pipette placed just below the liquid surface) 1.0 mL of the supernatant to a clean sterile container containing 4.0 mL of sterile saline and mix contents well. Use this 1:5 dilution of the test culture to inoculate the test medium in tubes containing the anti-TB agent.

3.7.2 Using inoculum from growth on solid media

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

Add 4 mL of sterile saline (0.85% sodium chloride) to a short glass bottle with capacity of 15ml with 2–3 mm glass beads (about 8–10 beads).

Obtain a representative portion of growth by scraping off as many colonies as possible. Be careful not to scrape off any medium because residual medium will give false turbidity readings.

Transfer the growth to the tube with the normal saline and glass beads. Tighten the cap and vortex the tube for 1–2 minutes to break up any clumps. If a vortex is not available, mix thoroughly by hand

to homogenize the suspension. The turbidity of the suspension should be greater than the McFarland number 1.0 standard. See Annex B for information on the McFarland turbidity standards.

Let the suspension stand undisturbed for 30 minutes to allow large clumps of bacteria to settle.

Use a pipette to carefully transfer (aspirate liquid carefully from just below the liquid surface) the supernatant suspension to another sterile tube. Avoid transferring any growth that has settled to the bottom.

Let this tube stand undisturbed for 15 minutes.

Use a pipette to carefully remove the supernatant without disturbing the sediment; transfer the supernatant to another sterile tube. The turbidity of this suspension should be higher than the McFarland 0.5 turbidity standard. See Annex B for information on the McFarland turbidity standards.

Adjust the turbidity of this suspension to the McFarland 0.5 standard by adding sterile saline and adjust by making a visual comparison. Do not reduce the turbidity below this standard. (See Annex B for information on preparing the McFarland standards.)

Dilute the above suspension to 1:5 by adding 1.0 mL of the suspension to 4.0 mL of sterile saline. Mix well and use this as the inoculum for tubes with medium containing an anti-TB agent.

Precautions

Make sure that the culture being tested is pure and free from mycobacterial or bacterial contamination. If there is any doubt, check by making an AFB smear and streaking a loop of the culture on a bacterial medium plate, such as blood agar. Mixed cultures of *M. tuberculosis* and other mycobacteria or contaminating bacteria will give either an X error or a false result showing resistance.

Always use fresh cultures. Old cultures may have low viability and may give false susceptibility results or other erroneous results.

The culture suspension should be well dispersed and homogeneous. Inoculum with large clumps may give erroneous results.

Over-inoculation may give false resistance results or an X400 error.

Insufficient inoculum may give false susceptibility results or an X200 error.

3.7.3 Inoculation and incubation

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

3.7.4 Adding to and inoculating the MGIT medium

The procedure should be performed in the following manner.

Aseptically add 0.8 mL of BACTEC 960 SIRE supplement or BBL OADC supplement to each of the MGIT tubes. Mix well.

Aseptically add 0.1 mL (100µl) of the anti-TB agent being tested to the appropriately labelled MGIT tube. Mix well.

The last step should be the inoculation of the test culture. Aseptically add 0.5 mL of the well mixed homogenized suspension (inoculum) to each of the tubes containing the anti-TB agent. Do not add inoculum to the control tube.

For the control, first dilute the test culture suspension to 1:100 by adding 0.1 mL of the test culture suspension to 10.0 mL of sterile saline.

For PZA DST a 1:10 growth control is prepared by adding 0.5 mL of the test culture suspension to 4.5 mL of sterile saline.

Mix well by inverting the tube 5–6 times or by using a vortex mixer. Add 0.5 mL of this diluted suspension to the control tube.

If it is difficult to measure 0.1 mL accurately, make two 10-fold dilutions by taking 0.5 mL of the suspension and adding it to 4.5 mL of sterile saline. Each 10-fold dilution should be mixed thoroughly.

Tighten the caps on all tubes, and mix the inoculated broth well by gently inverting the tube several times.

3.8 DST set carriers

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

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

- 2 tubes: this allows testing of a single concentration of one agent; it comprises a tube for the anti-TB agent and a control tube.
- 3 tubes: this set allows testing of two agents or two different concentrations of the same agent along with a control tube.
- 4 tubes: this set allows testing of three agents or three different concentrations of the same agent along with a control tube.
- 5 tubes: this set allows testing of four agents or four different concentrations of the same agent along with a control tube. E.g. used for first line DST (SIRE)
- 8 tubes: this set allows testing of seven agents or seven different concentrations of the same agent along with a control tube.

3.8.1 Entering the DST set carrier into the MGIT instrument

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

To run the susceptibility test, the barcode on the carrier is scanned and then the carrier is placed into the instrument using the entry feature for the susceptibility test. (See the user's manual for the

instrument and the DST instructions for further details.) Ensure that the tubes are placed in the correct order in the carrier, following the manufacturer's instructions.

The software is programmed to interpret results for only those set carriers that have been previously entered into the instrument. Default settings normally utilized for testing of first-line anti-TB agents do not apply when testing second-line agents. However, there is an option to enter a carrier as containing an unknown agent or "Unspecified Drug". In this case, the instrument will monitor the growth and GU values of all the tubes in a set. Once the GU of the control reaches 400 GU or more, the instrument terminates the test and flags it as completed but does not interpret the results. The results will need to be interpreted manually.

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

Precautions

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

Duration of the test

When the DST set carrier is entered as an unknown agent, the instrument continuously monitors the test set. The protocol terminates once the control reaches 400 GU or more (within 4–13 days). Once the test has been completed, the instrument indicates that results are ready. The carrier is then removed, its barcode is scanned, and the report is printed. The report will show GU values for all of the tubes in the set but will not have interpretations for the results.

3.9 Interpreting and reporting results

When the DST carrier set is loaded into the BACTEC MGIT, the instrument continuously monitors the test set. The protocol terminates once the Growth Control reaches 400 GU or more (within 4-13 days). Once the test has been completed, the instrument indicates that results are ready. The carrier is then removed, its barcode scanned, and report printed. The report will show GU values for all the tubes in the set but will not have interpretations for the results. Results are interpreted as described below.

- **Resistant:** a strain is described as resistant when the GU value of the control reaches 400 or more and the GU value of the tube with the agent being tested is 100 or more.
- **Susceptible:** a strain is described as susceptible when the GU value of the control reaches 400 or more and the GU value of the tube containing the agent being tested is less than 100 (Usually no GUs are observed for the drug containing tubes). We recommend repeat testing if any GU are observed for the new and repurposed drugs.
- **X errors:** the X error message indicates that results are indeterminate when certain conditions occur that may affect the test such as:

X400 error: this error occurs when the GU of the control reaches 400 or greater in less than 4 days. If this occurs, the instrument terminates testing. This error indicates that the test culture is contaminated or has been overinoculated. In such situations, the test should be repeated with pure, actively growing and properly diluted culture that has been confirmed to be *M. tuberculosis* complex;

X200 error: this error occurs if the inoculum contains only a low number of viable organisms. In this case, the control will not have reached the required number of GU within 13 days; this indicates that the culture has been insufficiently inoculated. Since the duration of the protocol is set for 13 days, the instrument will terminate the test at that time. Ensure that fresh, actively growing culture is used and that the suspension has been carefully prepared.

3.10 BD EpiCenter™ TB eXiST module

The BD EpiCenter™ microbiology data management system TB eXiST module (Becton, Dickinson and Company, Sparks, USA) provides advanced data management for BD microbiology systems, which includes the BACTEC MGIT automated mycobacterial detection system for susceptibility testing. This system negates the need for carriers and has the flexibility of testing multiple drugs simultaneously using a single growth control tube. The module may be useful for DST, including aiding in interpreting results and providing guidance on increasing the incubation period if necessary.

3.11 Important considerations

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

Certain resistant strains grow very slowly in the MGIT medium, and the results may not be achieved within 13 days using standard inoculum. In such cases, in order to achieve reportable results, the amount of inoculum used should be increased by decreasing the dilution of the culture suspension (for example, by using without dilution MGIT tubes that are positive on day 2 or day 3, or by using tubes that are positive on day 4 or day 5 with a 1:2 dilution).

When the interpretation is resistance, check the medium visually, and make sure that the test culture is not contaminated (look for turbidity, then put 1 drop of the medium on a blood agar plate or a nutrient agar plate). If the test culture is non-tuberculous mycobacteria or is *M. tuberculosis* but it is mixed or contaminated with non-tuberculous mycobacteria or other microorganisms, then it may falsely be reported as resistant. Prepare a Ziehl–Neelsen smear and look for the clumps and cord formation typical of the TB complex.

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

Results must be reported as soon as they are available. When results are reported, it is important to include the method used, the agent tested and its concentration. In general, results are reported as

“S” (susceptible) or “R” (resistant). However, in some situations, such as when testing moxifloxacin, an agent is tested at different concentrations to establish the level of resistance. In such cases susceptibility may be reported at different test concentrations with the interpretation of S and a low level or high level of resistance.

3.12 Quality control

It is important to perform quality control of DST. The best practice is to run a quality control strain with each batch being tested. *M. tuberculosis* H37Rv can be used for quality control because it is susceptible to all anti-TB agents. It is further recommended to include testing a well characterised resistant strain in each batch even that may allow for the detection of major errors in the preparation of drug stock solutions. See Annex C for details of quality control testing and validation.

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Annexes

Annex A. Storing stock cultures.

All clinical isolates and reference strains of mycobacteria used for quality control should be stored in conditions that preserve their viability and specific strain characteristics. To avoid serial sub-culturing, which could lead to genetic drift and alter the phenotypic characteristics of the strains, it is advisable to store single-use aliquots of cultures in cryovials at -70 °C; these may be stored for 10–12 years or longer. The viability of tubercle bacilli decreases much quicker at -20 °C than at -70 °C. It is also important to note that a heavy bacterial suspension supports viability for longer. To revive frozen cultures, especially those that have been stored for a long time, it is important to subculture in a very rich medium. Generally, liquid medium supports revival better than solid medium. Suspensions of strains used for quality control should be stored at -70 °C and used within 1 year. After 1 year, viability may decrease.

1. Equipment and materials required for storing stock cultures

The following equipment is needed for storing stock cultures:

- Cryovials with screw caps (that is, with an external screw on the body of the tube);
- A freezer that can maintain temperatures of at least -20 °C, or -60 °C to -70°C; the preferred temperature for storage is -70°C;
- One of the following suspension media:
 - a. OADC-enriched Middlebrook 7H9 broth, prepared according to the manufacturer's instructions;
 - b. 10% skim milk, autoclaved at 121 °C for 10 minutes;
 - c. 5% glycerol in 0.85% sodium chloride solution, autoclaved at 121°C for 10 minutes.

2. Procedure

Use only cultures that have been freshly grown on solid medium and or liquid media. If the culture is older than 2 weeks subculture on a fresh slant, and incubate at 37 °C. As soon as good growth appears, use this culture for making the suspension.

Scrape as many colonies as possible from the solid medium, and make a homogeneous suspension using one of the media mentioned above. Aseptically transfer approximately 1.5 mL into several 2 mL cryovials. If cryovials are not available then 1.5 mL Eppendorf tubes may be used.

A freshly grown MGIT culture can also be used transferred to cryotubes for frozen storage. Tighten the caps, label the tubes with the preparation date and culture information. Transfer the prepared cryovials to a suitable storage container (e.g. cryobox) which is then transferred to the freezer for long-term storage.

Keep a record of the cultures made and the dates they were frozen.

Annex B. McFarland Turbidity Standards

Already prepared McFarland Turbidity Standards may be commercially available.

If commercial standards are not available, follow the procedure described below.

- Prepare a 1% solution of chemically pure sulphuric acid in water by slowly adding 1.0 mL sulphuric acid to 99.0 mL water. CAUTION: Always add sulphuric acid to water.
- Prepare a 1% solution of chemically pure barium chloride in water by adding 1 g barium chloride to 100 mL of water.
- Mix 1.0 mL of the barium chloride solution with 99.0 mL of the sulphuric acid solution in a clean, properly washed flask. Mix well, and transfer to 1 or 2 clean tubes.
- Seal the tubes with tape or wax. This tube is equal to the McFarland number 1 standard.
- Add 0.5 mL of barium chloride solution to 99.5 mL of sulphuric acid, or add 5 mL water to 5 mL of a well-mixed suspension of the McFarland number 1 standard. Seal the tube. This will yield the McFarland 0.5 standard.
- Accurate measurements are critical to achieving workable McFarland standards. When comparing the turbidity of a culture suspension to a McFarland standard, ensure both the McFarland standard and the test suspension have been shaken well. Use the same type and size of tube for both the test and the McFarland standard.

Annex Table 1 gives information about the different amounts of barium chloride (BaCl_2) and sulphuric acid (H_2SO_4) needed to achieve McFarland Turbidity Standards for use with bacteria other than mycobacteria and their correlation with the number of bacteria per millilitre. The number of mycobacteria (that is, the number of colony-forming units) will differ for *M. tuberculosis* since *M. tuberculosis* forms clumps, and turbidity is affected by the size and shape of the bacteria.

Annex Table 1. Amounts of barium chloride and sulphuric acid needed to achieve different McFarland Turbidity Standards, and their correlation to the number of Escherichia coli per millilitre of suspension ³³

McFarland Turbidity Standard number	Volume (mL)		Number of bacteria/mL (x 10 ⁸)
	BaCl ₂ (1% concentration)	H ₂ SO ₄ (1% concentration)	
0.5	0.5	99.5	1.5
1	1.0	99.0	3
2	2.0	98.0	6
3	3.0	97.0	9
4	4.0	96.0	12
5	5.0	95.0	15

³³ Global Laboratory Initiative. GLI standard operating procedures on Preparation of reagents for culture and drug-susceptibility testing. Geneva, 2017.

Annex C. Quality control testing

Quality control strains: The table below provides characterized strains that are recommended to be used.

If the American Type Culture Collection (ATCC) or Belgian Coordinated Collections of Microorganisms/Institute for Tropical Medicine (BCCM/ITM) reference strains cannot be obtained, well characterized strains by both phenotypically and genotypically derived from a patient's isolate can be used instead. It is preferable to use a strain that has been fully sequenced and shown to have a wild type pattern for all known genes associated with TB drug resistance or mutations classified by WHO as associated with resistance (WHO mutations catalogue – reference).

Annex Table 2. Recommended quality control strains (37)

Drug grouping	Drug	ATCC	BCCM/ITM	Lineage	Resistance mechanism ^b
	Susceptible H37Rv strain	27294 ^a	500735	4	none
First-line	Rifampicin-R	35838 ^a		4	<i>rpoB</i> S450L ^{c,d}
	Isoniazid low-level-R	BAA-812 ^a		not known	<i>inhA</i> C-15T ^e
	Isoniazid high-level-R	35822 ^a		4	complete deletion ^{c,d,f} <i>katG</i>
	Ethambutol-R	35837 ^a		4	not known
	Pyrazinamide-R	35828 ^a		4	<i>pncA</i> G132S ^c
Group A	Fluoroquinolone high-level-R		500831 ^g	2	<i>gyrA</i> D94G ^h
	Bedaquiline-R		500807	4	<i>atpE</i> A63P
	Linezolid-R		501291 ^g	4	<i>rplC</i> C154R
Group B	Clofazimine-R		500861 ^g	1	<i>Rv0678</i> Y92Stop ⁱ
	Cycloserine-R		501136 ^g	4	<i>alr</i> D320N ^j
Group C	Delamanid-R/		501095	2	<i>ddn</i> Q58Stop ^k
	Carbapenems-R	No DST method exists			
	Amikacin-R		501330 ^g	4	<i>rrs</i> A1401G
	Streptomycin-R	35820 ^g		4	<i>rpsL</i> K43R ^d
	Ethionamide/prothionamide-R	BAA-812 ^g		not known	<i>inhA</i> C-15T ^e
	Para-aminosalicylic acid-R	DST not recommended			
Other	Pretomanid-R		501095	2	<i>ddn</i> Q58Stop ^k

ATCC, American Type Culture Collection; BCCM/ITM: Belgian Coordinated Collections of Microorganisms/Institute for Tropical Medicine.

^a Clinical and Laboratory Standards Institute. Performance standards for susceptibility testing of mycobacteria, *Nocardia* spp., and other aerobic actinomycetes. 1st ed. CLSI supplement M62.

^b Unless otherwise stated, the resistant control strains should test reliably resistant at the relevant critical concentration as the mutation in question confers a sufficiently large MIC increase with an associated MIC distribution that is not or not significantly divided by the critical concentration (i.e. the positive predictive value of testing should be high).

^c Ismail N, Omar SV, Ismail NA, et al. *In vitro* approaches for generation of *Mycobacterium tuberculosis* mutants resistant to bedaquiline, clofazimine or linezolid and identification of associated genetic variants. *J Microbiol Methods*. 2018;153:1-9. doi: 10.1016/j.mimet.2018.08.011.

^d Lee RE, Hurdle JG, Liu J, et al. Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux. *Nat Med*. 2014;20(2):152-158. doi: 10.1038/nm.3458.

^e Madison BM, Siddiqi SH, Heifets L, et al. Identification of a *Mycobacterium tuberculosis* strain with stable, low-level resistance to isoniazid. *J Clin Microbiol*. 2004;42(3):1294-5. doi: 10.1128/JCM.42.3.1294-1295.2004.

^f The complete deletion of *katG* was confirmed by Ismail et al. using whole genome sequencing, even though Lee et al. reported *katG* S315T as the isoniazid resistance mechanism.

^g Tentative control strain.

^h This high-level resistant mutant should test reliably test resistant at the critical concentrations for levofloxacin and moxifloxacin but not at the clinical breakpoint for moxifloxacin (World Health Organization. Optimized broth microdilution plate methodology for drug susceptibility testing of *Mycobacterium tuberculosis* complex).

ⁱ Phenotypic clofazimine DST is challenging and *Rv0678* mutants in general, including this mutant, are known not to test reliably resistant. The reasons are not understood fully (e.g. whether the critical concentration is not set optimally, the technical variability for clofazimine is inherently larger than other drugs or whether *Rv0678* confer smaller relative MIC increases for clofazimine than for bedaquiline).

^j Using *alr* start from Strych et al. (Characterization of the alanine racemases from two mycobacteria. *FEMS Microbiol Lett*. 2001;196(2):93-8 doi: 10.1111/j.1574-6968.2001.tb10547.x) that WHO considers to be correct. Corresponds to *alr* D344N using current H37Rv annotation (GenBank AL123456.3).

^k Bateson A, Canseco JO, McHugh TD, et al. Ancient and recent differences in the intrinsic susceptibility of *Mycobacterium tuberculosis* complex to pretomanid. *J Antimicrob Chemother*. 2022;77(6):1685-1693. doi: 10.1093/jac/dkac070.

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

4.3.1. Preparing the culture suspension

Subculture the reference strain on several Löwenstein–Jensen slants.

Incubate the slants at 37 °C ± 1 °C.

Observe growth visually. Growth should appear within 10–15 days of sub-culturing. As soon as there is good, confluent and pure growth, use it to make the suspension. The growth should be used as soon as possible (that is, within the 10–15 days). Older cultures do not give reliable results.

Remove growth from the slant by carefully scraping the colonies from it with a sterile loop or a sterile spatula made from wooden applicator sticks. Be extremely careful not to scrape off any culture medium, which will give false measurements of turbidity.

Transfer the growth into **Tube A**, a sterile glass tube with a screw cap that contains 4 mL of sterile Middlebrook 7H9 broth and 1–2 mm glass beads (about 6–10 beads); the glass beads will help to break up any clumps. Sterile normal saline or sterile deionized water may also be used.

Vortex the tube for 1–2 minutes. Make sure the suspension is well dispersed and has turbidity greater than the McFarland number 1 standard.

Let the suspension stand undisturbed for 30 minutes.

Using a pipette, carefully transfer the supernatant to **Tube B**, another sterile glass tube with a screw cap. Avoid transferring any sediment. This suspension should have a higher turbidity than the McFarland number 1 standard.

Let **Tube B** stand undisturbed for 15 minutes.

Carefully transfer the supernatant from **Tube B** into **Tube C**, another sterile glass tube with a screw cap; do not transfer any sediment.

Visually adjust the turbidity of the suspension in **Tube C** to McFarland Turbidity Standard number 1.0 or 0.5, whichever is needed, by adding Middlebrook 7H9 broth, sterile saline or deionized water; mix well. If the suspension is too turbid, transfer some of the suspension to another sterile tube and adjust the turbidity. This will be the working suspension for quality control testing. This suspension may be frozen in small aliquots (1–2 mL) in appropriate tubes or vials at -70 °C ± 10 °C. The frozen suspension may be used for up to 1 year. Once thawed, it should not be refrozen.

4.3.2 Validating a test procedure

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

4.3.2.1 Procedure

Comparing a new method with an established method.

Select 10–15 cultures, one which should be H37Rv. Cultures should be selected from clinical isolates, some of which are completely susceptible to all anti-TB agents and some of which are known to be resistant to the second-line agents being tested. If cultures from the supranational regional laboratory used in the most recent round are available, use these instead of laboratory isolates because these cultures will have been tested by several laboratories, and judicial results will already have been established and be available.

If information on resistance to second-line agents is not available, select cultures from patients whose samples was resistant to isoniazid, rifampicin and perhaps other first-line and second-line agents.

Prepare fresh subcultures as described earlier.

Following the recommended standard techniques for DST, use the selected cultures to validate the testing of the second-line agents. Test the cultures using the new procedure as well as with the laboratory's established DST procedure for second-line agents.

The tests using the reference method and the new method should be set up at the same time using the same culture suspension.

The concentrations of the agents used in the tests should be those that are recommended for each procedure; test concentrations may vary according to which procedure is used.

Expected results

If you are comparing DST results from two methods and the results are found to be discordant, the test should be repeated using both methods simultaneously.

There should be more than 90% agreement (preferably 95% agreement) between the results obtained using the new method and results obtained using the established method (that is, the reference method). The quality control strain (H37Rv) should be tested along with each batch of samples, and should show complete susceptibility to all agents being tested.

If a laboratory does not have an established reference method, then validation may be performed by comparing results from the new test with results from previous DST obtained from other sources for the same agent. In this case, rigorous testing for reproducibility may be an option; the procedure for reproducibility testing is described in the next section.

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

4. 3.3 Reproducibility testing

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

4.3.3.1 Procedure

Select about 5–7 cultures, one of which should be H37Rv, 2–3 of which should be susceptible and 2–3 of which should be resistant to the agents being tested.

Set up susceptibility testing at least five times on all of the cultures.

Repeat testing should be done on different days.

The standard procedures outlined in this manual should be followed for DST for all cultures, that is, testing should be performed on all cultures in one batch under exactly the same conditions and using the same procedures for all tests.

Standardized frozen suspensions may be used for repeat testing but it is preferable to make a suspension of fresh culture each time so that the reproducibility of suspension preparation is also assessed. For DST using liquid media, fresh growth in MGIT medium should be used as described (see Section 3).

Expected results

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

Annex D. Preparing and storing solutions for anti-TB drugs

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

The potency value is usually supplied by the manufacturer and should be included in the certificate of analysis; analyses are usually performed with a high-performance liquid chromatography (HPLC) assay, and are based on water content (for example, as assessed by Karl Fischer analysis or by weight loss on drying) and the salt/counter-ion fraction (if the compound is supplied as a salt instead of free acid or base). The potency may be expressed as a percentage or in units of $\mu\text{g}/\text{mg}$ (w/w). If a certificate has not been included, try to obtain the necessary information from the manufacturer. If information about the potency is not available and it must be calculated, analyse the water content, and determine whether the agent is presented in salt form, such as a phosphate, sulphate or hydrochloride. These factors must be taken into account when determining the potency. It is always better to obtain the information from the manufacturer or supplier if the potency is not given or if any value is unknown or cannot be clearly determined from the certificate.

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

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

Calculations

To calculate the amount of powder needed, first find the specific activity of the agent; then, incorporate the activity factor into the total amount weighed.

For example, if the activity of an anti-TB agent were 740 $\mu\text{g}/\text{mg}$, the weighing factor would be:

$$1000 \mu\text{g} / 740 \mu\text{g} = 1.35.$$

Use one of the following formulae for weighing the quantity of an anti-TB agent in a given volume of diluent necessary to achieve the desired stock solution.

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration}(\mu\text{g/mL})}{\text{Potency}(\mu\text{g/mg})}$$

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

$50 \times 415 / 904 = 22.9$ mg of the agent.

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

In a 100 mL flask add 50 mL diluent.

Weigh 22.9 mg of the agent using an analytical balance.

Add the powder to the flask and mix well.

Suppose that for some reason you weigh out a different amount of an agent, say 23.2 mg. In this case you would need to determine the volume of diluent required to have 415 µg/mL, using the formula shown below.

$$\text{Volume (mL)} = \frac{\text{Weigh (mg)} \times \text{Potency (}\mu\text{g/mg)}}{\text{Concentration (}\mu\text{g/mL)}}$$

For 23.2 mg of an agent with a potency of 904 µg/mg that needs 415 µg/mL of a stock solution:

$23.2 \times 904 / 415 = 50.5$ ml diluent

For additional information, see *Susceptibility testing of mycobacteria, nocardiae, and aerobic actinomycetes: approved standard*.

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

Storing stock solutions of anti-TB agents

Small volumes of the sterile stock solutions of antimicrobials should be dispensed into sterile polypropylene or polyethylene and polystyrene for bedaquiline vials appropriate for low-temperature storage, such as cryovials; the vials should be carefully sealed, labelled, dated and stored. Solutions of anti-TB agents should be frozen, preferably at $-70\text{ }^{\circ}\text{C} \pm 10\text{ }^{\circ}\text{C}$. Freezing at $-20\text{ }^{\circ}\text{C}$ is also acceptable.

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

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

Important considerations

Store pure agents in a cool, dry place, preferably in a desiccator, and refrigerate to protect and from heat and light sources if indicated by the manufacturer.

Use only the pure form of the agents. Impurities will cause unreliable results.

Check the activity (potency) of an agent and calculate the total weight of the agent required by taking into account the actual active component of the agent. Calculations for the amount of an agent to be weighed may differ because the potency of agents varies from lot to lot.

Weigh the agent (at least 2000 µg) extremely carefully, using a reliable and calibrated high-quality analytic balance appropriate for weighing small quantities.

Mix the solution containing the agent extremely carefully and ensure that the agent is completely dissolved before filtering it or making further dilutions.

All of the agents should be filter sterilized. Use a polycarbonate filter to minimise loss of the agent caused by absorption to the filter. If polycarbonate filters are not available and another kind must be used, discard the first 10-15 % of the filtered solutions, and collect the remaining solution for use.

Keep track of the expiry date for each agent and solution. Do not use an agent after its expiry date.

Record the weights used, and the calculations and dilutions made.



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