ORGANISMO ANDINO DE SALUD - CONVENIO HIPÓLITO UNANUE

PROGRAMA "FORTALECIMIENTO DE LA RED DE LABORATORIOS DE TUBERCULOSIS EN LA REGIÓN DE LAS AMÉRICAS"

TECHNICAL GUIDE
FOR BACTERIOLOGICAL DIAGNOSIS OF TUBERCULOSIS

PART 3 Susceptibility Tests

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First Edition, April 2018

This publication is part of the execution of the Program “Strengthening the Network of Tuberculosis Laboratories in the Region of the Americas”, funded by the Global Fund to Fight AIDS, Tuberculosis and Malaria, which has the Agency as Principal Recipient Andean Health - Hipólito Unanue Agreement (ORAS - CONHU); and as Sub-recipients to the Executive Secretariat of the Council of Ministers of Health of Central America and the Dominican Republic (SE COMISCA) and to the Pan American Health Organization (PAHO / WHO).

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PREFACE

The emergence of resistance to drugs used to treat tuberculosis and, in particular, multiple drug resistance has become a major public health problem and, in some countries, an obstacle to disease control.

Drug-resistant tuberculosis can be prevented and controlled only if infectious patients are diagnosed and cured without delay. A patient with untreated or inappropriately treated resistant tuberculosis is a source of transmission of resistant bacillus which, in turn, generates an increase in treatment costs and mortality

In order to identify, treat adequately, know the situation and guide the programmatic management of drug resistant tuberculosis, the support of the network of tuberculosis laboratories is indispensable. This network is responsible for conducting rapid susceptibility testing, culture, identification and characterization of the complete *Mycobacterium* tuberculosis susceptibility profile. In order for the response to be adequate, the network must be able to perform susceptibility tests for all cases in which bacteriological confirmation is possible, ensuring reliable results in a timely manner

This update of the Guide is intended to facilitate the increase in the supply of the M. tuberculosis susceptibility test and the acceleration of its results, through the incorporation of working algorithms, the systematization of technical procedures and the innovation of microbiological methods, contemplating the required biosafety and quality. It is dedicated to laboratories that have previously ensured competence, quality and biosafety to perform smear microscopy and, in the case of conventional susceptibility tests also for culture. So this third part of the Guide of Bacteriology of Tuberculosis presents procedures that are additional to those described in Parts 1 (Smear microscopy) and 2 (Culture).

For the drafting of this Guide, the rules that preceded it, mainly those used in Latin America, have been taken into account and the technological development subsequently achieved, up to the moment of its drafting. In relation to controversial issues or recent developments, priority has been given to the consideration of published evidence. However, when literature was limited, the opinion of the Supranational Laboratories and experts of the Region was considered.

DEFINITIONS

Biological risk containment area	One or more laboratories that meet the biosafety requirements defined by WHO to handle live cultures of Mycobacterium tuberculosis.
Isolation	Positive culture of acid-fast bacilli.
Vortex Shake	To shake with stirrer that generates a swirl effect in the solution or suspension, to homogenize it vigorously.
Amplicon	Product of nucleic acid replication or amplification.
Target	Site of action of an antibiotic.
Borderline	Term often used to describe the results of a laboratory test that are located near the limit value defined to classify the results or positive / negative, or sensitive / resistant.
Wild strain	Strain never exposed to a particular drug.
Clon	Genetically identical bacilli that descend by multiplication of a single bacillus, and form a colony in a culture medium.
Critical concentration	Lower concentration of drug that inhibits 95% of wild strains of M. tuberculosis but which, at the same time, does not inhibit those that have been isolated from patients that do not respond to therapy and are considered resistant.
Minimum inhibitory concentration	Lower concentration of drug that inhibits 99% development of the isolation investigated.
Dysgonic	Poor development, delayed, barely visible.
Eugonic	Clearly visible in the expected times, even when it vital development with few colonies.
Extensively resistant	Resistant to at least rifampicin, isoniazid, a quinolone and one of the following injectables: kanamycin, amikacin or capreomycin.
LiPA	Nucleic acid amplification system and hybridization in strips with immobilized probes (line probe assay).
“Clean” material	Without pathogenic or potentially infectious agents.
“Dirty” material	With (suspected) pathogens or potentially infectious agents.

MGIT	*Mycobacterial growth indicator tube*
Multidrug-resistant	Resistant to at least rifampicin and isoniazid.
Pansensitive	Sensitive to all anti-tuberculosis drugs.
Parameters indicating the accuracy of a sensitivity test	Sensitivity: accuracy to identify resistant isolates correctly (true resistant among all resistant identified by the test, expressed as a percentage). Specificity: accuracy to identify sensitive isolates correctly (true sensitive among all the sensitive identified by the test, expressed as a percentage). Efficiency: accuracy to identify sensitive and resistant isolates correctly (true resistant + true resistant among all investigated by the test, expressed as a percentage).
Prime	A short sequence of nucleic acid having base pairs complementary to a template strand to be copied or amplified, and which acts as a starting point for the addition of nucleotides (by its English name often used to name a primer).
Primary culture	Isolation obtained immediately after inoculating a sample of clinical origin (without previous subculture).
Pro-drug	A drug that is administered inactive or not very active and which, when metabolized in vivo, is activated.
Critical proportion	Percentage of resistant mutants in a standardized inoculum of Mycobacterium tuberculosis, which may grow in a culture medium containing the critical concentration of a drug, above which the strain is classified as resistant and below which it is classified as susceptible. Usually it is 1%.
Cross resistance	Resistance to two or more antibiotics that share at least one mechanism of activation or action.
Probe	A small nucleic acid fragment used as a tool to recognize the presence of complementary sequence DNA or RNA. To display this recognition, the probe may be labeled with some signal-emitting reagent, generally chromogenic or fluorescent.

ACRONYMS AND ABBREVIATIONS

A	Amikacin
AFB	Acid-fast bacilli
BK	Smear microscopy
BRCA	Biological risk containment area
C	Capreomycin
CFU	Colony Forming Units
DNA	deoxyribonucleic acid
MIC	minimum inhibitory concentration
e.q.f.	enough quantity for (indicates that the required amount of liquid to be added until the indicated volume is reached)
E	Ethambutol
FQN	Fluoroquinolone
HTC	Thiofen-2-carboxylic acid hydrazide
I	Isoniazid
K	Kanamycin
Lfx	Levofloxacin
LJ	Löwenstein Jensen medium base
LiPA	Line Probe Assay
Mfx	Moxifloxacin
MGIT	*Mycobacterial Growth Indicator Tube*
MDR	Multidrug resistant
NRL	national reference laboratory
PAS	p-amino salicylic acid
PCR	Polimerase Chain Reaction, polymerase chain reaction for nucleic acid amplification
PNB	p-nitrobenzoic acid
R	Rifampicin
RNA	ribonucleic acid
RR	rifampicin resistant
S	Streptomycin
SOP	standard operating procedure
DST	drug susceptibility testing
TB	Tuberculosis
XDR	extensively resistant
Z	Pyrazinamide

Note: International abbreviations for antibiotics are not always used to facilitate labeling. In general, they have been limited to only one letter and have been considered frequent use in laboratories

BASE AND USEFULNESS OF THE SUSCEPTIBILITY TEST

The treatment of tuberculosis and the generation of resistance

With few exceptions, broad-spectrum antibiotics are not effective against *Mycobacterium tuberculosis.* Some characteristics of this microorganism explain this natural resistance. Its highly hydrophobic wall prevents the penetration of drugs. In addition, it is difficult for antibiotics that act on the metabolism, synthesis or multiplication of the germs to find their target in this bacillus that multiplies very slowly and is able to persist in dormant state inside the macrophages, under microaerophilic conditions. In addition, the bacillus prefers to be housed in certain compartments of the host (pulmonary caverns, empyema, and solid caseous material) where the arrival of antibiotics is difficult and its action is very limited by very low PH.

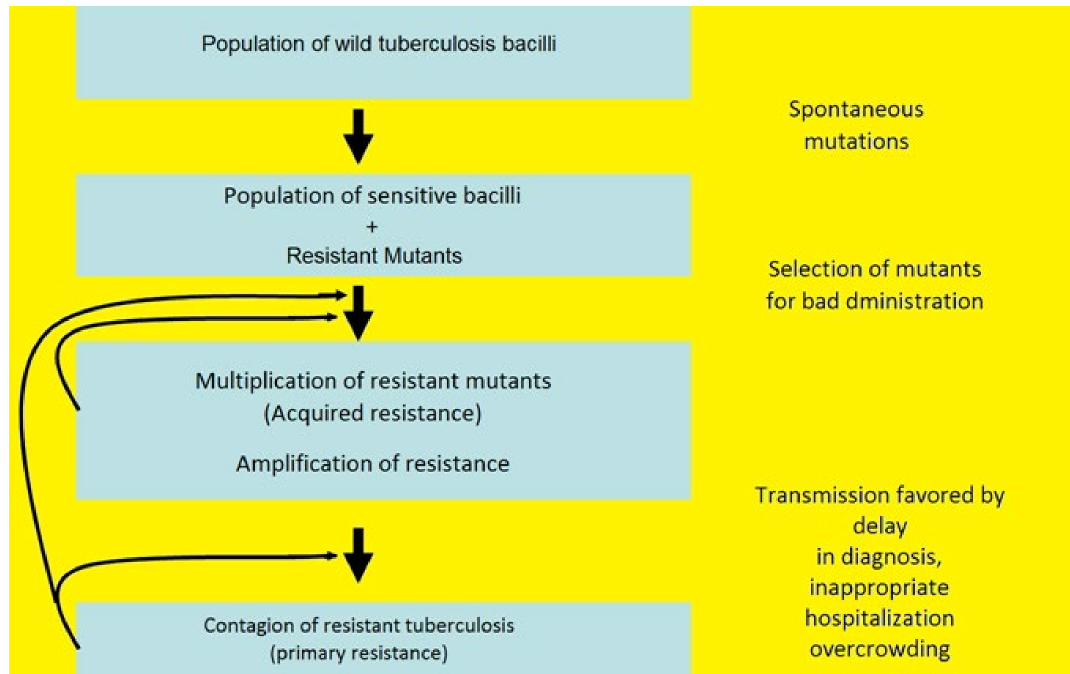
Therefore, it was very difficult to form an effective therapeutic scheme for TB. It took more than 20 years of experimentation to produce solid information indicating that combining the most active antibiotics discovered could cure more than 95% of patients within 6 months with minimal relapse. The antibiotics that are currently part of the internationally standardized optimal anti-tuberculosis treatment are: I, R, Z, and E. These drugs are combined during a first intensive phase of approximately 2 months and then the treatment is completed for another 4 months with I and R.

When the bacillus multiplies, mutants naturally resistant to these antibiotics may appear. If therapeutical schemes are poorly prescribed (administering drugs or combinations of inappropriate drugs), or if they are not met regularly or completely, the population of susceptible bacillus can be killed but not the resistant one, which continues to multiply. First-line treatment has a high probability of failure when selecting mutants of the bacillus, which are RR, and the probability of failure is higher if those mutants are simultaneously resistant to I and R, that is to say MDR. Faced with another type of resistance in which neither I nor R are involved, standard first line treatment still has high potency to heal the patient.

For these reasons, it is strongly recommended to apply standardized schemes of demonstrated efficacy, and directly supervise that the patient takes the drugs regularly, until complete. In addition, it is recommended not to hospitalize a case with TB, unless it is indispensable. If hospitalization is necessary, it should be performed in isolation, in adequate rooms to avoid the spread of respiratory infection.

A patient diagnosed with TB by BK who is affected by a bacillus strain that is not resistant to the key drugs for therapeutic success begins to respond to first-line treatment. This is evidenced by the decrease in the degree of positivity of BK until, at the end of the second to fourth month of treatment, the BK becomes negative. Most cases do so at the end of the second month. If the BK persists positive at that time, it is advisable to perform a culture to verify if the bacillus strain affecting the patient is viable and a rapid ST to know if it is resistant or not, anticipating a possible treatment failure. For programmatic purposes, treatment failure is only diagnosed when BK persists or is positive by the end of the fourth month, or later.

Appearance and resistance amplification to anti-tuberculosis drugs



The cases with the highest risk of drug-resistant TB are the failures and contacts of cases with resistant TB. Then are cases recovered after loss of follow-up and relapses.

When MDR TB is diagnosed or failure of the first line regimen due to drug resistance, schedules with antibiotics with anti-mutant bacilli activity are formed. Combines drugs such as FQN (Mfx, Lvx), injectables (K, A, C, possibly S), ethionamide, PAS, cycloserine and others of more recent development. Persistence in the misuse of drugs during retreatment may lead to the addition of MDR resistance to other drugs that are pivots for the treatment of MDR TB, such as FQN and aminoglycoside or C. then compared to the so-called XDR TB that has even worse prognosis.

Mechanisms of action of drugs and resistance of bacillus

Although the bacillus multiplies and evolves very slowly, it adapts quickly against antibiotics. As has been said, when it is active and multiplies, mutations naturally occur in its DNA, which are usually point changes of some basis. Some of these mutations may confer drug resistance because they cause changes in the composition or conformation of the proteins to which the antibiotics bind. These proteins are usually enzymes that activate the drugs or enzymes that intervene in some vital biological process of the bacillus. Other times the mutation causes overexpression of a gene that compensates for the damage done by the drug or governs some mechanism that can expel the antibiotic, such as a reflux pump.

The effect of the mutations is very variable. Some mutations cause resistance that manifests even with low concentration of the antibiotic, others can generate resistance also at high concentration. At the same time, the resistance may be cross-linked for several drugs or not. On the other hand, the resistance generated by a mutation can be enhanced or nullified by other mutation (s). Finally, not all mutations in genes related to drug activity generate resistance. Understanding these biological events is necessary to properly interpret laboratory findings.

The following table summarizes, in a very general way, the main mechanisms of action and resistance for each drug, and the accepted methods to perform the ST up to the moment of writing this Technical Guide. It should be clarified that drugs not considered essential (such as cycloserine, thiacetazone, clofazimine and PAS) and those recently developed and / or used for the treatment of TB (bedaquilin, delamanid, ß-lactam) have been less investigated. In all those cases there are mechanisms not yet known.

Main mechanisms of action and resistance determinants of antituberculosis drugs
Characteristics of the susceptibility tests

Drug	MIC versus sensitive bacillus µg / ml	Main biological process of the bacillus that affects	Mechanisms most frequently involved in resistance						Susceptibility Tests	
			Gen	Molecule that modifies		effect of mutation	conspicuous mutations	known implication	Phenotype	Commercial Genotype
Isoniazid	0,02-0,2	Synthesis of wall	*KatG*	catalase-peroxidase	activator of I	1	S315T	High level of resistance (MIC> 1mg / l) affects only I most frequently in MDR-TB; and when not associated with RR, is a predictor of TBMDR	reproducible predicts drug activity under treatment	Available for the most frequent mutations
Ethionamide	2,5-10		*inhA*	reductase	I white and ethionamide	2		Low level of resistance to I (MIC <1mg / l), affects I and etio / protionamide more frequently in I mono-resistant TB	less reproducible	
Thiacetazone	0,1-0,5		*ethA*		Activator of ethionamide and thiacetazone	1		affects resistance to etio / protionamide and thiacetazone	With uncertain clinic value	Not available
			operon hadABC		drug target	1		affects the etio / protionamide		
Rifampicin	0,05-1	Synthesis of RNA	*rpoβ*	RNA polymerase	drug target	1	S531L y H526Y/D	More prevalent high level of resistance to all rifampicins	reproducible predicts drug activity under treatment	Available High sensitivity and specificity
							H526L	Low level of resistance to R may not be detected by phenotypic methods		
							L533	low level of resistance to all rifamycins	The MGIT device frequently fails to detect low level of resistance	
							D516	affects R and much less rifabutin		
Quinolones	0,5-2,5	DNA conformation	*gyrA*	DNA girasa	drug target	1	D94G	High level of resistance to all FQN	Reproducible	High sensitivity needs to be supplemented with phenotypic test against each quinolone in use to elucidate cross-resistance
			gyrB	DNA isomerase						
IInjectables	2-8	Protein synthesis	*rrs*	ribosomal protein	drug target	1	A1401G	high level of resistance to A and K and low level to C unless certain mutations are summed in tlyA	Reproducible	high sensitivity needs to be complemented with phenotypic test to elucidate cross resistance
Streptomycin Kanamycin Amikacin			*rpsL*	ribosomal protein						
Capreomycin			*eis*	promoter				Low level of resistance K		

Main mechanisms of action and resistance determinants of antituberculosis drugs
Characteristics of the susceptibility tests (Continuation)

Drug	MIC versus sensitive bacillus ug / ml	Main biological process of the bacillus that affects	Mechanisms most frequently involved in resistance						Susceptibility Tests	
			Gen	Molecule that modifies		effect of mutation	conspicuous mutations	known implication	Phenotype	Commercial Genotype
Pyrazinamide	16-50 (ph 5,5)	Energy in the membrane	*pncA*	pyrazine midasa	drug activator	1		M. bovis - BCG are naturally resistant	no precise reference method has been identified	
			RpsA	ribosomal protein	drug target	1				
Ethambutol	1-5	Synthesis of wall	*embB*	transferase	drug target	1	M306Mut	low or moderate level of resistance	less reproducible than the I and R tests	insufficient precision
Cycloserine	≤16	Synthesis of wall	*alr*	racemase	drug target	2		BCG is naturally resistant	reproducible in LJ not in liquid medium or agar	not available
Linezolid	0,5-8	Protein synthesis	*rplC*	ribosomal protein	drug target	1	T460C		insufficiently assessed	not available
Clofazimine	≤1	Release of oxygen compounds and membrane disruption	*Rv0678*	effluent pump	drug target	2		bedaquiline cross-resistance	Non-standardized	not available
Bedaquiline		Energy generation	*atpE*	ATP synthetase	drug target	1			Non-standardized	not available
			Rv0678		effluent pump	3		cross-resistance with clofazimine		
Delamanid	≤0,05	Synthesis of wall	*ddn, Fbi A, Fgd1*	Nitroreductasa activity	drug activator	1			Non-standardized	not available
PAS		Synthesis of folic acid, iron uptake	thyA	synthetases	drug targets	1			standardized insufficiently evaluated reproducibility	not available
			folC			1				
β Lactamic amoxicillin, meropenem		Wall Disruption							Non-standardized	not available

(a) 1 inhibits the union of the drug with the target 2 over-expression of a mechanism that compensates for drug damage 3 overexpression of an ejection system (efflux pump)

Nomination of mutations Example S531L serine has been replaced by leucine at position 531

Drugs for which cross-resistance can occur

Isoniazid, ethionamide / protionamide, thioacetazone

All three are pro-drugs. To be effective, they must be activated by enzymes of the bacillus that also act in the degradation of superoxigenated compounds produced by the macrophages of man in response to the infection. Once activated, these drugs attack bacillus enzymes that intervene in different stages of the synthesis of mycolic acids that, in turn, are essential components of the bacillus wall. They can also affect other biological processes. In particular, I, which has a very simple structure, is one of the most complex antibiotics by its mechanism of action; its power is surely due to the effect on multiple targets. It is active against bacilli that actively multiply in the lung caverns. It is bactericidal at very low concentration and in the early stages of the disease. That is why it rapidly reduces the number of live bacilli in the patient's sputum and therefore the risk of transmission.

It is activated by catalase *(katG)* and primarily attacks two enzymes involved in the reduction *(kasA)* and elongation *(inhA)* of mycolic acids. In addition, they interfere with DNA formation and could also affect NAD and, therefore, energy production. Certain mutations that occur in *katG* make the bacillus resistant to I because it cannot be efficiently activated. It can also become resistant by some mutation in the genes encoding the white enzymes of the drug, mainly *inhA.* Most often, mutations occur in very limited regions of these genes. Mutations in *katG* are the most prevalent, generate a high level of resistance, and are associated with MDR TB. *InhA* mutations, on the other hand, generate low levels of resistance to I, confer resistance to ethio / protionamide, and are relatively more frequent in I-mono resistant isolates. More rarely, other mutations occur outside these regions or in other genes, even in some as yet unidentified.

On the other hand, ethio / protionamide and thiacetazone are activated by the same enzyme (ethA). Once activated, thiacetazone inhibits a methyltransferase mediated transformation pathway (mmaA) and the hadABC operon, both involved in resistance to that drug.

In summary, I and ethionamide are activated by different molecules, but they have a common target. Ethio / protionamide and thiacetazone are activated by the same enzyme but have different targets. Thus cross-resistance between I and etio / protionamide and between etio / protionamide and thiacetazone may occur, depending on the mutations that appear to affect the activators or target the drugs.

Rifamycins (rifampicin, rifabutin)

Because they are lipophilic they rapidly penetrate through the envelope of the bacillus and interfere with the synthesis of messenger RNA, joining the polymerase encoded by the rpoß gene. The R is bactericidal and still acts on bacilli that are slowly metabolizing, kills the persistent ones and finishes to sterilize the sputum of the patients.

In about 95% of the isolates that are resistant to R, the rpoß gene is mutated in a small region. Only 3 mutations are found in more than 70% of the resistant isolates, and one of them (S351L) is the most frequent globally. These mutations confer a high level of resistance, greatly elevating MIC, and cross resistance with other rifamycins. Some less frequent mutations confer a low level of resistance to R, which is often not detected by conventional PS, especially those performed with the MGIT system, but also associated with drug ineffectiveness in the therapeutic regimen.

Fluoroquinolones

They inactivate enzymes responsible for the conformation of bacillus DNA. Resistance is mainly associated with point mutations in genes encoding gyrase DNA (gyrA and gyrB). The most prevalent are presented in a small fraction of the first. Other mechanisms are less frequent and appear to confer low resistance. In general, multiple mutations in one or both genes raise the level of resistance. But it has also been shown that some mutation can negate the effect of another (s) and make the insulation hypersensitive.

There is cross-resistance between old and new generation FQNs (such as that caused by the D94G mutation in gyrA). But knowledge about this phenomenon is incomplete so the pattern is difficult to predict.

Injectables: aminoglycosides (streptomycin, kanamycin, amikacin) and capreomycin

Aminoglycosides and C, which is a cyclic polypeptide, bind to different subunits of the ribosome and cause defective RNA reading affecting protein synthesis. Certain changes in the sequence or conformation of RNA or ribosomal proteins (rpsL, rrs, gidB, tly A, eis) generate high, moderate or low resistance of the bacillus to one or more of these drugs. It seems that certain mutations in *rrs* are associated with cross-resistance, that others in the gene are good markers of resistance to K and some in *tly* A are preferentially found among isolates resistant to C. However, in the bacilli may occur one or multiple mutations that confer resistance to one or more of these drugs, with a pattern that is difficult to predict.

Bedaquiline and clofazimine

Clofazimine affects a phospholipase and certain potassium transporters of the bacillus membrane. It could also block the respiratory chain, leading to the release of compounds that cause oxidative stress in the bacillus.

Bedaquiline inhibits the ATP synthetase encoded, in part, by the atpE gene that governs the generation of the energy required by the bacillus. Certain mutations in this gene have been identified as a cause of resistance.

Both drugs have different targets, but two mechanisms have been identified that confer low resistance to clofazimine and cross-resistance with bedaquiline. One is overexpression of an efflux pump (Rv0678), and the other is mediated by mutations in the pepQ gene whose function is not well known, probably involved in the hydrolysis of peptides.

ß-lactamics

They bind to various enzymes involved in the synthesis and lysis of the bacillus wall. M tuberculosis is naturally resistant to this class of antibiotics not only because its wall prevents entry but also because it produces broad-spectrum ß-lactamases that destroy them. However, in the presence of ß-lactamase inhibitors (clavulanate or sulbactam), the susceptibility of clinical isolates is variable. In vitro, the combination is effective against MDR and XDR isolates. Certain mutations in the gene encoding a ß-lactamase (BlaC) could confer hypersusceptibility of the bacillus to ß-lactamics.

Other drugs

Pyrazinamide

Its introduction into the first-line regimen allowed shortening treatments lasting from 18 to 12 months to 6 months and appears to be a mainstay for the development of shortened regimens for the treatment of MDR-TB. Because it acts at acidic pH, it can kill semi-dormant bacilli within necrotic foci during active inflammation; does not provide benefit in the second stage of treatment. It is not fully active in the neutral culture medium. In spite of the high activity it shows in vivo, in vitro it has a very high MIC, still at pH 5.5 and shows a slow bacteriostatic effect on the bacillus. The following mechanism of action has been proposed. Z enters by passive diffusion into the bacillus, is converted by the cytoplasmic pyrazinamidase of the microorganism to pyrazinoic acid that is the active molecule. The pyrazinoic acid exits the bacillus by passive diffusion and by a weak efflux mechanism. Outside, at acidic pH, a small proportion of the pyrazinoic acid is converted to an acidic conjugate, with no charge; it crosses the membrane of the bacillus easily and accumulates in the cytoplasm. Upon entering, it introduces protons that could acidify the cytoplasm of the bacillus and inhibit vital enzymes. It could also deplete the membrane and affect the transport of substances through it. Z has several similarities with I: it attacks multiple targets; it is a pro-drug that requires a transformation to be an active drug, and is very specific for the TB bacillus. Other bacteria, including other mycobacteria including *M. bovis* and its variant BCG, are not sensitive because they have weak or no pyrazinamidase activity or efflux systems that expel the drug. Several mutations have been identified in the pyrazinamidase gene (*pnc*A) and in its promoter as a mechanism of resistance. Pyrazinoic acid binds with much affinity to the ribosomal protein S1 (*Rps*A) that could also be involved between resistance mechanisms, along with others little known or unknown.

Ethambutol

It is bacteriostatic. It acts on the bacillus that is multiplying, in the biosynthesis of arabinogalactan, the main polysaccharide in the wall of the mycobacteria. Inhibits the polymerization of intermediates that accumulate. The target may be in several arabinosyl transferases encoded by the operon *emb.* Certain mutations in *M. tuberculosis emb*B present in most resistant isolates confer a high level of resistance, whereas in less frequent isolates with a low resistance level no mutations are found in that gene, suggesting that there are other mechanisms of resistance. Some resistant mutants have defective cell wall, which could support the administration of E in the treatment of conditions caused by some mycobacteria that in vitro are resistant to it.

PAS

Its mechanism of action is not well known. It is postulated that, like sulfonamides, it competes with p-aminobenzoic acid or interferes with the biosynthesis of mycobactin (proteins from the mycobacteria that carry the iron into the bacteria), which would explain its reduced range of action. Mutations occurring in the genes involved in these processes appear not to be the only mechanisms of resistance.

Cycloserine

It blocks the synthesis of peptide-glycans by competing with D-alanine for D-alanine racemase *(alr)* and d-alanyl-d-alanine synthetase (d-alanine ligase) *(dd*lA). In the resistant clones a point mutation was found in the *alr*A promoter leading to overproduction of the enzyme.

At the time of writing these guidelines, 4 classes of drugs that had not previously been used to treat TB are investigated (diarylquinolines such as bedaquiline, nitroimidazole derivatives such as delamanide and petromanide, ethylene diamines, and oxazolidinones such as linezolid) were investigated. Knowledge about its mechanisms of resistance that occur against these drugs is still incipient.

Probability of appearance of resistant mutants

It is very unlikely that patients with anti-tuberculosis treatment naive are affected by resistant strains of bacillus, except that have been infected with some cases with drug-resistant TB, which can be suspected when the contact is known. As already mentioned, among those with a history of previous treatment, it is the failures that are most likely to be affected by resistant bacilli.

Since mutations appear as the bacillus multiplies, the more it has been reproduced the greater the chance that resistant mutants will appear or are added. That is why they are more frequent in very advanced pulmonary lesions and in the lymph nodes that are characterized by a high number of bacilli. In contrast, they are rare in poorly advanced lesions and in most extra pulmonary TB. This principle explains why single drug prophylaxis (I) is indicated to prevent the progression of latent infections, and why irregularities in the first stage of treatment can lead dangerously to select resistant mutants, whereas those that occur later difficult they do it.

Mutations that lessen the ability of the bacillus to survive and multiply, or those that are favored by other biological factors, may predominate.

It is more likely that I-resistant mutants will appear than to R. Therefore, under the selection pressure of these drugs, resistance generally first appears to I and then to R.

In other words, the majority (over 90%) of R-resistant bacilli are already resistant to I, or MDR. Then RR is an MDR marker and its rapid detection is very useful because it allows to warn about the need to redesign the therapeutic scheme.

The frequency with which resistance to other drugs appears is greater between isolates that have already been made MDR, and more so among the XDR. This was demonstrated for Z, linezolid, clofazimine and certainly has relation with the use of the drugs in these cases.

In general the relative frequency of the different mutations is similar in the world. Even so, the prevalence of certain particular mutations could vary in different geographic regions, and in different lineages of the bacillus. This could limit the sensitivity of molecular tests in some special scenarios.

Indication and usefulness of the susceptibility test of Mycobacterium tuberculosis.

The detection of resistance to R and / or I allows

- *To identify cases with MDR-TB or with a high risk of MDR-TB that requires readjustment of the therapeutic regimen to improve their prognosis.*
- *Saving Patient Lives.*
- *Interrupt the transmission of strains of the bacillus that cause a form of disease difficult to cure.*

This is why it is necessary to perform DST early and quickly when identifying patients at high risk for drug-resistant TB.

Ideally, all cases diagnosed with bacteriologically confirmed TB should have access to ST, at least for the drugs that are key to the success of treatment (I and R). In the process leading to this goal, the ST should be prioritized for cases with the following characteristics that increase the risk of drug resistance:

- Treatment failure.
- History of previous treatment, irregularity in compliance with a treatment or prescription of an incomplete or inadequate schedule,
- exposure to drug-resistant TB infection, especially in overcrowding, in
  - health centers, prisons or other institutions where such cases have been registered, particularly if the exposed persons are immunocompromised or workers of those institutions.
  - the home, work or social environment
  - in a geographical area with a high level of resistance to anti tuberculosis drugs.

Among cases with a history of treatment, those classified as failures and chronic are more likely to be affected by drug-resistant strains of the bacillus. In addition, other cases with increased risk could be identified, such as those treated by some component of the health system that administers treatments inappropriately.

HIV-infected patients are particularly vulnerable to hospital infections, and are especially exposed to TB when treated at referral hospitals, which also focus on TB care. The situation is even more serious, even though those hospitals treat cases with drug-resistant TB and when infection control measures are not effective.

In countries with a low prevalence of resistance, they can be defined as groups exposed to immigrants from countries with a high resistance rate or, in the absence of this information, from countries with programs that operate poorly (with deficiencies in treatment supervision, stocks of medicines or in their administration).

As a preventive measure it is beneficial to include among the groups prioritized for the knowledge of the susceptibility profile to the following cases

- patients under treatment that delay the conversion of BK, even though they have a favorable evolution
- those who do not tolerate some or several first-line drugs, in case it is necessary to resort to schemes other than the standardized,
- patients who may have a very poor outcome in the event of an eventual failure of treatment (i.e., immunecompromised).

The reference laboratory and the conduction of the National TB Control Program must agree on and disseminate the norms that govern the supply of ST, and design and implement appropriate forms for the request for bacteriological studies. Standards and tools for data collection should clearly identify risk groups for resistant TB, drugs to be tested according to standardized therapeutic regimens, reliability of adopted methods for ST and reported resistance levels.

If TB RR or MDR is detected, it is necessary to know the susceptibility to the rest of the first and second line drugs.

Until reaching the goal of performing DST on anti-tuberculosis drugs for all microbiologically confirmed cases, the following are the priorities

At the time of diagnosis of patients

- *with a history of treatment with the following order in relation to the risk: failures, recovered after loss of follow-up, relapses.*
- *case contacts with drug-resistant TB (home residents, boarders or workers of health institutions or prisons where TB MDR-TB cases are recorded).*
- *Immunecompromised (HIV positive and diabetic).*
- *with antituberculosis drugs intolerance.*

During the treatment control of TB cases

- *with smear positive at the end of the second month of treatment or later.*
- *Diagnosed with negative smear and converted to positive smear*
- *Poor adherence to treatment.*

The knowledge and registry of the susceptibility profiles to the anti-tuberculosis drugs of the diagnosed cases also allows to keep the level of resistance under constant surveillance.

Principles of methods

The result of the Susceptibility Tests should not be informed if previously has not been identified to the isolation investigated as M. tuberculosis

If it were another unidentified mycobacteria properly the error is serious for the patient

Other mycobacteria may not require any treatment (in case they were contaminants or transient colonizers or require a very different treatment to the employee for TB (in the case where they were causing pathology)

A rapid method of molecular identification or the detection of *M. tuberculosis* antigens that can be performed very simply by lateral flow immunochromatographic assays is required to no delay ST, or its report.

Methods investigating the growth of bacillus in medium with antibiotics (phenotypic)

To know if the drugs are active against the bacillus present in a clinical sample, they are culture in medium containing each of the drugs that are to be tested, separately, and it is observed if the bacilli develop or not after they have been exposed to the action of antibiotics (resistant or sensitive phenotype, respectively). It is always necessary to cultivate it also in drug-free medium, as a control of the viability and purity of the germ and to be able to compare

the development that it presents with and without exposure to the drug.

The ST commonly used for other bacteria in solid medium, such as those using antibiotic impregnated disks or strips, which diffuse on the culture medium, are not useful. Due to the slow development of the bacillus, the need to accurately estimate the proportion of bacillus clones resisting the action of drugs and the biological risk involved in their manipulation in the laboratory, it was necessary to design peculiar tests that are not normally performed in a laboratory of general microbiology.

The population of bacillus with which they work must be actively multiplying to obtain reliable results, i.e. it is not advisable to work with aged crops.

The action of the antibiotic on the bacillus occurs in the first hours and days of contact. However, prolonged incubation is required so that the development or lack of development with each drug tested is evident, thus evidencing whether the bacillus has resisted or not.

In both solid and liquid medium it is possible to include various concentrations of each drug or serially dilute it to determine its MIC. But for the general clinical application, the methods have been simplified by choosing a critical concentration that allows differentiating sensitive bacillus isolates from the resistant ones, as demonstrated by exhaustive investigations by Canetti, Rist and Grosset.

Proportions method in solid medium

The most widely used method in Latin America is the economic and simplified variant of the proportions method, standardized by the aforementioned researchers in the LJ medium, but it can also be performed on Middlebrook agar. The critical concentrations of antibiotics may be different depending on the culture medium used, depending on the inactivation they undergo. By standardizing the methods, we have found concentrations for each medium that allow to reproduce the results in all of them.

When agar is used, the plates or tubes can be inspected under a microscope or magnifying glass to detect development earlier.

The proportions method allows counting the number of CFU that grow in medium. When the amount of CFU in the medium with antibiotic exceeds 1% of that developed in the control, the isolation is considered resistant. In order to be able to detect this resistance limit, in the drug-free medium, at least 100 colonies must have been developed.

The method is laborious but very precise and continues being considered the reference to elucidate inconsistencies, uncertainties or doubts that arise with the results obtained with other tests

Inoculum

In order to have a result that shows the susceptibility profile of the bacilli affecting the patient at the time of the investigation, it must be based on a recent sample of sputum or other type.

Direct Testing.

To speed up the result, ST can be performed directly from the clinical sample, if it contains abundant bacillus, ie if it has positive BK, preferably 2+ or 3+. With fewer bacillus, development is likely to be insufficient to detect resistant clones that may be in low proportion. The sample should be decontaminated with the usual procedure if it is not, it is probably a naturally sterile tissue. If the direct test is performed and the development is insufficient it is necessary to repeat it from the development obtained in the medium without antibiotic (control), because the antibiotics select resistant clones.

Indirect Testing.

When working from cultured bacillus, it is always convenient to start from the primo culture and not from residues, to avoid the selection of clones. In order to accelerate the results and avoid the aging of the crop, the ST should be prepared as soon as development is observed, although the colonies are still small, unless very few colonies have been detected and it is presumed that with a few additional days incubation development will be more abundant. In part 2 of this Manual dedicated to Culture has expressed the idea that a minimum number of colonies developed in

the primo culture to carry out the ST is necessary. It should be clarified that this applies when working with a sample investigated for diagnosis and that has BK positive 1+ to 3+. This type of sample must produce an abundant number of colonies if properly transported and cultured. However, patients with poorly advanced extra pulmonary lesions who are HIV positive or children and cases already under treatment may release a small number of viable bacillus in the samples and in those cases it is advisable to perform the TS, even from cultures with few colonies, and inform the treating physician that the study has been performed under these conditions. The peal of few colonies of a primo culture to obtain greater development does not increase its representation, since only the few clones initially isolated are multiplying. To be more certain with the results of ST of this type of patients it is advisable to cultivate 2 or 3 successive samples, if this is possible, and send to the lab that performs the ST all positive tubes.

In order to prepare the inoculum it is necessary to take most of the bacillus present in a sample or in culture, so that the result is representative of the population of bacillus that affects the patient. For this, work is done with all the precipitate obtained with a decontaminated sample or the bacillary mass taken from the entire surface of the development obtained by culture. When the development of a crop is scarce, it is necessary to take colonies of all the developmental tubes with which it is counted, even if they are from different samples of the patient planted consecutively.

To obtain the proper development for the ST to be valid, it is necessary to standardize the inoculum and seed suitable dilutions to count the CFU. In the simplified variant of this method, three dilutions are seeded in the drug-free medium and two dilutions in the drug containing medium. When starting from a decontaminated sample, dilutions are planted which are larger the higher the degree of BK positivity of that sample. When starting from insulation, the procedure is started with a suspension of bacillus with a standardized opacity, and with it are dilutions. For the calculations to be valid, the dilutions must have been made with suspensions very homogeneous, with great precision, changing pipettes every time you move from a concentrated suspension to a more diluted one and the volume sown in each tube must be exactly the same.

Incubation and Reading

The inoculated bacillus suspension in solid medium should be homogeneously distributed to allow accurate counting of CFU, and thus tubes with solid medium should remain tilted until absorbed. Absorption may take 2 to 20 days depending on the type of tubes and medium being used. This prevents the peal, or "overshoe", when moving the tubes. This effect may lead to false estimates of the bacillary load sowed.

The method requires quantifying the inoculum in the control medium, without drug. Usually, the development that is obtained with the more concentrated suspension sown is difficult to quantify, because it is abundant. Then, knowing the dilutions made, from the number of CFU observed in the tubes seeded with more diluted suspensions, it is possible to infer the amount of CFU observed with the most concentrated. Then the CFU developed in drug media tubes can be counted, and calculate whether or not this number exceeds 1% of the amount of inoculated bacilli.

Intermediate inspections should be carried out to check if development is sufficient and no undesired contamination has occurred. If any resistance appears, it must be informed at the time of the intermediate inspection. In most cases, resistance can be detected within the first three weeks of incubation. In the particular case of the two key drugs for the success of antituberculosis treatment, I and R, the resistance is detected in more than 95% of the cases in that period. If resistance does not appear, incubation is continued until the month or 40 days, according to the medium used, to confirm that the strain is sensitive to the drugs that inhibited the development during the first weeks. This is done because infrequently it is possible that late resistant mutants.

In order to interpret the final result of the ST it is necessary that the means used are of good quality and that three conditions are simultaneously fulfilled.

Condition	Reason
sufficient development (at least 100 colonies) in the control tubes, without drug, seeded with a suspension of concentrated bacilli	to make it possible to detect resistant mutants that are in that inoculum in low percentage, close to 1%,
separate and accounting colonies in the control tubes seeded with more diluted suspensions	to make it possible to infer. from the number counted in these tubes, the number of colonies developed with the most concentrated suspension (usually difficult to count or countless)
Correlation between the number of developed colonies with each of the suspensions seeded, according to the dilution factor applied	so that the inference from the previous point are valid

Nitrate reductase test

An economical alternative to accelerate the results of TS in egg-based medium is to add sodium or potassium nitrate. It is then possible to reveal early the nitrate reductase activity of the bacillus by the Griess method. This is the same biochemical test used to identify TB bacillus and other mycobacteria (see TB Manual for Bacteriological Diagnosis of TB, Part 2 Culture). It will be positive in the medium with and without drug, in the case in which the isolation resists the antibiotic, and only in the medium without drug in the case in which it is sensitive.

Ideally, the method should be performed directly on samples with freshly collected positive BK or as soon as development in the primo culture is obtained. With aged samples or cultures, it is possible that the enzymatic activity deteriorates and therefore it is not possible to obtain interpretable results.

With this test, it is not possible to count colonies. As experienced to obtain high reproducibility with the conventional proportions method, the inoculum should be seeded 10 times more diluted in the medium without drug. The intensity of the color of the reaction, and hence the development, of the drug containing medium with which it is not contained is then compared. If the first, the color is more intense than in the second, it is inferred that a high percentage of resistant bacilli has grown and therefore that the isolation is resistant.

To determine the time at which the test can be revealed, the inoculum is seeded in several control tubes, containing medium without drug. The developer solutions are periodically added to each of them until a positive reaction is detected by the appearance of color. At that time development is considered sufficient to interpret the results and the tubes are revealed with antibiotics. Most of the results are obtained at two weeks of incubation.

Its accuracy is very high, similar to that of conventional methods, to determine the susceptibility to I and R. It is not recommended; however, it can be applied to investigate the activity of other drugs of first line because the agreement of results is smaller.

Adaptation of the proportions method in liquid medium

The use of broths has disadvantages from the biosecurity point of view, as will be discussed below, but allows accelerating results, especially when resources are used to detect the development of the

bacillus before it is clearly visible. For this, the broths may contain some detector of viability of the bacillus (indicators of consumption of O_2, CO_2 release or redox activity) that change color or emit fluorescence when the bacillus develops.

The inoculum is also standardized from a homogeneous sample of the primary culture that has reached a sufficient number of CFU in the broth so that the reader emits positive signal. The ideal is to make the ST quickly, otherwise, in addition to delaying the results, it is necessary to adjust the dilutions, according to the time elapsed since the signal was detected, or to make a previous peal in the same broth.

In liquid media it is not possible to count colonies. Then, based on the same principle of the proportions method, the inoculum 100 times more diluted than in the medium with antibiotic is sown in the medium without antibiotic. Thus, if greater growth is detected in the second than in the first, more than 1% of resistant clones have grown and the isolation of the bacillus is determined to be resistant to the drug in question.

As explained in the Culture Manual, the industry has developed equipment that periodically monitors and compares the development detected in the medium with and without drug. The MGIT 960/320 device interrupts the ST when the development of the drug-free medium reaches the threshold to emit the positive signal or, when this threshold is not reached, at the end of the two weeks of incubation. This automatic interruption of the test is disadvantageous for cases of disgonic isolation or that have a low percentage of resistant clones, close to 1% established as a limit, which may require a slightly longer incubation. When it is suspected that this type of isolates is present, it is convenient to repeat the test with the conventional method that allows quantifying the proportion of the population of bacillus that is resistant. Another feature to avoid automatic interruption of the ST is the use of special software that allows the operator to make the decision when it ends.

In "home" systems, the most diluted inoculum in the medium without antibiotic is also sown, as it has been standardized to obtain results coincident with those of the conventional reference method. In these cases several tubes or wells of microplates containing medium without antibiotic, as control, are sown. Periodically one of them is revealed until obtaining a positive signal. At that time the development is sufficient to interpret the test and then proceeds to reveal the rest of the tubes or holes planted with antibiotics.

Critical points of DST performed in culture medium (phenotype)

a) the purity and conservation of antibiotics

b) the quality in the preparation and conservation of culture medium with antibiotics

c) the viability of the bacilli to be investigated in the sample or culture

d) the standardization and homogeneity of the inoculum

e) the interpretation of results

Pyrazinamide susceptibility test

As mentioned, Z is more active at pH close to 5. At this pH a not insignificant percentage of isolates of *M. tuberculosis* does not develop or do so in very disgonic form in culture media, an effect that is most noticeable in the means based on eggs. It has been said that commercial systems that use liquid media are the reference for determining susceptibility to this drug. However, false or inconsistent results have also been reported.

On the other hand, it is known that strains that have a high level of resistance to Z have lost the activity of the enzyme pyrazinamidase, by mutations that appear in the gene that codes it. It is possible to distinguish these strains by investigating whether the bacillus has the ability to act on the Z and release pyrazinoic acid. This can be done through a very simple and inexpensive biochemical test (Wayne's method). If the result is negative it is inferred that the strain is resistant to Z.

Knowledge about the in vitro susceptibility of bacillus to this drug is not yet complete. There are strains with low level of resistance to the concentration of Z that is used in the means of culture, which have enzymatic activity. And there is a percentage of isolates for which the results of the molecular test are not correlated with that of susceptibility performed in culture.

Molecular methods

They are applied to identify mutations that have been associated with drug resistance. For this it is not essential that the bacillus is multiplying or viable, so that the conditions of the culture or the sample from which they work are not so critical.

To verify if the bacillus carries any of these mutations it is possible to amplify and sequence the gene segment where they occur. Several sets of primers are used to amplify, on the one hand, a specific segment of *M. tuberculosis* to identify the bacterium, and on the other the segment of the gene or genes where the mutations associated with resistance to a given drug are concentrated. Each amplified product is called an amplicon, after they can be sequenced on an automatic device. This is the most accurate method to characterize the amplicons because it allows them to accurately identify the mutations produced and to verify whether they are associated or not with the drug resistance in question. Although technological development is becoming more and more accessible to the sequencing, not only of amplicons but also of the entire genome of the bacteria, at the time of writing of these Guides its use is not generalized in institutions dedicated to the detection and management of cases of TB.

For clinical application, commercial kits have been developed that provide primers and reagents to characterize the amplicons, without sequencing. These kits allow determining if the *M. tuberculosis* complex is present and if the isolation carries some of those mutations that more frequently they are resistant to a particular drug, most often investigate mutations associated with RR.

When LiPA is employed, amplicons, generated from primers with a chromogenic tag, are hybridized on a strip having immobilized nucleic acid segments characteristic of *M. tuberculosis*, without mutation, and with the more frequent mutations that determine resistance. It is possible to see with which immobilized segments the amplicons hybridize by their brand that develops color when the test is revealed. The process can be completed in no more than 48 hours, starting from a positive culture or a highly positive BK sample.

In addition, the biotechnology industry has been able to develop PCR equipment and software in real time to capture the signal emitted by the probes by hybridizing in a solution, quantifying them and representing them in a curve, thus showing the progress of the reaction. Moreover, it has been possible to develop and simplify all this process inside a closed and disposable cartridge, which contains all the necessary reagents and enzymes. Thus the risk of transfer of amplified material from one sample to the other is minimized, which may be more likely to occur with the LiPA or other “open” molecular system. It only requires treating the sample with a decontaminating solution and loading it into the cartridge. The first of these fully automated systems (Xpert) employs probes of the wild-type *rpo*B gene, i.e. the tuberculosis bacillus without mutation. When no hybridization occurs, *M. tuberculosis* is not detected. If hybridization occurs with all probes it is interpreted that *M. tuberculosis* exists in the sample and that it does not have any of the mutations that most frequently make it resistant to R. If any probe that investigates the mutations associated with resistance does not hybridize or hybridize late, the *M. tuberculosis* strain detected in the sample is interpreted to be resistant to R. The process is completed in a couple of hours.

Accuracy and clinical significance of the results

With standardized and validated methods to investigate the susceptibility of *M. tuberculosis* to first line drugs in culture, laboratories with good quality of work can achieve very high efficiency with I (97%) and R (99%). With these drugs the results are clear and reproducible because:

a) inhibit the bacillus at a very low concentration, much lower than that used in the clinic and the one chosen for ST

b) it is rare to isolate bacilli that have a very low percentage of resistant clones or resistance to low levels of these drugs

It has been described that the border resistance to R of some isolates of *M. tuberculosis* may not be detected by the MGIT320 / 960 systems because, as explained, it interrupts the reading at a given time, and therefore does not evidence the late development that can present this type of insulation.

When validated molecular methods are applied to quality until the publication of this Manual, it is possible to detect the presence of the bacillus in practically all samples that have high bacilli (BK positive) load and in most but not 100% of lung samples with negative BK and positive culture. In other words, they are less sensitive than culture. When the presence of the bacillus and the mutations associated with resistance to R or I is detected, the resistance can be diagnosed with high certainty, especially in scenarios or population groups with a high prevalence of resistance to these drugs. In general, when it is inferred that resistance exists without identifying the mutation that has occurred there is a certain risk. In fact, at low frequency, a mutation that does not determine drug resistance or "silent". On the other hand, when these mutations are not found, they may still exist and are not detected by commercial systems, because they only investigate the most frequent among known ones. As already discussed, all mutations that cause resistance have not been discovered. In addition, drug resistance may be more complex than the simple presence or absence of mutations in certain genes. In the R case, about 95-97% of the resistant isolates of the bacillus that appear in clinical practice can be detected with commercially available equipment. Sensitivity is much lower with other drugs. Particularly is the case of I, because of the number of genes involved in resistance, the approach is more complex. By these considerations, the molecular ST are useful for accelerating the detection of resistance, but must be complemented with the conventional ones made in culture medium.

In sum, the evidence holds that the resistance determined by the laboratory at R first, and then at I, have high precision and correlation with the outcome of the first-line treatment. However, commercial systems developed to molecularly assess I activity have more limited sensitivity.

The result of ST on other drugs becomes important when faced with cases of intolerance to first-line drugs, with I-resistant TB, and even more so in cases with RR, MR or XDR TB. Most of the other drugs are less active than the I and R against the bacillus, they may have an effect close to that used in clinical practice and / or it is more frequent to have isolates with moderate or intermediate resistance. All this conditions the precision and reproducibility of the ST. The exercises between supranational laboratories have demonstrated that it is possible to achieve high efficiency, similar to I and R, to test aminoglycosides and FQN, and a little lower for capreomycin. For Z, there is no reference ST that is totally reliable, for all the peculiarities described about the mechanisms of action and resistance.

Evidence about the value of ST to drugs other than I and R to predict drug effectiveness in therapeutic regimens is scarce, inconsistent, or difficult to interpret because each drug is used in combination with others. For these reasons, individual results should be carefully interpreted by specialized and experienced laboratory and medical professionals in relation to the history of treatment, the results of previous ST, and the clinical and bacteriological evolution documented for the case. The results acquire consistency when the corresponding ones correspond to different

isolations of the same patient, or they evolve towards the resistance in agreement with inadequate schemes of treatment or non-compliance. Undoubtedly, the complete susceptibility profile to alternative, auxiliary or second-line drugs defines the difficulty each case presents for its treatment, depending on the number and type of drug for which resistance is detected.

The predictive value of any test decreases when the phenomenon they investigate is rare. Thus, in scenarios with low prevalence of resistance to anti tuberculosis drugs, it is advisable to confirm all resistant results. In this way, it is possible to minimize the incidence of falsely resistant results caused by some error.

As resistance to Z occurs more frequently between RR isolates (MDR, preXDR and XDR) the "resistant" result has a higher predictive value if the test is performed for RR cases. It is convenient to consider this, especially since there is no reference ST for this drug.

Faced with results that must be interpreted, the laboratory report should not be limited to "sensitive" or "resistant", but should add evidence-based considerations that guide the physician.

DST quality assurance is essential
False resistance can deprive the patient of the best drugs available and false sensitivity may mask the need to make corrections to the therapeutic scheme. Both of these errors can have serious consequences for the patient and the community.

ORGANIZATION OF THE OFFER OF THE SUSCEPTIBILITY TEST IN THE LABORATORIES NETWORK

Logistics

The NRL, together with the conduction of the Tuberculosis Control Program, should regulate and organize the ST offer for all patients, prioritizing those who have an increased risk of developing drug resistant TB until achieving universal coverage. They must also lead the process so that access is equitable and timely and the results are reliable for the good clinical management of the cases.

In order to ensure timely results of the ST, the health system must provide and use adequate basic tools such as:

- Forms (formats) to identify patients at risk of resistance,
- Containers for safe transport of samples and insulation
- Regular transport and with adequate biosecurity,
- Efficient network telecommunication system (telephone, fax, computer or mobile devices with Internet connection and electronic mail).

All delays have harmful consequences, beginning with the prejudice that can have the patient who is receiving an ineffective or sub-optimal treatment. The delay in sending the samples can lead to contamination that disables ST, which is more critical when using liquid medium. It can also reduce the viability of the bacillus resulting in a higher percentage of invalid results with any phenotypic method.

Then, the network of laboratories is involved, which, in order to ensure speed and quality, must be highly organized, integrated and connected to the health system and the TB Control Program, equipped, supplied with highly trained personnel and a system quality management.

Accelerate laboratory procedures

There are some misconceptions that, for themselves, lead to delays in diagnosis, such as that there is no urgency for TB cases or that a ST inexorably requires at least 4 months. The possibility, for example, that an HIV positive patient is affected by a strain of the MDR bacillus requires an urgent response from the laboratory, because the life of the patient may depend on it. Likewise, the prognosis of any case of TB depends on the administration of an adequate treatment and, therefore, on the rapid detection of drug resistance that are key to the success of these schemes. To rectify these concepts, training and organization designed for the laboratory network should consider key points such as:

- distinction of samples and urgent isolations whose study should be prioritized
- consequences of delays in the evolution of the patient
  - accumulation of unprocessed samples or isolations or referral to the reference laboratory
  - infrequent inspection of culture
  - performing unnecessary subcultures s before investigating isolations
  - delay in reporting results

It is necessary to minimize the avoidable delays that must be distinguished from those that are inherent to the available methods, and which are presented in the following table.

Average time taken by WHO-supported methods
tto produce ST results

	Time to produce result	
Method	Resistant (a, b)	Sensitive (c)
Proportions in	3 weeks	40 days
Lowenstein Jensen		
agar Middlebrook 7H10	2-3 weeks	3 weeks
MGIT	5 to 14 days	5 to 14 days
Fast "home" for I and R	7 to 14 days	7 to 14 days
Molecular	2-3 hours to 2 days	-----------

(a) To investigate samples with positive BK, or bacillus isolates. If samples with negative BK are taken, the time necessary to obtain a previous positive culture is added, depending on the amount of bacillus present, is approximately 30-60 days in solid medium, and half of that time in liquid medium.

(b) Most MDR-TB cases can be detected and reported within the time frames listed. To inform antibiotic susceptibility it is necessary to prolong the incubation during the period mentioned in c) because it is necessary to ensure that they do not develop late resistant clones.

Selection of methods and laboratories

The MGIT medium, the reading equipment and the molecular systems recommended by WHO, are commercial. The remainders are methods whose reagents are prepared completely or partially in the laboratory ("home").

As has been said, Latin America has extensively used the proportions method, which is why it was selected in this Manual among the "landlords" to test all drugs. The nitratase method for I and R rapid research has also been selected because it is the easiest application in Latin American laboratories that do not have the conditions or resources to use the MGIT system and requires reagents and equipment commonly available in laboratories that perform culture in the middle of LJ. By employing solid medium, it is more advantageous than MODS for laboratories that do not have a high biosafety standard.

As described below, phenotypic ST require significant infrastructure reinforcement and biosafety measures in the laboratory because they involve the manipulation of large numbers of live bacilli and the concentration of isolates that are resistant to the drugs. On the other hand, they are demanding enough human resources and trained to perform laborious and relatively complex techniques. For these reasons, in most countries they remain concentrated in the NRL. This situation may be satisfactory in countries with low TB burden and in particular MDR-TB, limited territorial extension and with an easily accessible NRL and well communicated with the health system.

In recent years, methods have been developed that allow intermediate laboratories to quickly identify cases with RR TB or MDR TB and select them so that they can then be further investigated in the NRL. This organization may be appropriate in areas with high TB burden, with difficult access to **population of wild tuberculosis bacilli** and a network of laboratories with adequate resources. It must be taken into account that, in order to maintain the quality of the ST, it is necessary to perform it with a certain frequency (a minimum average of 8 monthly tests). In addition, these laboratories must be initially qualified and then integrated into a regular external quality assessment program conducted by the national reference level. Although ST on drugs other than I and R is also decentralized, isolates of resistant TB cases I, RR or MDR detected by the intermediate level of the network should be sent to the LRN to confirm the resistance, investigate the susceptibility to other drugs and concentrate the information necessary for MR TB surveillance. It is also necessary to send to the LRN the isolates of patients with drug intolerance so that the knowledge of the complete susceptibility profile contributes to guiding alternative treatments, if they were necessary.

When it is decided to decentralize ST to facilitate access to rapid diagnosis of MDR-TB, the implementation of the most rapid test that is feasible in hospitals or reference centers for the treatment of MDR-TB and HIV-positive patients should be prioritized. Especially those who provide care to patients at higher risk of TB, such as poor or other types of vulnerability).

Advantages and disadvantages of the decentralization of ST in the network of laboratories.

Level of the network in which the ST is implemented	Advantage	Disadvantages
A single central laboratory	simple quality management training	work overload and time-consuming results
A central and other qualified laboratory	the NRL can devote more time to other demanding activities Increased coverage and speed for the detection of TB RR / MDR	Complex quality management training

Methods recommended by WHO for conducting the susceptibility test

In culture medium (phenotypic)

with first and second line drugs

- *Method of the proportion in solid medium*
- *The adaptation of the proportions method in MGIT*
- *Ratio (or proportion) of resistance*
- *Absolute concentrations.*

only with I and R

- *Nitrate reductase or nitrates, the Griess method*
- *Colorimetric methods or REMA (resazurin microplate assay)*
- *MODS (microscopic observation drug susceptibility)*

Molecular (genotypic)

for R, I, FQN and second line injectables

- *LiPA*

for R

- *Automated real-time PCR in cartridges (Xpert MTB / RIF).*

For its part, the NRL must have implemented both fast and conventional methods. The rapid method can be used to detect resistance to R and possibly also to I, and some second line drugs, and to select cases that will then be subjected to more complete and costly investigation. As mentioned the fastest are the molecular ones. They are also useful for investigating isolates that carry mutations that limit their reproduction in vitro or that are dead due to poor preservation of the sample. Then, conventional methods will be used to solve doubts, investigate the susceptibility to other drugs and strains with borderline or abnormal behavior. For these purposes it is recommended that the NRL maintain its technical capacity and demonstrated quality to perform the reference method of the proportions in solid medium. This method is also the reference method for evaluating new methods. MIC determination is useful for evaluating new drugs, whose ST is not yet standardized, or assessing the level of resistance to any particular drug in conflicting cases.

Some general characteristics of the methods developed by the industry that need to be considered when selecting and combining them are compared below.

Characteristics of the methods developed by the industry and recommended by WHO for the implementation of the ST

	Xpert MTB /RIF	LiPA	Liquid medium with automated reading
Application	Detection of the complex *M. tuberculosis* and its resistance to R	Detection of *M. tuberculosis* complex and its resistance to injectable R, I and FQN Identification of other mycobacteria	Detection of all mycobacteria in culture ST of *M. tuberculosis* to drugs of 1st and 2nd line.
Susceptibility to detect the bacillus of the tuberculosis	greater than BK lower than the culture	high with samples with BK + limited with samples BK-	Maximum detects more cases even than the conventional culture in LJ
Time for production of results	in the day (The reaction is complete in 2 hours)	two days	For detection of TB bacillus: 2 to 4 weeks (depending on the amount of bacillus present in the sample) and 1 or 2 additional weeks for ST
Number of samples that can be processed per day of 8 hours	12 with the team of 4 modules can be processed by a single person	depends on the available human resources (HHRR) and molecular biology equipment	Usually the limit is not given by the team but by the available HHRR (a)
Personal time per processed sample	less	medium	higher
Biosafety level required	low	high	high
Sample Requirements	volume of at least 1 ml; it is not so critical the temperature and the delay since the collection	The temperature and the delay since the harvest are not so critical	fresh not overexposed to heat
Calibration / Maintenance	at a distance, faulty modules can be replaced (replacement delays have occurred)	Normally available in the country for generic equipment	requires technical assistance from the signature in the country, in case of malfunction

(a): The MIGT 960 can incubate 960 tubes, the MGIT 320 can incubate 320 tubes

HHRR: Human Resources

Long-term interventions and, therefore, diagnostic methods that can be sustained over time are necessary to control TB. Then, the rapidly increasing demand for rapid diagnosis should be satisfied after a rigorous analysis of the alternatives in light of:

- the strategies of the National TB Program
- the resources and organization of the laboratory network
- the experience and acceptance of laboratory staff
- the initial investment and expenditures to sustain in the long term
- (even after the withdrawal of potential donors or partners)

  basic logistics,
  the regular supply of inputs,
  the maintenance of infrastructure, biosafety and laboratory equipment,
  quality management

It is necessary to validate each method adopted in the scenario where it is to be used and to include it in the continuous quality management system. Any innovation should be induced in laboratories that have demonstrated competence, are accessible to the health system, and can concentrate the research of patients prioritized to apply innovation.

Infrastructure and equipment

In general, a laboratory that performs ST requires

- continuous and reliable power supply
  (Automated culture reading equipment in liquid medium may have its own battery that keeps them operating for a limited time but not the rest of the equipment; if interruptions are frequent, a UPS must be installed for each critical equipment or generator. back-up for the whole laboratory, the BSC must have UPS or be connected to a generator).
- room temperature regulation.
  to 24-26°C, for the comfort of the personnel, for the equipment and to store the commercial reagents / equipment that do not require refrigeration but that can be altered to very high temperatures. Air conditioners should be more powerful in areas where heat dissipating equipment (autoclave, refrigerators, etc.)
- spacious warehouse for supplies .

The World Health Organization has defined the following minimum biosecurity requirements for each method

Minimum requirements for biosafety recommended by WHO for tuberculosis research

Biological Risk Level	Activity	Essential, minimum infrastructure to reduce and control risk	
		Biological	amplicon transfer
Low	Preparation and treatment of samples for BK Culture n with Ogawa Kudoh method direct molecular tests on closed cartridges with automated systems (eg Xpert MTB / RIF)	a) Laboratory separate from public access and administrative areas. b) Waterproof, smooth surfaces. c) Ventilation by windows or mechanics to renew the air. d) If there are air conditioners, *split* type, properly located so as not to cause airflow in the work area.	
Moderate	Treatment and centrifugation of samples for Culture ST Direct (By any bacteriological means or LiPA)	a, b, d … Plus e) extraction of air to an open area, non-traffic, air renewal (6 to 12 changes per hour), without recirculation of air to other areas of the plant. f) Autoclave in the risk containment laboratory or in a contiguous laboratory. g) Centrifugal with detachable tube holder and lid h) CSB class I (EN12469/NSF49) o II A2 (NSF49) o class II (EN12469) Correctly installed recertified at least annually connected to an emergency generator (battery) that provides at least 15 minutes of autonomy	3 separate environments with restricted access for LiPAs or other "open" molecular methods
High	Processing of positive cultures and / or suspensions of viable *M. tuberculosis* for: ID Direct ST (By any bacteriological or molecular means)	a, b, d, f, g, h . Plus i) Anteroom (double door entry to containment laboratory) j) Air addressing from clean areas to the contaminated ones and from there to the exterior after passage through HEPA filter (Via the BSC pipeline connected to the outside if there is no other system)	

Note that it is within the moderate risk level of direct DST (the nitrate or proportions method) as long as tubes with positive cultures are not opened in the laboratory. This implies that laboratories that only meet requirements to operate at moderate risk should not identify the microorganism developed in culture medium. An alternative for these cases is to include in direct ST I, R and PNB that is an inhibitor of the *M. tuberculosis* complex. If the culture has different characteristics of *M. tuberculosis and / or drug resistance*, it should be sent to the reference laboratory to verify its identification and susceptibility. Molecular methods, on the other hand, ensure the identification of low-risk (closed-label) or moderate-risk (LiPA) *M. tuberculosis* complex if done directly from clinical samples.

Any procedure involving the opening of positive cultures of *M. tuberculosis* is framed within the high-risk level.

Equipment and supplies

Requirements of the methods selected in these Technical Guides for the realization of the ST

Requirements	Non-commercial methods		Commercial methods		
	Proportions in LJ Nitrate reductase	Proportions in Agar	With liquid mediums	LiPA	Real-time PCR in cartridges
Equipment (*)	Incubator Coagulator		Automated system for detection of growth and software (optional)	Water system Ultrapure free of nucleases Thermal Cycler Sonicator water bath heater block Microcentrifuge Cabin DNA free	Equipment Computer and software micropipettes
Supplies	Availables	may need to be managed in some countries	It may be necessary to manage the authorization of some input in some countries		
	economics	Medium cost	Greater Cost		
	stables long-term (except eggs)	expiration medium term	Maturity in the medium term		
Commercialization	Several local manufacturers and suppliers		Dependence of a single manufacturer (and possibly supplier) of reagents, equipment and software		

(*) In addition to the basics necessary for all methods, for the preparation of reagents and samples or isolates: distiller, refrigerators, freezer, electronic scale, vortex stirrer, centrifuge, BSC UPS, autoclave

These marketing requirements and characteristics need to be carefully analyzed, and the difficulties previously solved before adopting a method, particularly in scenarios with low or variable budgets from one year to the next, lack of efficient support for importation, disorganized procurement procedures or not reliable, local distributors with no interest in licensing and importing reagents, or with complex customs interventions. It should be considered that the cost of imported reagents can result to be 2-5 times higher than the catalog price, according to the taxes and marketing conditions that are applied in each country.

For the case where it is necessary to acquire inputs with expiration date, the demand for delivery of inputs with an appropriate useful life must be established in the purchasing processes according to the frequency with which the purchase is made. A convenient alternative is to agree purchases with partial deliveries of the inputs, in dates scheduled during the year. On the other hand it should also be considered that some antibiotics and some inputs used for commercial methods require maintaining the cold chain during transport and storage. The Xpert MTB / RIF system requires plenty of space with adequate temperature for storing cartridges.

For commercial methods it is very important to establish good contracts with companies to ensure equipment installation, staff training, software updating, regular calibration and maintenance, and immediate technical assistance in the event of a malfunction (even in laboratories away from the capital of the country in the case where the ST is decentralized).

The ST laboratory must have relatively complex equipment and refrigerators that maintain their temperature strictly to ensure the preservation of culture medium and reagents. Therefore, it is necessary that you have a regular preventive and corrective maintenance service. Any disruption in the operation of essential equipment can lead to discontinuation of the ST supply and, therefore, to the abandonment of the assistance of patients who require this result to receive the most appropriate treatment.

Human Resources

Profile and training of personnel required to carry out ST

	Non-commercial methods		-commercial methods		
	Proportions in LJ Nitrate reductase	Agar Ratios	With liquid mediums	LiPAs	Real-time PCR in cartridges
Support for					
Purchasing Processes	1 time per year	frequent	frequent	frequent	frequent
Preparation of medium and reagents	permanent	permanent	no	no	no
Training and Experience	Culture and ST		Culture and ST	Sample Processing	
				Molecular Techniques	
			Software Operation		Software Operation

Registration system

Regardless of the method adopted, the laboratory should have one or more computers and a database that allow it.

- record material input,
- prepare lists of tests to be carried out,
- record all laboratory results,
- to track at all times the studies carried out with each material, its results and those responsible for each study,
- prepare and print reports,
- analyze information from each patient, the set of patients or groups of patients selected as required for epidemiological surveillance.

To optimize the time and avoid errors, the laboratory should avoid the multiplicity of written records and the repetition of entry of the same information in several registers.

The laboratory database should contain the minimum information required by the TB Control Program application form, including the identification of risk factors for drug-resistant TB. It must also contain the identification and characteristics of the samples or insulation received.

The database should allow the analysis of the information to provide the following minimum information for MDR TB surveillance.

Minimum Information for Surveillance of MDR-TB

	Number of cases						
	TOTAL	with ST to R	RR	with ST to IR	MDR	with ST to DSL	XDR
TOTAL							
Risk Category							
Failure							
Recovery after loss of follow-up							
Relapse							
Contact MDR case							
HIV Positive							
Deprived of freedom (prisoners)							
…………………………..							
…………………………………….							
Delay in diagnosis (*)							
Number of cases with information							
Average (days)							
Minimum (days)							
Maximum (days)							

(*) Days between the identification of the patient with risk and the availability of the result of the ST for I and R
According to laboratory records

It is considered a day of identification of the patient with risk to that in which
TB is diagnosed if universal ST is offered or
The bacteriological result indicating failure of treatment or
TB is associated with HIV
the result of the laboratory detected -MDR TB in the focus of that case

Note that surveillance requires the analysis of the ST results of the TB cases identified in the corresponding period and not of the samples tested. For the cohort evaluation, it will also be necessary to know the number of MDR-TB cases with bacteriological conversion, before and after 6 months of treatment.

The information system of a reference laboratory should also be useful for monitoring in real time:

- the request, reception, quality and stock of inputs
- the delay in releasing the results of the diagnostic tests.
- the results of internal and external quality controls
- the stratified workload according to origin and patient characteristics, type of test performed, etc,
- incidents and laboratory accidents
- development of human resources (training).

NRL prepared for high biological risk should have one or more computers located within the BRCA and networked with one or more computers located outside. It is also convenient to have a scanner inside the BRCA to capture images of documents, save them and make them available on the network. In this way, the written records within the BRCA are minimized and even eliminated. At the same time, technical and professional staff can observe results, prepare and print reports and analyze information in clean areas, without needing to remove records from the BRCA.

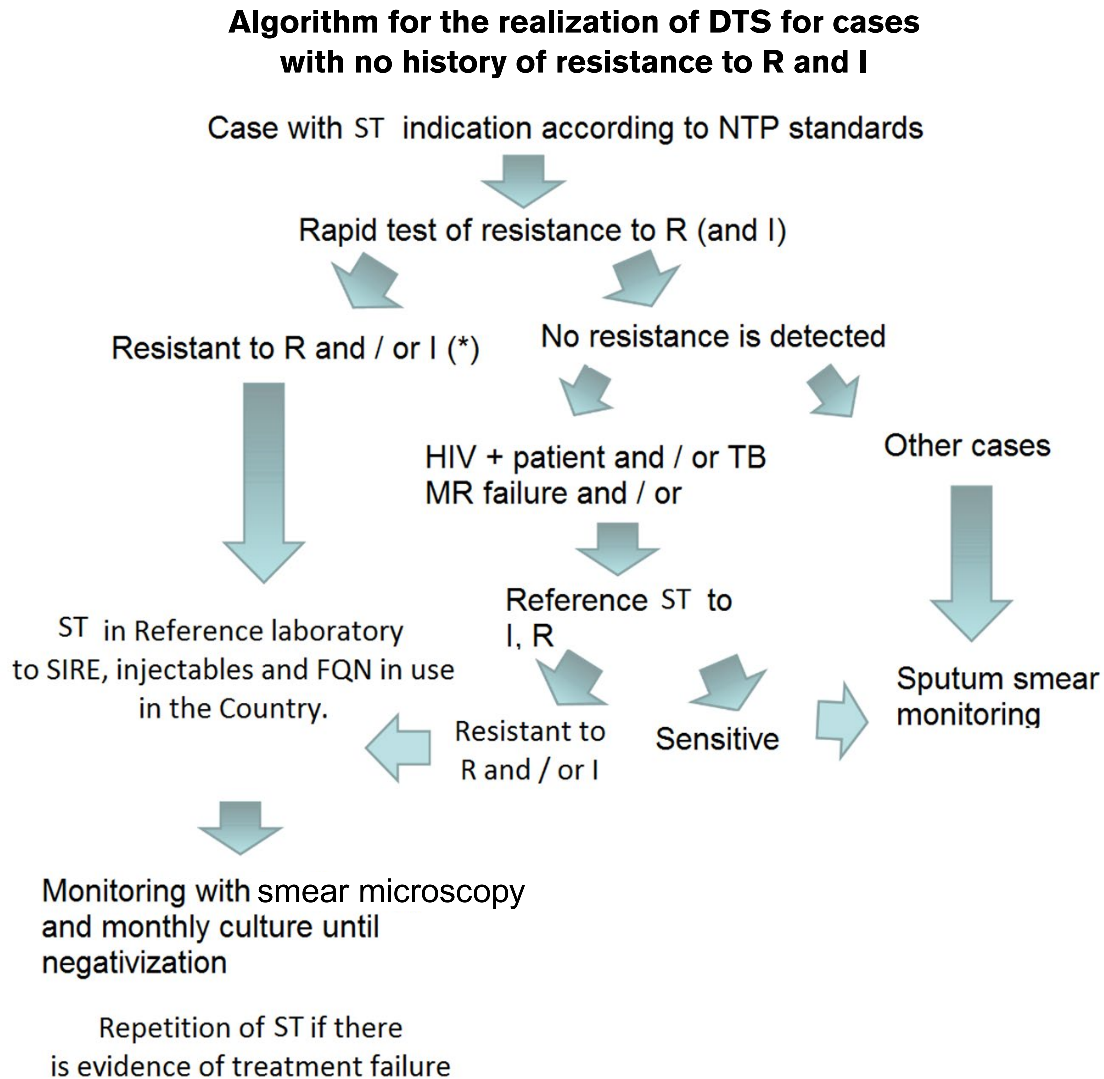
Automated culture reading equipment software can be optional acquisition and allow visualization of growth curves of the isolates. This may be superfluous for detecting bacilli in cultures, but it is of greater importance for ST reading. The curves allow easy identification of isolates that have a borderline behavior towards an antibiotic, or anomalies in the tests. It is possible to have remote access, from a clean area, to the information produced by these equipment, in real time.

When adopting commercial methods, dependent on a computer and software, it is necessary to ensure in the contracts the absolute ownership for the health system of the information that is entered in the database and retention of that information in the case in which it is decided not maintain the system. For the latter purpose, it may be necessary to request the assistance of the companies to create an interface between the software of the equipment and the employee usually by the laboratory, so that the first one transfers and stores the information in the second. Another option is to request the adaptation of the equipment software to contain all the aforementioned minimum information and ensure its export to a database that can be stored and analyzed by the laboratory staff. Also, the connectivity of the NRL base with the National TB Control Program should be sought, and the systems that may exist online in the country for case management and / or epidemiological surveillance.

The information in records on paper and in databases should be safeguarded for as long as required by institutional and national standards. The databases should be stored and protected in backup copies, one of which should be kept outside the building occupied by the laboratory, in the event of an accident. All records must be made available to the health system in the long term. At the same time it is necessary to protect the confidentiality of stored data and avoid undue alterations. To this end, rules should be established to safeguard the data and, in the case of databases, the adoption of passwords for authorized personnel.

Work Algorithm

In light of the available knowledge and treatment schemes applied up to the time of writing of these Guidelines it is advisable to investigate the susceptibility to the drugs following the following sequence.



For areas or populations in which the prevalence of RR is very low, it is advisable to quickly verify results with a second rapid test performed with a new sample of the patient.

The initial ST to I and R should be carried out directly, by guaranteed bacteriological methods, whenever a positive BK sample is available. In other situations, the sample of the patient must first be cultured and then the indirect DST. Laboratories without suitable conditions for culture with methods that require centrifugation, away from a reference laboratory or that do not have a frequent sample transport system, can sow the samples using the Kudoh-Ogawa method, and send the tubes sown to the laboratory reference as soon as possible.

For patients with MDR TB the set of drugs to be tested may include ethionamide or protionamide and those that are more recently being tested include bedaquiline, delamanid, linezolid and clofazimine. It is necessary to keep the updated knowledge on the evidence published in relation to the accuracy and value to predict success or failure of the therapeutic scheme that DST have with these drugs.

As in all TB cases, BK and especially culture have a critical role to monitor the treatment of a case with MDR TB that is receiving a second line drug regimen. With the schemes recommended at the time of writing this Manual, ST should be repeated at the 6th month of treatment with second line drugs to guide the continuation of therapy. And then, if the patient remains on positive culture, it should be repeated 6 months after each change of Schedule.

Access to diagnosis and good guidance for the treatment of MDR-TB depends on

- *an alert and agile health system*
  to detect and document risk factors,
  take and transfer samples to the laboratory
  with the information needed to trigger rapid
  studies
- *networks of organized laboratories, with sufficient resources,*
  clear working algorithms, quality assured
  work,
  agile channels of communication with
  health
  services
  and with the TB Control Program
- *efficient national reference laboratories with*
  highly trained human resources, good
  infrastructure

BIOLOGICAL MATERIAL TO BE INVESTIGATED

Sampling and preservation

The indications concerning biosafety, containers, collection and number of samples to be collected, according to the location of the lesion, have been described in detail in the first part of this Manual dedicated to BK. All the recommendations presented there are valid to facilitate and expedite the receipt of samples, to ensure their correct identification and registration.

Selection of isolates

The laboratory carrying out the ST can receive samples to perform the ST directly from them, and / or isolates obtained by other laboratories that perform cultivation.

The laboratories of the network that cultivate must know the instructions to select and prepare the positive cultures that must be referred to the laboratory that performs the ST, observing the following recommendations:

- Prefer primary culture and not a subculture, since in the subculture can be lost some clone present in the original isolation.

- Send the culture to the reference laboratory as soon as it develops, either in solid or liquid medium, to avoid delays in the diagnosis.

- In the case where it uses only solid medium, the following recommendations will be taken into account to benefit the representativeness of the results.

Cultivated material	Tubes to be sent to the NRL for ST
a sample investigated for diagnosis with abundant bacilli (positive AFB result 1+ to 3 +)	at least one tube with abundant development (1+ to 3+) If, exceptionally and due to technical failures, the development was smaller, send several tubes that have at least 20 colonies
a sample with few bacilli (negative AFB result or 1-9 AFB / smear) or a patient undergoing treatment) with culture with development less than 1+	all tubes in which there are colonies

- If the laboratory that has carried out the culture has adequate biosafety conditions for high biological risk, verify by microscopy the presence of mycobacteria and the absence of contaminants (bacteria, fungi, yeasts, etc.) when the appearance of development is not typical of M. tuberculosis. If contaminated, proceed to decontamination prior to shipment, and cultivate a new patient sample, in case there is no other positive culture of the same patient.

If a ST is to be repeated or another drug is performed with additional drugs and no further patient isolation is available, the control tubes of the first ST should be operated, never with drug-containing media because resistant clones were selected there.

Transport of biological material

- Verify that the sent tube (s) is clearly labeled (if the medium is solid, place the label on the opposite side of the colony development)

- Complete the ST application form

- Condition the tubes in the appropriate containers and transport them as described in Appendix I part 2 of the Manual (culture) and in Appendix I of this part 3 (Susceptibility Test).

INCOME AND ORGANIZATION OF THE MATERIAL

Work should be systematized to minimize risk, avoid confusion and be able to track procedures in case of doubt.

Written records cannot graduate from the BRCA without being previously sterilized. Thus, from the outset, it is convenient to record the information in databases, and scan applications or other documents, using computers connected in network outside and within the BRCA.

- Register in the database the information contained in the application form for bacteriological studies corresponding to the case and the material received.

- Record the results of identification tests and ST performed by the laboratory that sends the material (if it would have done them).

- Check in the database the antecedents that are known of the patient.

Admission to the BRCA must respect the relevant SOP (see Appendix I).

Inspect material received within a BSC with good functioning certified

- Display, verify and record the following characteristics of the material received

    - Number assigned by the laboratory submitting the insulation
    - Date of specimen that was cultivated
    - Date of culture.
    - Indication of if it is primo primary culture or reseed
    - Culture medium in which the isolation is received
    - Degree of culture positivity (number of colonies or crosses, according to standards), if it is a solid medium.
    - Aspect of culture.
        - Features compatible with M. tuberculosis
          (rough and non-chromogenic colonies in solid medium, or non-chromogenic flocs in liquid media)
        - Features not compatible with M tuberculosis
        - Contaminated
        - Dry
        - With excess of water

  - Assign an own registration number of the laboratory that performs the ST to any material that enters. This number will be unique for all procedures and reports that correspond to each material received.

  - Record the numbers of samples or isolates that cannot be processed (due to spillage, poor condition, contamination) and arrange for new material to be requested immediately, whenever it is possible to take a new sample.

  - Elaborate a list of work with the tests to be performed for each sample or isolation, following the algorithm adopted by the country (see as guide the one presented in the previous chapter).

- Classify biological material, depending on whether the sample is for direct ST, or isolation for indirect ST.

- Sort by number, on a tray, samples requiring ST directly to R, or to I and R.

- Sort by number, on a tray, samples that require direct ST to 1st and 2nd line drug.

- Order in a rack, according to their number, pipes with insulation requiring ST to R or to I and R.

- Sort in racks, according to their number, the tubes of the insulation that require ST to 1st and 2nd line.

- Verify that the material necessary for testing is available before starting the tasks, controlling the list of materials contained in each SOP laboratory. In general, for all methods employing culture media, the following material should be routinely available in the risk containment laboratory, preferably in an amount greater than that strictly necessary for any contingency.

Material and equipment to be available in the BRCA

- Markers with ink for glass.

- Latex gloves.

- Autoclavable flasks for plastic tip disposal (if autoclaving of material is immediate after completion of tasks, in an autoclave located inside the BRCA or in an adjoining area, the use of hypochlorite is not necessary).

- Waterproof, autoclavable bags of different sizes for the disposal or recycling of material that will be previously autoclaved.

- Bags and containers suitable for disposal of sharps

- Absorbent paper to cover the surface of BSC
- Alcohol 70 % to disinfect BSC.

- Sodium hypochlorite 1% to disinfect other surfaces

- Disposable cloths for surface cleaning.

- Bacteriological handles, preferably disposable, sterile

- Very transparent, disposable glass or plastic tubes with screw cap, 15 ml capacity, sterile.

- Plastic tubes with 1-2 ml capacity, screw cap, sterile.

- Glass bottles and tubes with distilled, sterile water
- Graduated plastic pipettes, individually wrapped, from 1 to 10 ml, sterile.

- Single-wrapped graduated bulb-shaped plastic pipettes, 1 to 3 ml, sterile

- Pro pipettes or automatic pipettors
- Racks made of stainless steel or polypropylene with adequate capacity.

- Trays made of stainless steel or polypropylene, disinfectable.

- Stainless steel or polypropylene container, with lid, to locate the material that must be autoclaved and discarded.

- Stainless steel or polypropylene container, with lid, to locate material that must be autoclaved and recycled.

- Container of stainless steel or polypropylene, with lid, to locate the pipettes that must be autoclaved and discarded.

- Stainless steel or polypropylene container with lid to locate the pipettes to be recycled.

- Disinfectable boxes, preferably autoclavable, capable of incubating the total of tubes or plates of 1 line or 2 line ST (tubes in upright position or stacked plates, according to the method used).

- First aid kit for incidents / accidents (see Appendix I).

The following basic equipment, with good certified performance, must be available: autoclave located in the same BRCA or in an adjacent area, refrigerators, BSC, incubator or stove or vortex type agitator.

The characteristics of the necessary equipment and equipment are described in Appendix II and III of Parts 2 (Culture) and 3 (Sensitivity Test) of this Manual.

In describing each method will mention the additional material or equipment to the above mentioned that particularly require. For molecular testing using LiPA, it is necessary to equip other areas of work, outside the BRCA.

Perform all procedures that require the opening of tubes with M. tuberculosis isolates within a BSC located in the BRCA that meets the WHO minimum requirements

Extreme precautions to minimize biological risk and cross-contamination

In the page 49, part 2 of this Manual on Culture, outlines recommended practices to minimize risk and cross-contamination. In Appendix I to this part 3 dedicated to the ST the additional safety measures for the BRCA are presented. It is recommended to review them in detail.

Identification of Mycobacterium tuberculosis

- Check the purity of the cultures and identify if the microorganism to be investigated is M. tuberculosis by a rapid test, before performing the indirect ST, or after obtaining the development of a direct ST.

In Part 2 this Handbook (Culture) detailed how to analyze the morphology to preliminarily identify the characteristics compatible with M. tuberculosis and some basic biochemical tests to confirm the identification.

This section describes other useful methods that can be performed from the bacterial mother suspensions, immediately prior to or simultaneously with the ST

Growth inhibition test with p-nitrobenzoic acid in LJ medium.

Principle

PNB has been shown to inhibit the growth of species that make up the M. tuberculosis complex and other species of mycobacteria. However, it has also been reported that, exceptionally, some isolates from other

species *(M. kansasii, M. marinum M. xenopi and M. gastri,)* may also be inhibited.

In laboratories that can only operate with medium risk, and that perform ST directly from samples, the inclusion of this inhibitor facilitates the identification of the germ, without the need to open tubes with the culture already developed.

Biological material to be investigated

- Mother suspensions prepared from samples with positive BK Isolation from solid or liquid medium

- Control strains:

Sensitive	*reference strain of M. tuberculosis sensitive to drugs, preferably M. tuberculosis H37Ra*
Resistant	*reference strain of M. avium.*

Mediums of culture

For each isolation and control strain to investigate

- 1 tube with LJ medium
- 1 LJ tube with PNB (LJ-PNB)
  belonging to the same batch, with controlled quality, labeled with the number of each material to be investigated.

Procedure

The test can be performed simultaneously with ST directly or indirectly to anti tuberculosis drugs.

- Inoculate a drop of the stock suspension or culture obtained in liquid medium in the LJ tube, and a drop in the LJ-PNB tube.

- Distribute the inoculum from the apex to the bottom of the tube.

- Incubate tubes upright at 37 ° C for 15 days.

Reading and interpretation of results

Compare the growth of the LJ and LJ-PNB tubes of each strain and interpret as follows:

LJ tube	LJ-PNB Tube	Inhibition development	It is identified as
≥ 20 colonies	without development	Yes	*M. tuberculosis* complex
≥ 20 colonies	more than 20 colonies	No	environmental mycobacteria
< 20 colonies or negative	-----	----	uninterpretable

- Ensure this interpretation by observing the morphology and pigmentation of the colonies.
- If in doubt, perform a definitive test (niacin, lateral immune chromatography or molecular test), or send the isolation to the reference laboratory

Detection of MPT64 antigen by lateral flow immunochromatographic assays

Principle

The MPT64 antigen is specific for the M tuberculosis complex and can be identified by lateral flow immunocromatographic assays in a commercially available device consisting of a cassette containing a membrane with 4 areas:

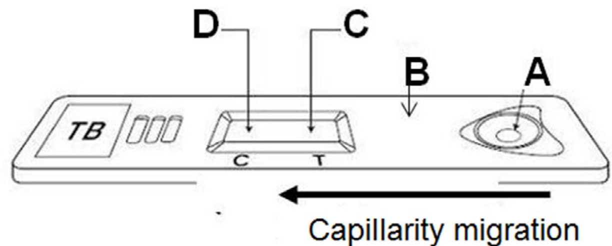
A. intended to deposit the bacterial suspension

B. contains labeled anti-MPT64 mouse antibody which binds to an epitope of that antigen, if it is present in the investigated isolate

C. has immobilized on the membrane a second anti-MPB64 mouse antibody which recognizes another epitope of the antigen and traps the complex formed in area B

D. has immobilized on the membrane a polyclonal anti-mouse immunoglobulin antibody which captures the excess of monoclonal antibody that moves from area B; allows you to check if that antibody is in good condition and if you have successfully migrated.

Bacterial suspension and non-immobilized antibodies migrate by capillarity.



Limitations

The equipment does not detect the mixture of environmental mycobacteria with the M. tuberculosis complex.

Additional Materials

1 ml glass graduated pipettes may be used, but it is more practical to use micropipettes since the volume to be dispensed is small.

- Automatic micropipette 10 to 100 µl
- Automatic micropipette 100 to 1000 µl
- Tips for dispensing 200 µl, with aerosol protection
- Sterile distilled water
- Plastic tubes of 0.5 to 1 ml capacity, with pressure cap or thread
- Rack for tubes of 0,5 to 1 ml.

Biological material to be investigated

- Alcohol-resistant bacillus isolates
  If cultures grown in liquid media are investigated directly employ them.
- If isolates obtained from solid media are investigated, proceed as follows:

- Take with handle the colognes and transfer to the plastic tube
- Add, with micropipette or pipette, 200 μl of sterile distilled water or buffer provided by the manufacturer
- Close and shake the vortex.

Take with handle the colognes and transfer to the plastic tube
Add, with micropipette or pipette, 200 μl of sterile distilled water or buffer provided by the manufacturer
Close and shake the vortex.

- Control strains:
Positive: *M. tuberculosis* H37Ra
Negative: *M. avium* reference strain

Reactive

- Equipment of commercial origin

Procedure

Follow the manufacturer 's instructions. In general, proceed as follows:

- Open the envelope containing the cassette at the time of use
- With micropipette, place 80-100μL of the bacterial suspension, or culture in liquid medium in area A
- Place the cassette in a tray in a horizontal position and allow to stand at room temperature for 15 minutes.

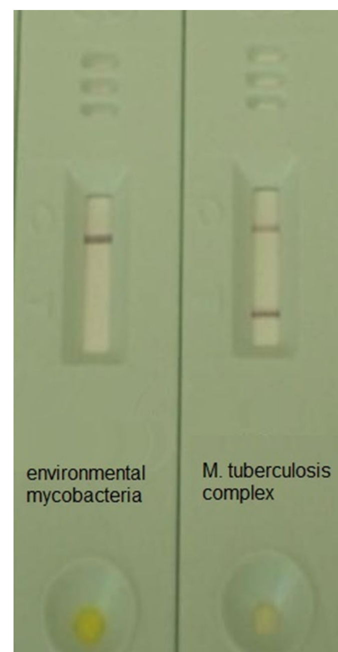
Reading and interpretation of results

Do not read after 60 minutes of placing the material to be investigated, as there may be changes in the results when the support dries.

Apparition of band in area		It is identified as
C	D	
yes	Yes	Complex *M. tuberculosis*
no	yes	environmental mycobacteria
--	no	Uninterpretable

If the result is not interpretable, repeat the test. There could be deterioration of reagents or procedural errors. If again it is not interpretable, or in case of doubts due to inconsistency of results, perform another definitive test (molecular test, niacin).

Always ensure the interpretation of the result by observing the morphology and pigmentation of the colonies.



Susceptibility test to antituberculosis drugs and chemical inhibitors

The *M. tuberculosis* complex is composed of highly related species: *M. tuberculosis, M. africanum, M. canetti, M. bovis (*and the BCG strain that supposedly derived from *M. bovis), M. microti, M. caprae and M. pinnipedii.*

M. tuberculosis, M. africanum, M. canetti, M. bovis can cause TB in man, and BCG can cause some post-vaccination complications. M. tuberculosis is the prevailing species among those that originate TB in humans. M. bovis causes TB in various types of domestic and wild animals and can be transmitted by them to man; it is possible to find it, particularly, in individuals with TB dedicated to the breeding or processing of bovine products. M. africanum is a heterogeneous group, with intermediate properties between M. tuberculosis and M. bovis, which has been isolated mostly in equatorial Africa. M. canetti has been very exceptionally isolated. M. microti, M. caprae and M. pinnipeddi have as main hosts to certain animals (rodents, goats and seals respectively). However, a case of TB has been described in man caused by M. microti, associated with immunosuppression.

The differentiation of the species within the *M. tuberculosis* complex is not determinant for the selection of the scheme to treat TB in humans; the treatment depends mainly on the susceptibility profile of the microorganism against antibiotics. However, it may be of epidemiological interest to differentiate TB caused by *M. bovis.* On the other hand, a complication caused by the BCG vaccine is usually not treated with antibiotics, so its differentiation is of clinical interest.

The PNB tests, detection of MPT64 by lateral flow immunochromatographic assay, Xpert and the LiPA described below, allow the identification of *M. tuberculosis* complex, differentiating it from environmental mycobacteria. But the distinctions of the species that integrate this complex require additional genetic or phenotypic tests. In particular, the addition to ST performed in LJ tubes with certain antibiotics and chemical inhibitors is useful to resolve the differentiation between *M. tuberculosis, M. bovis* and BCG. This is appropriate when using this medium for ST and is facing a case of TB with exposure to *M. bovis* infection or a child (or more exceptionally an adult) with manifestations and antecedents indicating a complication caused by the BCG vaccine. Thus, it is possible to solve most of the clinical situations in which the doubt arises that an isolation identified as belonging to the *M. tuberculosis* complex is not of the species *M. tuberculosis.*

Useful tests for the differentiation of the complex M. tuberculosis and the species that integrate it that are more frequent in the human clinic

	Differentiation of the *M. tuberculosis* complex from the rest of the mycobacteria		Differentiation of the most common *M. tuberculosis* complex species in human clinical samples		
	Mycobacteria environmental	Complex *M. Tuberculosis*	*M. tuberculosis*	M bovis	BCG
Development speed	slow or fast	slow			
macroscopic aspect of the colony	smooth /may have pigmentation	AFB arranged in rope	rough pigmented	smooth, flattened, non-pigmented	rough pigmented
microscopic appearance	Long and filamentous or short AFB, mostly disaggregated				
niacin	negative		positive	negative	negative / positive
catalase at 68 ° C	positive	negative	positive	negative	negative
nitrate reduction	negative/ positive				
GeneXpert or other molecular test designed to detect markers of the *M. tuberculosis* complex	negative	positive			
Detection of MPT64 by immunochromatography	negative	positive			
Susceptibility to chemical inhibitors and antibiotics					
PNB	resistant	sensitive			
PAS	resistant	sensitive (*)			
TCH			resistant	sensitive	sensitive
Cycloserine			sensitive (*)	sensitive	resistant
Z			sensitive (*)	resistant	resistant

PNB: p-nitrobenzoic acid or 4-nitrobenzoic acid
PAS : p-amino salicylic acid
TCH : hydrazide of thiophene-2-
Z: Pyrazinamide

(*) result corresponding to the wild-type strain, but may become resistant if exposed to the action of the drug.

As can be seen in the table, it is possible to quickly and simply identify the *M. tuberculosis* complex with a molecular test or by detecting the MPT64 Ag, and then differentiate *M. tuberculosis, M. bovis* and *BCG* by adding to the ST performed on LJ TCH and cycloserine. The niacin and nitrate reductase tests can support the results of these tests.

ST to PAS, TCH and cycloserine in LJ are performed by the proportions method following the procedures described in this Handbook (see Löwenstein-Jensen's Medium Measurement of Antituberculosis Drug Testing in Solid Media, Proportion Method). Also described there are tests with Z and PNB.

The niacin and nitrate reductase tests were described in part 2 of this Culture Manual *(see Identification of M. tuberculosis).*

SUSCEPTIBILITY TESTS IN SOLID MEDIA

Preparation of bacillary master suspensions

From samples
(for direct ST)

Biological material to be investigated

Sample of sputum or other with positive AFB 2 (+) or 3 (+) processed following the procedures for cultivation detailed in part 2 of this Manual (Culture). Samples that most commonly meet this requirement are those of sputum and biopsies or ganglion secretions.

Depending on the case, working with:

- the precipitate obtained after the decontamination process of the sample, if it is a sample that must be decontaminated before being inoculating in the culture media
- the precipitate obtained after a centrifugation of 15 minutes at 3000 g, in the case of a liquid with a volume greater than 3 ml, taken with asepsis.
- the maceration of a tissue sample taken with asepsis.

Procedure

- Suspend the material to be investigated in a tube containing 1 ml of phosphate buffer pH 6.8 labeled with the sample number.

From isolates obtained in culture media

(for indirect ST)

Additional Materials

By culture to be processed

- 1 bottle or tube of glass very resistant, or of very transparent plastic, with capacity for 15-20 ml, with screw cap, containing 5-10 glass beads, sterile, labeled with the number of each insulation to investigate.
- Mc Farland 1 or BCG 1mg / ml turbidity pattern.

Biological material to be investigated

- Isolates identified as *M. tuberculosis*

Procedure

For culture in solid media

- To take with the handle bacillary mass, trying to take material of all the colonies, avoiding drag medium of culture. In the case of isolation with poor development, take bacillary mass from all available tubes.
- Place the material in the tube or jar with glass beads. Discard the handle.
- Ensure that the lid of the tube or bottle is closed and vortexed until completely disassembled, checking that there are no lumps.
- Add 1 ml of sterile distilled water.
- Ensure the closure of the tube or bottle cap, and vortex until homogenized.
- Leave to rest 15 to 30 minutes, to sediment lumps and decrease the risk of dispersing aerosols.
- Verify that there are no lumps in the supernatant; if detected and can not be disassembled by repeating the two previous steps, prepare the suspension again from the beginning.
- With a pipette, gently remove the supernatant (without touching the lumps that may have decanted) and transfer it to a tube with screw cap. Discard the pipette.
- Add sterile distilled water, little by little, until the opacity matches, at first glance, the Mc Farland 1 or BCG 1 mg / ml turbidity pattern.
- Allow settling for at least 15 minutes before proceeding with dilutions.

For cultures in liquid media

- Let the tube or bottle containing the isolation settle for 15 to 30 minutes
- Remove with a pipette approximately 5ml of the suspension (without touching the sediment) and transfer it to the tube or jar with glass beads. Discard the pipette.
- Ensure that the lid of the tube or bottle is closed and vortexed until the suspension is homogeneous, free of lumps.
- Leave to rest 15 to 30 minutes, to sediment lumps and decrease the risk of dispersing aerosols.
- Verify that there are no lumps in the supernatant; if detected and cannot be disassembled by repeating the two previous steps, prepare the suspension again from the beginning.
- With a pipette, gently remove the supernatant (without touching the lumps that may have settled in the bottom of the tube or bottle) and transfer it to a tube with screw cap. Discard the pipette.
- With a pipette, gently remove the supernatant (without touching the lumps that may have settled in the bottom of the tube or bottle) and transfer it to a tube with screw cap. Discard the pipette.
- Allow settling for at least 15 minutes before proceeding with dilutions.

Dilution of bacillary the stock suspension

In the following descriptions the nomenclature with superscripts is used to mention the dilutions:

	dilution
10^{-1}	1:10
10^{-2}	1:100
10^{-3}	1:1.000
10^{-4}	1:10.000
10^{-5}	1:100.000
10^{-6}	1:1.000.000

Additional Materials

By ST to perform

- 6 tubes with screw cap containing 9 ml of sterile distilled water with the labels 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, arranged in series on a rack, leaving two (one to locate the tube containing the stock suspension and one to systematically run each tube after completion of each dilution, so that errors are avoided).

Note:

For ST in agar medium dilution 10^{-6} is not necessary

For direct ST, dilution 10^{-6} is not necessary and 10^{-5} may also be unnecessary depending on the outcome of the AFB (see below the description of direct ST)

Biological material

- Bacillary suspensions prepared with the samples or isolates to be investigated using ST.

Procedure

- Smoothly and without touching any sediment that could be observed, pipette 1 ml of the stock suspension into the tube containing 9 ml of distilled water labeled 10^{-1}. Close the tube cap securely. Discard the pipette. Vortex Shake.

- To pipette 1 ml of the tube with dilution 10^{-1} into the tube containing 9 ml of distilled water labeled 10^{-2}. Close the tube cap securely. Discard the pipette. Vortex Shake.

- To pipette 1 ml of the tube with dilution 10^{-2} into the tube containing 9 ml of distilled water labeled 10^{-3}. Close the tube cap tightly. Discard the pipette. Vortex Shake.

- To pipette 1 ml of the tube with 10^{-3} dilution into the tube containing 9 ml of distilled water labeled 10^{-4}. Close the tube cap securely. Discard the pipette. Vortex Shake.

- To pipette 1 ml of the tube with dilution 10^{-4} into the tube containing 9 ml of distilled water labeled 10^{-5}. Close the tube cap securely. Discard the pipette. Vortex Shake.

- To pipette 1 ml of the tube with dilution 10^{-5} into the tube containing 9 ml of distilled water labeled 10^{-6}. Close the tube cap securely. Discard the pipette. Vortex Shake.

Note:

For indirect ST, dilutions can be made using micropipettes and reducing to 4 the number of tubes with distilled water to be prepared (2 with 9.9 ml and 2 with 9 ml). Proceed as follows:

- Transfer 100 microliters of the master suspension into a tube containing 9.9 ml of distilled water (10^{-2} dilution). Close the tube cap securely. Discard the pipette. Shake with vortex.

- Transfer with pipette 1 ml of dilution 10^{-2} suspension into a tube containing 9 ml of sterile distilled water (10^{-3} dilution). Close the tube cap securely. Discard the pipette. Vortex Shake.

- Transfer 100 microliters of dilution 10^{-3} into a tube containing 9.9 ml of distilled water (10^{-5} dilution). Close the tube cap securely. Discard the pipette. Vortex Shake.

- Transfer with pipette 1 ml of the suspension to a 10^{-5} dilution containing 9 ml of sterile distilled water (dilution 10^{-6}) tube. Close the tube cap securely. Discard the pipette. Vortex Shake.

Proportion method

Principle

The method developed by Canetti et al., and then modified, quantifies the percentage of clones that resist the action of each drug tested, existing in a population of *M. tuberculosis* bacillus present in a sample of a patient.

In the simplified version, a single (critical) concentration of each antibiotic is tested. The concentration is selected in such a way as to predict with the greatest possible certainty the activity of the drug when it is

delivered in the treatment, as assessed clinically or by indirect evidence.

Using a standardized inoculum, compare the development obtained in culture medium containing each drug with that obtained in medium without drug. Isolation is classified as resistant if the percentage of clones surviving the drug activity is equal to or greater than 1%.

In order to obtain sufficient development and, at the same time, to count colonies, the inoculum is seeded with 2 dilutions. Each dilution is seeded in drug-free medium (control) and with each drug to be tested. It is convenient to add a third dilution to plant only the drug-free medium. This third dilution allows to count colonies when the inoculum has been high and thus to avoid repetitions. The inoculum must be very homogeneous and the dilutions and volumes sown must be accurate so that the calculations made are correct.

The agar medium allows acceleration and visualization prior to development and, therefore, has a less prolonged incubation than the egg-based media.

Note

In each case, the necessary drug tubes will be selected according to the adopted work algorithm. For example, I and R tubes were selected for initial screening of patients, and tubes with S, E, quinolone, injectables, and other drugs used in the second line regimens, in cases where has detected resistance to I and / or R.

The culture media to be used must belong to the same batch, perfectly identified with its number, with the drug contained in each tube (in the case where it is not control without drug). The lot must have been subjected to quality control.

In Löwenstein-Jensen culture media

Inoculation and incubation
Direct method

By sample to investigate

Biological material

- Dilutions performed with the suspension obtained from the decontaminated sample, selected as the result of the AFB, as presented below.

Result of AFB of the sample to be investigated	Dilutions of the inoculate initial suspension		
	I	II	III
(++)	10^{-1}	10^{-3}	10^{-4}
(+++)	10^{-2}	10^{-4}	10^{-5}

Culture media

Select and label the tubes with the corresponding ST number and the dilutions to be sown (depending on the result of the AFB) as indicated below:

Result of the acid fast bacilli (AFB) of the sample to be investigated	Tubes with culture media labeled with the sample number and dilution				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
(++)	2 tubes without drug 1 tube with each drug to investigate		2 tubes without drug 1 tube with each drug to investigate	2 tubes without drug	
(+++)		2 tubes without drug 1 tube with each drug to investigate		2 tubes without drug 1 tube with each drug to investigate	2 tubes without drug

Identify the date of completion of the ST. This can be done in the control tubes (without drug) or, if the ST number to be performed is high, in the tray and container where each test will be placed during the incubation.

Order the tubes by placing in a rack, in series, each dilution to be planted and the tubes with medium corresponding to that dilution. Leave a free space at the beginning to systematically move each tube after seeding, so that omissions or double seeding in the same tube.

Additional material

- A petri dish or a disposable absorbent cloth.
- A tray or plate with a glass dipstick or other support, or racks that allow the tubes to be incubated (with the top off the tube raised approximately 15 º).

Procedure

When performing the following steps observe the following precautions:

- discard with vigorous movement the aqueous exudate that may have remained in each tube with LJ after coagulation, on an open Petri dish or an absorbent cloth, without touching any surface with the top off the tube
- not open a tube without having closed the previous one..
- Proceed as follows for each ST
- Take the first tube with a medium to plant, open the lid
- To pipette exactly 0.2 ml of dilution I into each of the tubes labeled with this dilution (2 LJ tubes without a drug, and one tube with each drug to be investigated) Discard the pipette,
- To pipette exactly 0.2 ml of dilution II into each of the tubes labeled with this dilution (2 LJ tubes without a drug and one tube with each drug to be investigated) Discard the pipette,
- To pipette exactly 0.2 ml of dilution III into each of the tubes labeled with this dilution (2 LJ tubes without drug),
- Take the first seed tube, gently distribute the seeded suspension over the entire surface of the medium, making a smooth rotary motion
- Check tube cap closure without tightening; tilt immediately on tray or rack by lifting the top of the tube approximately 15º. Ensure that the tube does not rotate after it is located,
- Complete the previous two steps with all the tubes seeded,
- Incubate to 37 °C.

Indirect method
For the isolates to investigate

Biological material

- Dilutions 10^{-3}, 10^{-5}, and 10^{-6} previously prepared with the stock suspension obtained from a positive culture of *M. tuberculosis.*

Culture media

	tubes to be seed (labeled with ST number and dilution)		
	I	II	III
	10^{-3}	10^{-5}	10^{-6}
LJ without drug (controls)	2	2	2
LJ with every drug to investigate	1	1	--

- Order the tubes by placing in a rack, in series, each dilution to be seed and the tubes with medium corresponding to that dilution. Leave a free place at the beginning to systematically move each tube after seeding so that omissions or double seeding in the same tube,

- Identify the date of completion of the ST. This can be done in the control tubes or, if the ST number to be made is high, in the tray and box or container where each test will then be placed during the incubation.

Additional Materials

- A petri dish,

- A tray or plate with a glass dipstick or other support, or rack allowing incubates inclined tubes (with the mouth of the tube raised approximately 15 °).

Procedure
When performing the following steps observe the following precautions:

- Discard with vigorous movement the aqueous exudate that may have remained in each tube with LJ after coagulation, on an open Petri dish or an absorbent cloth, without touching any surface with the top off the tube,
- not open a tube without closing the previous one

Proceed as follows for each ST

- Pipette exactly 0.2 ml of dilution 10^{-6} into each of the tubes labeled with this dilution (2 LJ tubes without drug). Discard the pipette,

- Take the first tube, gently distribute the suspended seed over the entire surface of the medium,

- Check tube cap closure without tightening; tilt immediately on tray or rack by lifting the top of the tube approximately 15°. Ensure that the tube does not rotate after it is located,

- Complete previous operation with all seeded tubes,

- Incubate at 37 °C.

Initial tube inspection

Procedure

- Inspect the tubes every two days to verify that the inoculated suspension has been absorbed by the medium.

At that moment:
- Adjusting the screw cap of each tube,

- If the space is limited in the culture stove, put the tubes upright in boxes, grouping each dilution neatly and ST,

- Identify, record and discard any tube that shows contamination,

- In the case where there is widespread contamination in a ST that disables its subsequent reading, arrange to report this finding immediately, repeat the ST if there is another tube with the isolate in good condition, or request new sample whenever may be possible. Discard contaminated tubes.

Reading and recording results

Additional Materials

- Manual counter (optional)
- Magnifying glass (optional)

Procedure

- Perform two readings
  at 20-22 days (3 weeks)
  at 40-42 days (6 weeks)

- If, exceptionally, tubes are detected with unabsorbed water in the lower part, keep them in a vertical position during the reading, to prevent the water from repeating the colonies that have already developed by drag,

- Examine the development and verify that there are no abnormalities (contamination or different appearance of *M. tuberculosis),*

- Count the colonies that are presented separately. The magnifying glass and the manual counter can be used to facilitate this process. Do not count the colonies that only grow on the flute peak or thin edges of the culture medium in the drug tubes, since the drug may be inactivated there,

- Record the results as follows:

Observed development	to register
0-100 colonies, separated	The number of colonies
100-200 colonies, separated	2+ (approximate number of colonies)
More than 200 separate colonies	2+
confluent development	3+

- At the end of the third week of incubation, proceed as follows:

Observation	Process
Development of some tube with drug	If this development is equal to or greater than 1% of the inoculated in the control tubes (see calculations and interpretation of lower results). If it is greater, report the resistance of the drug immediately. The report of the results for the drug (s) for which resistance is not yet observed should be allowed up to 6 weeks as they could develop resistant clones in the later days.
Poor development in control tubes inoculated with dilution I (20 colonies or less)	Provision that the ST be repeated immediately because there is a high risk that the development necessary to interpret the test results will not be achieved.
None of the above	Extend the incubation until 6 weeks.

Record observations

- At the end of the 6th week of incubation, record, analyze and interpret the results as indicated below (see Verification of test quality and Calculations and interpretation of results).

In Middlebrook 7H10 media

Inoculation and incubation

Direct method

Biological material

- Dilutions performed from the decontaminated sample, selected as the result of the AFB as shown below.

Result of the AFB of the sample to be investigated	Dilutions of the inoculate stock suspension		
	I	II	III
(++)	10^{-1}	10^{-3}	10^{-4}
(+++)	10^{-2}	10^{-4}	10^{-5}

Medium of culture

- Petri dishes prepared with agar without drug and with each of the drugs to be tested. Label them with the number of the sample and the dilutions to be sown in each quadrant (depending on the result of the AFB).

Additional material

- CO2 permeable plastic bags of adequate size to contain a Petri dish with culture medium.

Additional equipment

- Incubator at 37°C with atmosphere of 5-10% of CO2,
- Heat-sealing plastic bags.

Procedure:

- Order the tubes containing the dilutions of each sample in series on a rack,
- Sort the plates following the same sequence
- When opening, take care that the inside of the cover does not touch any surface
- To pipette exactly 0.1 ml of dilution I into the control (no drug) quadrants and into each of the quadrants containing the drugs to be tested labeled at dilution I. Discard the pipette,
- To pipette exactly 0.1 ml of dilution II into the control quadrant (without drug) and into each of the quadrants containing the drugs to be labeled with dilution II. Discard the pipette,
- Gently move the plates to distribute the seed on the surface of the medium,
- Place the plates on trays in a horizontal position. Leave at room temperature until the planting is absorbed and no condensation of moisture is observed on the lid,
- With a tape, ensure the attachment of the cover and base of each plate,
- Place each plate in a plastic bag and seal it,
- Condition the plates again in boxes or trays,
- Incubate at 37 °C in an oven with 5-10% CO2 atmosphere.

Indirect method

Biological material

- Dilutions 10^{-2} (dilution I) and 10^{-4} (dilution II) previously prepared from the stock suspension of each M. tuberculosis positive culture to be investigated.

Culture media

- Select the Petri dishes prepared with the agar without drug and with the drugs to be tested. Label them with the ST number and the dilutions to be sown in each quadrant.

Procedure

- Order the tubes containing the dilutions of each sample in series on a rack,
- Order the plates following the same sequence,
- When opening, take care that the inside of the cover does not touch any surface,
- To pipette exactly 0.1 ml of dilution 10^{-2} into the control quadrants (without drug) and into each of the quadrants containing the drugs to be tested labeled with dilution 10^{-2}. Discard the pipette,
- To pipette exactly sows 0.1ml dilution 10^{-4} in quadrant control (without drug) and in each of the quadrants containing drugs tested labeled with dilution 10^{-4}. Discard the pipette (*),
- Gently move the plates to distribute the seed on the surface of the medium,
- Place the plates on trays in a horizontal position. Leave at room temperature until the planting is absorbed and no condensation of moisture is observed on the lid,
- With a tape, ensure the attachment of the cover and base of each plate,
- Place each plate in a plastic bag and seal it,
- Condition the plates again in boxes or trays,
- Incubate at 37 °C in an oven with 5-10% CO_2 atmosphere,

(*) Note: It is advisable to also inoculate the 10^{-5} dilution in a medium-free drug quadrant to increase the chances of accurately counting colonies.

Initial plate inspection

Procedure

- Inspect the plates for a period of 2 to 7 days after sowing without removing them from their bags,
- Identify, record and any plaque that shows contamination,
- In the case where there is widespread contamination in a ST that disables its subsequent reading, arrange that this finding be immediately reported and request new material whenever possible,

Note: It is advisable to add a second inspection at the end of the second week and proceed as follows

Observation	Process
Development of some tube with drug	If this development is equal to or greater than 1% of the inoculated in the control tubes (see calculations and interpretation of lower results). If it is greater, report the resistance of the drug immediately. The report of the results for the drug (s) for which resistance is not yet observed should be allowed up to 6 weeks as they could develop resistant clones in the later days.
Poor development in control tubes inoculated with dilution I (20 colonies or less)	Provision that the ST be repeated immediately because there is a high risk that the development necessary to interpret the test results will not be achieved.
None of the above	Prolong the incubation until 6 weeks.

Reading and recording results

Additional equipment

- Magnifying glass or stereoscopic microscope

Procedure

- Carry out the final reading at 21 days (3 weeks) of incubation.
- Examine each plate under the magnifying glass or microscope, without removing them from the bags
- Examine the development and check that there are no abnormalities (different aspect of *M. tuberculosis).*
- Count the colonies that are presented separately.
- Record the results as follows:

Observed development	to register
0-50 colonies, separated	The number of colonies
50-100 colonies, separated	1+ (approximate number of colonies)
100-200 colonies, separated	2+ (approximate number of colonies)
200-500	3+
confluent development	4+

Verification of test quality

- Check if the following conditions are met:

1. Good quality of the medium with and without drug used, according to the results of the quality control performed with the reference strains (see chapter on Quality Management),
2. sufficient development (at least 100 colonies) in the control tubes or quadrants seeded with dilution I,
3. separate and countable colonies in the control tube or quadrant with at least one of the other dilutions seeded,
4. correlation between the number of colonies developed and the dilutions sown

Condition 1 is indispensable.

Condition 2 is necessary when no drug development is detected, because it may not have been given opportunity to develop clones resistant to that drug because the inoculum is scarce.

Condition 3 is necessary when development in the drug medium is detected because it is necessary to calculate if that development is equal, greater or less than 1% of the inoculum.

Condition 4 is necessary to be able to infer from the colonies that develop with a dilution, those developed with another dilution, following the following reasoning:

If, for example, LJ have been sown the following dilutions called I, II and III

		Dilutions seed		
PS	Processed Material	I	II	III
Direct	Sample AFB (++)	10^{-1}	10^{-3}	10^{-4}
	Sample AFB (+++)	10^{-2}	10^{-4}	10^{-5}
Indirect	Isolation	10^{-3}	10^{-5}	10^{-6}

Dilution II is diluted 1:100 with respect to I, and dilution III is diluted 1:1000 with respect to I.

Then it is observed:

- tubes with dilution I should show development approximately 100 times greater than those with dilution II and approximately 1000 times greater than those with dilution III,

- the tubes with dilution II should show development approximately 10 times greater than those of dilution III

Normally, with good quality of work, the 4 conditions are fulfilled, confluent development is observed in the control tubes inoculated with dilution I and accounting colonies in the control tubes corresponding to dilution II. In that case you can proceed to perform the calculations, interpret and report all the results.

If condition 1 is met, but conditions 2, 3 and / or 4 are not met, the TS should be repeated. Exceptionally, some of the results may be interpreted and reported as described below.

Calculation and interpretation of results

This last step should be done by highly trained, experienced and experienced staff to make the right decisions based on the results recorded and to ensure the quality of the report.

- If the quality of the ST has been verified and no development has been observed in any of the drug-containing tubes or quadrants, report that the isolation is sensitive to the drugs tested. In this case the excessive inoculum does not disable the interpretation of the test since it is not necessary to count colonies.

- If colonies are visualized in one or more drug-containing tubes or quadrants, in the first or second reading, it is necessary to calculate whether these colonies constitute more or less than 1% of the inoculated seed. In this case it should be possible to count colonies to make the corresponding calculation.

To do this, follow the following sequence:

- Average the number of the two control quadrants each dilution in which counting could be done (ie in tubes with separate and countable colonies),

- Based on the average obtained in the previous step, estimate the seed inoculated with each dilution, as follows.

				Process		
Situation	Colonies observed in control tubes or quadrants seeded with dilution			Average the colonies counted in the two control tubes or quadrants inoculated with the dilution	Estimated seed inoculated	
	I	II	III		With dilution I	With dilution II
1	Accountants			I	the average obtained	
2 (frequent)	Countless	Accountants		II	Average obtained with dilution II multiplied by 100	Average obtained with dilution II
3 (infrequent)	Countless	Countless	Accountants	III	Average obtained with dilution III multiplied by 1000	Average obtained with dilution III multiplied by 10

- Count or estimate colonies developed with each drug tested, with each dilution planted, as follows:

Situation	Colonies observed in the quadrant with drug inoculated with dilution		Estimation of the number of colonies they developed	
	I	II	With dilution I	With dilution II
1	Accountants	few or none	Number of colonies counted with dilution I	
2	Countless	Accountants	Number of colonies counted with dilution II multiplied by 100	Number of colonies counted with dilution II

- Taking the calculations corresponding to a dilution, calculate the percentage representing the number of colonies developed in the drug medium with respect to the average inoculum corresponding to that same dilution (see examples below),
- If that percentage of clones that have developed in the medium with drug is greater than 1% to classify the result as “resistant”, and if it is smaller as “sensitive”.
- If the verification of the quality of ST has not fulfilled condition 2, or 3 or 4 only some results can be reported in the following situations

The condition is met

1	2	3	4	Inoculum	Conduct to follow
yes	no	yes	yes	Insufficient	Report resistance to drugs with which more than 1% of resistant clones have grown. Repeat the ST for the rest of the drugs.
yes	yes	no	yes	Excessive	Inform the susceptibility to drugs for which no development was recorded with all dilutions inoculated. Repeat the ST with the drugs with which development was recorded.
yes	yes	yes	no	Poorly diluted	Inform the susceptibility to drugs for which no development was recorded with all dilutions inoculated. Repeat the ST with the drugs with which development was recorded.
yes	no	yes	no	Insufficient and poorly diluted	Repeat all ST

Are summarized below reports that can be released in time for each reading:

Requirements		Result report
PS	Tube with drug	
Complies conditions 1,2,3 May or may not meet condition 4	Absence of development or development less than 1% related to control Finished 3 weeks of incubation on 7H10 agar or 6 weeks on LJ	Sensitive
Complies with conditions 1,2 and 4 May or does not meet condition 3	Lack of development Finished 3 weeks of incubation on 7H10 agar or 6 weeks on LJ	Sensitive
Complies with conditions 1,3 and 4 May or may not meet condition 2	Development greater than 1% of the inoculum at the time of detection	Resistant

When a borderline result is recorded (ie the development of resistant clones in a smaller proportion but close to 1%), proceed as follows:

- if it is rifampicin, prolong the incubation one week (to see if there is evidence of the development of resistant mutants that take a little longer to multiply)
- in all cases,
  repeat the ST with the drug (s) that throw that result; if another positive culture of the same patient were already available, prefer the new culture
  inform the doctor that this result has been recorded.

The following examples may facilitate the interpretation of results:

Susceptibility test performed on Lowenstein Jensen medium

Example 1

<table>
<tr><td>N° xxxxxx
Sputum sample BK 3+
Seeding Date xxxx</td><td colspan="2">Lot of medium
xxxx</td><td colspan="7">Responsable of
ST xxxxxx</td></tr>
<tr><td rowspan="6">Reading 21 days | Date XXX</td><td colspan="2">Inoculated</td><td colspan="7">Reading</td></tr>
<tr><td rowspan="2">(*)</td><td rowspan="2">(**)</td><td colspan="3">Controls</td><td colspan="2">isoniazid</td><td colspan="2">rifampicin</td></tr>
<tr><td>Tube 1</td><td>Tube 2</td><td>Average</td><td></td><td>%</td><td></td><td>%</td></tr>
<tr><td>I 10^{-2}</td><td></td><td>++</td><td>++</td><td>150</td><td>65</td><td>43,3</td><td>5</td><td>3,3</td></tr>
<tr><td>II 10^{-4}</td><td>1/100</td><td>1</td><td>2</td><td>1,5</td><td>0</td><td></td><td>0</td><td></td></tr>
<tr><td>III 10^{-5}</td><td>1/1000</td><td>0</td><td>0</td><td></td><td colspan="2">resistant</td><td colspan="2">resistant</td></tr>
</table>

(*) relative to the master suspension
(**) relative to dilution I

At 21 days (first reading)

The colonies could be counted with the dilution II (in this case 10^{-4} of the master suspension prepared directly with the sample), and from it the development observed with the dilution I:

Average colonies developed with dilution II
in the control tubes = (1 + 2) / 2 = 1.5

Estimation of the average colonies developed with dilution I
in the control tubes = 1.5 x 100 = 150

At that time 65 colonies were already observed in the tube with isoniazid, which represents the following percentage in relation to the inoculum.

65 x 100/150 = 43.3%.
The development is superior to 1% of the inoculum, it is classified as Resistant to I.

Also 5 colonies were observed or in the tube with R representing the following percentage:

5 x 100/150 = 3.3%.
The development is superior to 1% of the inoculum, it is classified as RR.

Isolation is multi-resistant.

It is reported immediately, without waiting until 42 days. And it is also immediately available to make ST to E and second line drugs, taking material from the control tubes.

This second test had the following results.

N° xxxxxx Seeding Date xxxx	Lot of medium xxxx				Responsable of ST xxxxxx												
Reading 21 days | Date XXX	Dilution inoculated		Reading														
	(*)	(**)	Controls		Average	E		S		K		A		C		FQN	
			Tube 1	Tube 2			%		%		%		%		%		%
	I 10^{-3}		65	43	54	0		0		0		0		0		0	
	II 10^{-5}	1/100	0	0		0		0		0		0		0		0	
	III 10^{-6}	1/1000	0	0													

N° xxxxxx Seeding Date xxxx	Lot of medium xxxx				Responsable of ST xxxxxx												
Reading 42 days | Date XXX	Dilution inoculated		Reading														
	(*)	(**)	Controls		Average	E		S		K		A		C		FQN	
			Tube 1	Tube 2			%		%		%		%		%		%
	I 10^{-3}		++	++	200	0		0		0		0		0		0	
	II 10^{-5}	1/100	3	1	2	0		0		0		0		0		0	
	III 10^{-6}	1/1000	0	0		sensitive		sensitive		sensitive		Sensitive		sensitive		sensitive	

(*) relative to the master suspension
(**) relative to dilution I

First reading (21 days):

No development is observed in any of the drug tubes. But this result cannot be informed. On the one hand the inoculum in the controls has not yet reached the required minimum of 100 colonies, on the other hand it is still possible to appear resistant clones in the remainder of incubation.

Final reading (42 days):

Colonies of controls could be counted in dilution II (10^{-5} of the stock suspension)

Average colonies developed with dilution II in the control tubes = (3 + 1) / 2 = 2

Estimation of the average colonies developed with dilution I in the control tubes = 2 x 100 = 200

This means that development has exceeded the minimum required.

It was not observed any colony in drug tubes

Isolation is classified as sensitive to E, streptomycin, K, A, C and the FQN assayed. The selection of these drugs was made considering the second-line schemes employed in the country.

Example 2

Isolation No. XXX Seeding Date xxxx	Lot of medium xxxx				Responsable of ST xxxxxx				
Reading 21 days | Date XXX	Dilution inoculated		Reading						
	(*)	(**)	Controls			isoniazid		rifampicin	
			Tube 1	Tube 2	Average		%		%
	I 10^{-3}		25	40		0		0	
	II 10^{-5}	1/100	0	0		0		0	
	III 10^{-6}	1/1000	0	0					

Isolation No. XXX Seeding Date xxxx	Lot of medium xxxx				Responsable of ST xxxxxx				
Reading 42 days | Date XXX	Dilution inoculated		Reading						
	(*)	(**)	Controls			isoniazid		rifampicin	
			Tube 1	Tube 2	Average		%		%
	I 10^{-3}		48	130		0		0	
	II 10^{-5}	1/100	14	0		0		0	
	III 10^{-6}	1/1000	0	0		no interpret		no interpret	

(*) relative to the master suspension
(**) relative to dilution I

First reading (21 days):

No development is observed in any of the drug tubes. But this result cannot be informed. On the one hand the inoculum in the controls has not yet reached the required minimum of 100 colonies, on the other hand it is still possible to appear resistant clones in the remainder of incubation.

Final reading (42 days):

The colonies counted in the control tubes show that the inoculum has not been homogeneous. Indeed, the two tubes seeded the dilution I (10^{-3}) have a number of colonies that are very different from each other. The same applies to the two tubes seeded with dilution II (10^{-5}). In addition the number of colonies developed with dilution 10^{-3} does not appear to be approximately 100 times greater than those developed with the dilution10^{-5}. Thus, no averages or percentages can be calculated. "Not interpretable" is reported and the test is repeated.

This implies a very important delay and detriment for the patient who will not have a timely orientation for their treatment. That is why it is very important to take care and precision in the procedures of the PS.

Example 3

Lot of medium XXXX | Responsable of ST XXXXXX

IsolationNo. XXXX
Background: resistant to I R S
Sowing Date xxxxx

Reading 21 days | Date XXX	Dilution inoculated		Reading																		
	(*)	(**)	Controls		Average	I		R		E		S		K		A		C		FQN	
			Tube 1	Tube 2			%		%		%		%		%		%		%		%
	I 10^{-3}		~125	++		++		++		0		0		0		0		cont		78	
	II 10^{-5}	1/100	15	29	22	2	9,09	7	31,8	0		0		0		0				1	4,5
	III 10^{-6}	1/1000	0	0		Resistant		Resistant												Resistant	

Lot of medium XXXX | Responsable of ST XXXXXX

Isolation No. XXXX
Background: resistant to I R S
Sowing Date xxxxx

Reading 42 days | Date XXX	Dilution inoculated		Reading																		
	(*)	(**)	Controls		Average	I		R		E		S		K		A		C		FQN	
			Tube 1	Tube 2			%		%		%		%		%		%		%		%
	I 10^{-3}		+++	+++	6000	+++		+++		8(***)		0		+++		0		discarded		+++	
	II 10^{-5}	1/100	49	71	60	35	58,3	83	138	1(***)		0		2	3,33	0		0		39	65
	III 10^{-6}	1/1000	3	8	5,5	resistant		resistant		sensitive		inconsistent		resistant		sensitive		NI		resistant	

(*) relative to the masterk suspension

(**) relative to dilution I

(***) only at the peak of flute

We have investigated the isolation of a patient diagnosed with MDR TB, more precisely with a previous ST was determined to be resistant to I, R and S. Therefore, from the beginning, first and second line drugs are tested.

At 21 days (first reading):

The colonies could be counted with dilution II (in this case 10^{-5} of the master suspension prepared with the isolation).

Average colonies developed with dilution II
in the control tubes = (15 + 29) / 2 = 22

At the same dilution, two colonies were observed in the tube with isoniazid, which represents the following percentage in relation to the inoculum

2 x 100/22 = 9.1%
The development is superior to 1% of the inoculum, it is classified as Resistant to I.

Also, with the same dilution II, 7 colonies were observed or in the tube with R representing the following percentage

7 x 100/22 = 31.8%.
The development is superior to 1% of the inoculum, it is classified as RR.

With the FQN, 1 colony was observed which represents the next percentage of the inoculum
1 x 100/22 = 4.5%.

1 x 100/ 22 = 4.5 %.
The development is superior to 1% of the inoculum, it is classified as Resistant to the tested FQN.
Dilution I confirms the resistance detected with dilution II

Contamination is detected in a tube with C inoculated with dilution I:

The report is immediately issued confirming multidrug resistance and added resistance to the FQN.

At that same moment it is arranged that ST be made to a more recent quinolone among those employed by the TB Control Program and the repetition of the ST to C, since this information is important for this patient who, until this moment, is classified with TB at least pre XDR

At 42 days (final reading):

Colonies could be counted with dilutions II and III (10^{-5} and 10^{-6} of the master suspension prepared with the isolation).

Average colonies developed with dilution III
in the control tubes = (3 + 8) / 2 = 5.5
Average colonies developed with dilution II
in the control tubes = (49 + 71/2 = 60
Estimation of colonies developed with dilution I = 60 x 100 = 6000

The development is very abundant, the relation between the numbers of colonies counted with the different dilutions is good, and it has been possible to count or to estimate the development of colonies.

No colonies are observed with A. It is classified as Sensitive to A.

No colonies were also observed with S, so the isolate would also be sensitive to that drug. However, the patient has resistance to S with previous isolates. The result is not consistent and therefore this situation is reported and the ST is repeated to this drug, preferably with another isolation of the patient.

In the tubes with E some colonies with the dilutions I and II were observed. But these colonies grew only in the peak with flute and not in the rest of the surface. This development is not considered because in the thinnest area of the culture medium the drug has surely been inactivated.

With dilution II, 35 colonies were observed in the tube with I, 83 with R, 2 with K and 39 with the FQN assayed. In this case the percentages can be calculated directly in relation to the average inoculum of the control tubes corresponding to the same dilution II.

Drug	Calculation
I	35 x 100/ 60 = 58,3%
R	83 x 100/ 60 > 100%
K	2 x 100/ 60 = 3,3%
FQN	39 x 100/ 60 = 65%

In all three cases the development is superior to 1% of the inoculum, the MDR that has already been diagnosed is confirmed, and is classified as K Resistant and tested FQN. Resistance has spread and the patient has XDR TB.

At this same time the ST to S, C and the latest generation FQN made 21 days ago can be checked to see if resistance is detected early. In this critical case, it is important to expedite reporting as much as possibl.

Susceptibility test performed on Middlebrook 7H10 medium

Example 1

Sample N° xxx BK 2+ Seeding Date xxxx	Lot of medium xxxx			Responsable of ST xxxx				
Reading 21 days | Date XXX	Dilution inoculated		Reading Quadrants					
	(*)	(**)	Controls	isoniazid		rifampicin		
					%		%
	I 10^{-1}		2+(~150 col)	1	0,7	0		
	II 10^{-3}	1/100	0	0		0		
				Sensitive		Sensitive		

The colonies could not be accurately counted in the control quadrant, but were visually estimated at 150 with dilution I (in this case 10^{-1} of the master suspension prepared with the decontaminated sample).

At that same dilution, a colony was observed in the quadrant with isoniazid. The percentage relative to the control would be:

- 1 x 100/ 150 = 0.7 % (approximately)
- The percentage is less than 1% with certainty because the control quadrant has more than 100 colonies, even though it could not be accurately counted. Therefore the insulation is classified as Sensitive to I.

The percentage was less than 1% but close to that limit. In view of this result it is advisable to recommend to the physician the repetition of ST in the next patient's control, with a new sample, to verify if the percentage of resistant clones has not risen.

No development was observed in the quadrant with R, the isolate is classified as sensitive to R.

Example 2

Sample N° xxx Seeding Date xxxx			Lot of medium xxxx		Responsable of ST xxxx		
Reading 21 days | Date XXX	Dilution inoculated		Reading Quadrants				
	(*)	(**)	Controls	isoniazid		rifampicin	
					%		%
	I 10^{-2}		4+	1+ (108 col)		2	
	II 10^{-4}	1/100	54	35	64,8	0	
				Resistant		Sensitive	

(*) relative to the master suspension
(**) relative to dilution I

The percentage of colonies developed with isoniazid can be calculated considering the count corresponding to dilution II.

35 x 100 / 54 = 64. %

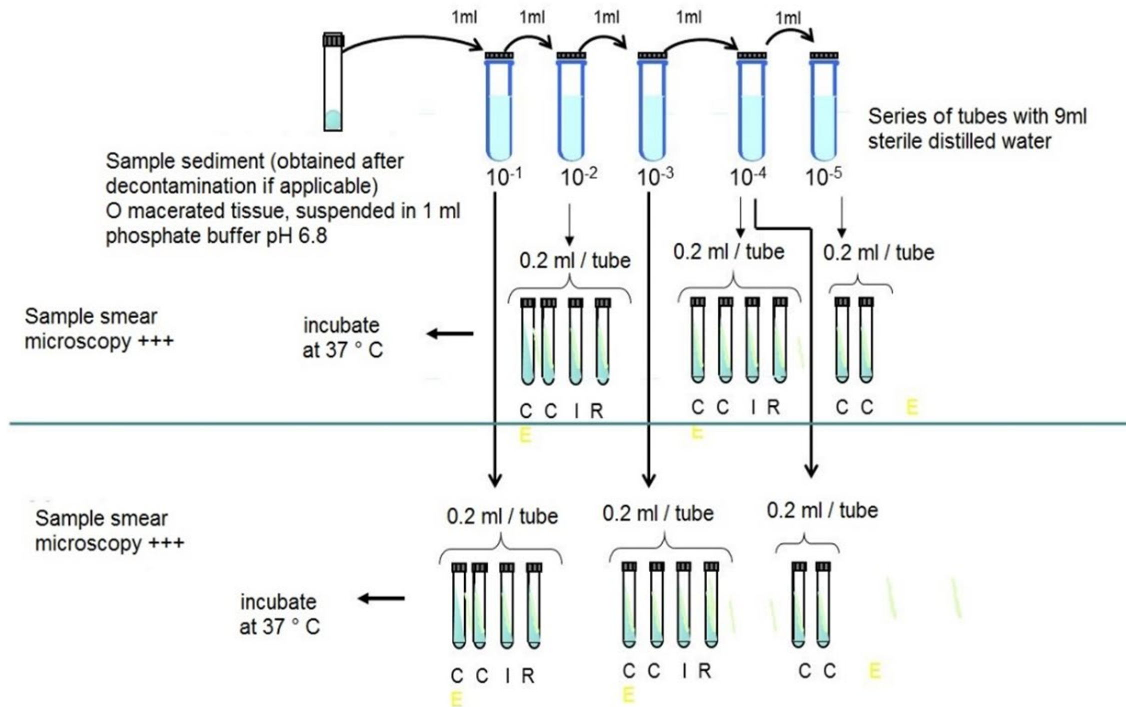
The development is superior to 1% of the inoculum; the isolation is classified as resistant to I

The percentage of colonies developed with R should be calculated with dilution I.

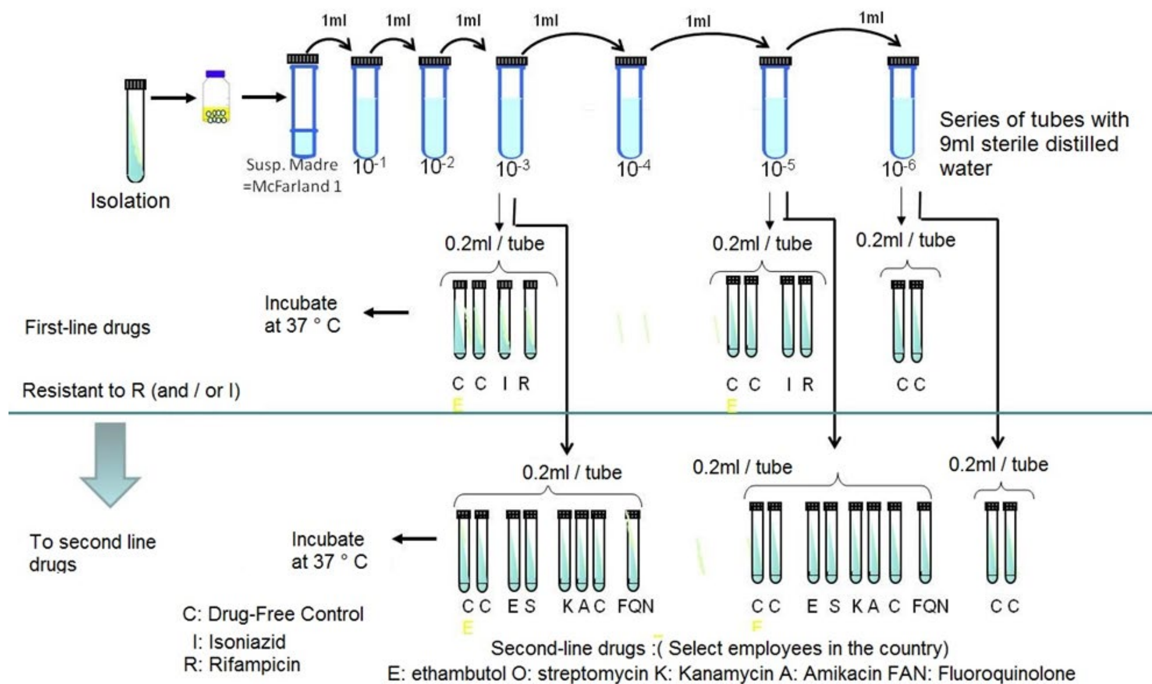
- Estimation of colonies developed with dilution I
- in the control quadrant = 54 x 100 = 5400
- Percentage of colonies developed with R = 2 x 100/5400 = 0.04%
- The development is less than 1% of the inoculum; the isolation is classified as sensitive to R.

Since the isolate was resistant to I, PS is available to second line drugs, in case it is considered necessary to use some of them (eg a FQN) to treat the case.

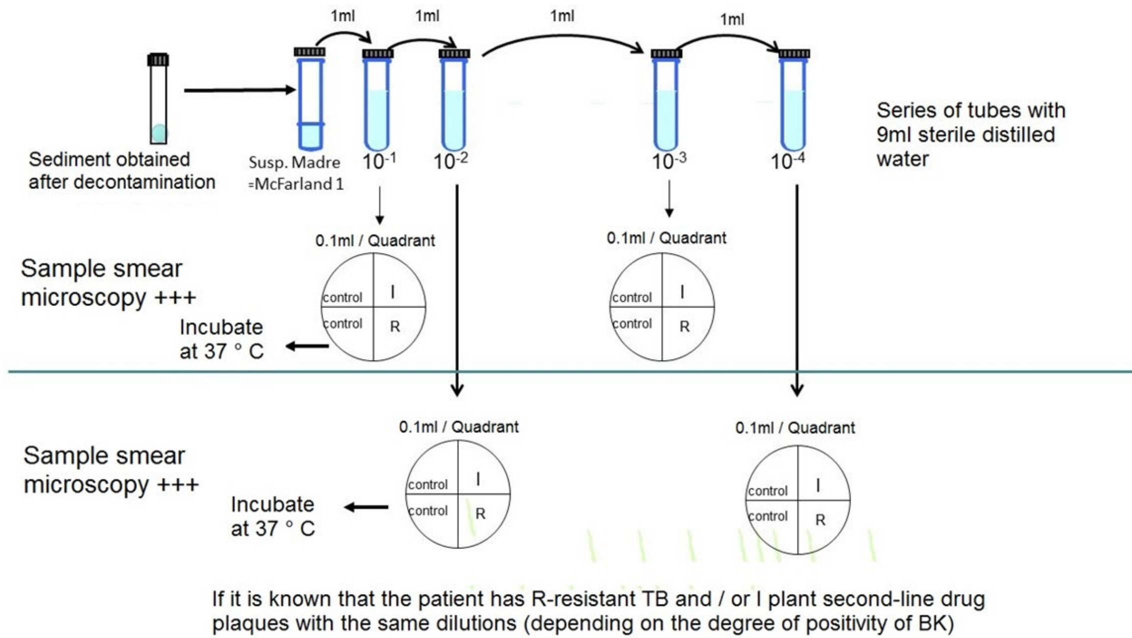
LOWENSTEIN-JENSEN DIRECT FOR FIRST LINE ANTITUBERCULOSIS DRUGS



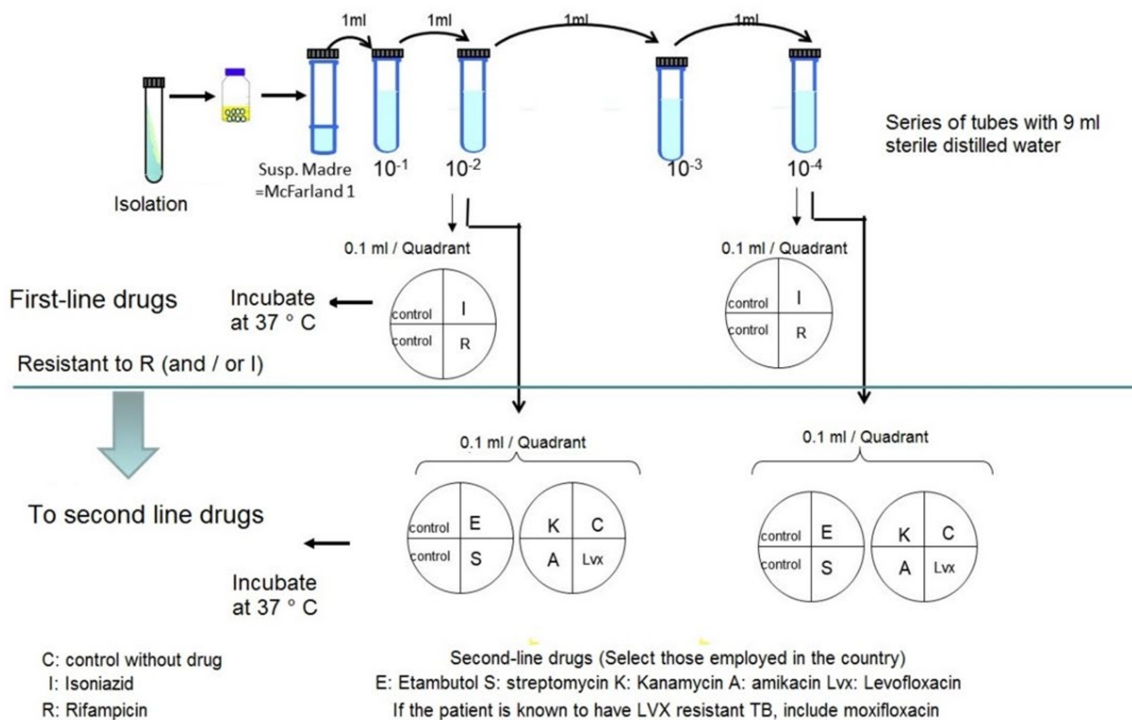
INDIRECT DST IN LOWENSTEIN-JENSEN MEDIUM



DIRECT DST FOR FIRST LINE DRUGS IN MIDDLEBROOK 7H10 OR 7H11 MEDIUM

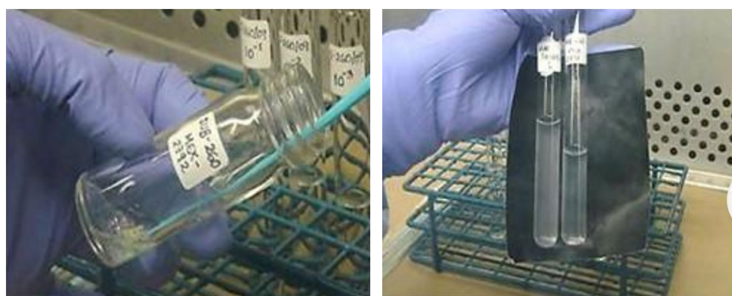


INDIRECT DST IN MIDDLEBROOK 7H10 OR 7H11 MEDIUM

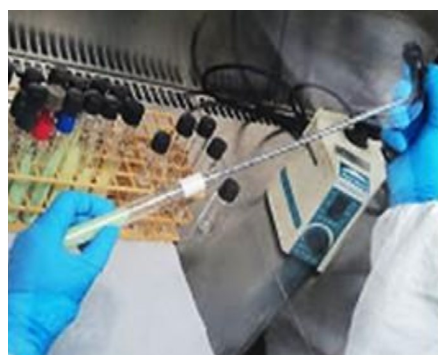


Method of proportion in solid medim

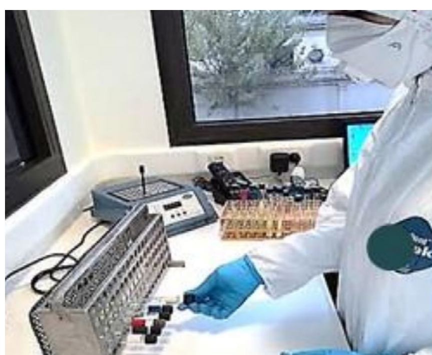
Preparation of the wort area



Preparation of the stock bacillary suspension



Inoculation in Lowenstein-Jensen medium



Place the inoculated tubes horizontally in the rack

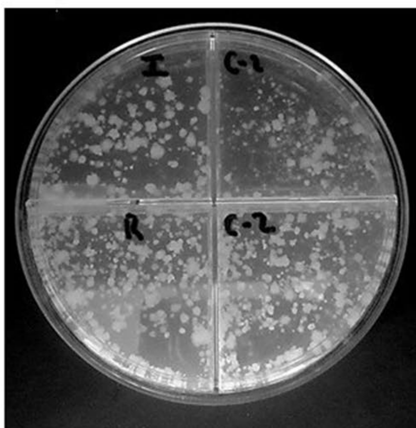
Quantification and registration of growth in Lowenstein-Jensen

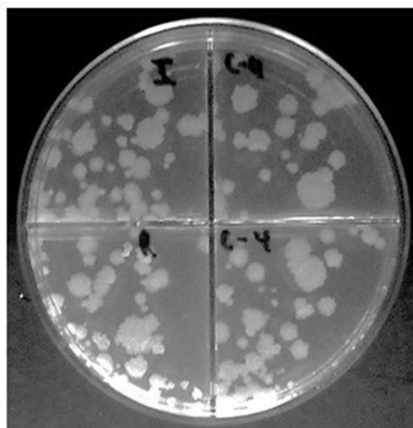
Lowenstein-Jensen proportion method

Strains susceptible to isoniazid and rifampicin.

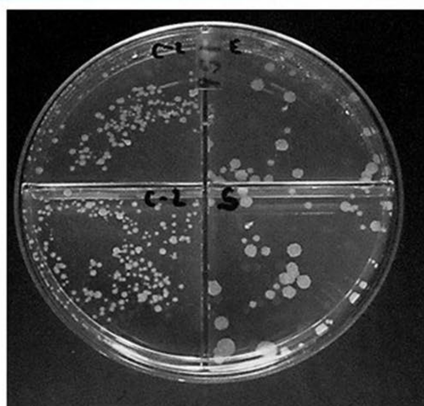
Strain resistant to isoniazid, rifampicin, streptomycin, fluoroquinolone, etambutol-sensitive kanamycin and capreomycin.

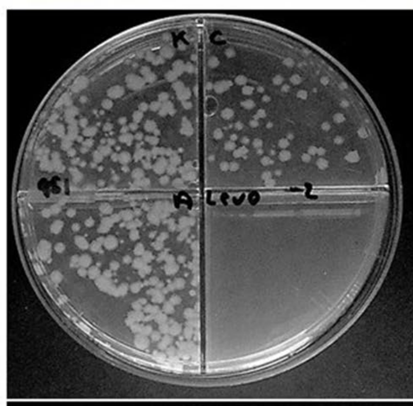
7H10 Proportion Method

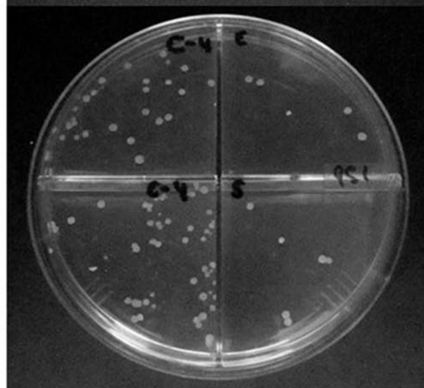


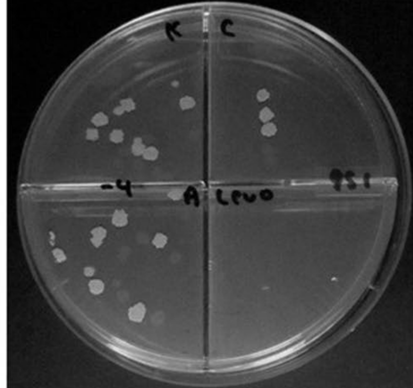


Isolation resistant to isoniazid and rifampicin









Strain resistant to ethambutol, streptomycin, kanamycin, capreomycin and amikacin susceptible to levofloxacin

Pyrazinamidase test

Principle

Isolates of *M. tuberculosis* that are sensitive to Z possess the enzyme pyrazinamidase that acts in the transformation of pyrazinamide into pyrazinoic acid, and it is this latter molecule that is active against the bacillus. In contrast, isolates that have a high level of resistance to Z have lost the activity of that enzyme, as a result of the mutations that appear in the gene that encodes it.

The Wayne method demonstrates the formation of pyrazinoic acid when the bacillus has multiplied in a Z-containing culture medium. To do this, iron-ammo-nium sulfate that forms with the pyrazinoic acid a pink ferrous salt is added. For this to be possible there must be abundant amount of bacilli in the active phase of multiplication.

Limitations

There are strains with low level of resistance to the critical concentration of Z that have enzymatic activity.

Biological material to be investigated

Young isolates (up to 3-4 weeks of development) obtained in solid or liquid medium (indirect method).

Control strains:

Positive: reference strain of *M. tuberculosis* sensitive to drugs, preferably *M. tuberculosis* H37Ra
Negative: strain of *M. bovis* BCG

Means and reagents

For each isolation and control strain to be investigated:

- 1 tube with screw cap with Half Dubos with Z, with controlled quality, labeled with the number of isolation or corresponding strain,

- 1% iron and ammonium sulphate solution.

Procedure

- Transfer abundant bacillary mass from each culture (with a disposable handle or pipette depending on whether it is a solid or liquid medium respectively), to the surface of a tube of Dubos medium with Z. The reaction may have a false negative result if the inoculum is low,

- Incubate at 37 °C for 7 days, in a vertical rack,

- After the incubation, add to each tube 1 ml of the 1% ammoniac ferrous sulphate solution,
- Allow 4 hours, in cooling at 2-8 °C,

- Place each tube on a white background and examine for the presence of a pink band

Reading and interpretation of results

Positive
Pink stripe appearance, whatever its intensity.

It is inferred that the isolation is sensitive to Z.

Negative
The pink band is not observed.

It is inferred that the insulation is resistant to Z.

When a culture is resistant only to Z, the differentiation of *M. bovis* or *M. bovis* CGB into the *M. tuberculosis* com-plex should be considered.

In the case of *M. tuberculosis,* consider that resistan-ce to Z not associated with RR is uncommon.

Nitrate reductase method (from Griess)

Principle

M. tuberculosis, like many other microorganisms, has the enzyme nitrate reductase involved in the reduction of nitrate to nitrite (possible source of nitrogen). To demonstrate this, the bacillus is grown in medium with sodium or potassium nitrate and then three solutions are added that trigger a reaction from the nitrite and finally produce a colored azo derivative (Griess reaction). The presence of nitrite indicates that the enzyme was active and therefore that the bacillus is viable.

Taking advantage of this characteristic, it is possible to culture the bacillus in the presence of nitrate, with and without anti tuberculosis drug, and then evidence of a short incubation if it remained viable or not after the action of the drugs.

The inoculum is seeded 10 times more diluted in the medium without drug, as determined by the experimentation developed to reproduce the results of the method of the conventional proportions. The color intensity of the reaction is then compared, and hence the development of the drug-containing medium with which it does not. If in the first one the color is more intense than in the second, it is inferred that a high percentage of resistant clones has grown and therefore the bacillus is resistant.

To determine the time at which the test can be revealed, the inoculum is seeded in several control tubes, containing medium without drug. The developer solutions are periodically added to each of them, until color is detected. At that time it is considered that the development is sufficient to interpret the results, and proceeds to reveal the rest of the tubes seeded with the antibiotics. Most of the results are obtained after two weeks of incubation.

It is thus inferred if the bacillus is sensitive or resistant to every drug being tested, more rapidly than with conventional tests.

Limitations

The test has been validated only with R and I,

When it is tried to identify borderline resistances it is necessary to resort to conventional methods in solid media that provide information about the proportion of bacillary population that is resistant to each drug.

Biological material to be investigated

- Master bacterial suspension prepared with
  Samples with positive BK 2 (+) or 3 (+) (direct method)
  Young isolates (up to 3-4 weeks of development) medium solid or liquid (indirect method).

  Control strains (young snakes):
  reference strain of M. tuberculosis sensitive to drugs,
  R-resistant and I-sensitive reference strain,
  reference strain resistant to I and R. sensitive

- Dilution 1:10 of each master bacillary suspension
  See above, under the title Preparation of master bacillary suspensions, how to prepare and dilute them.

Media and Reagents

For each sample, isolate or control strain to investigate

- The following tubes with screw cap containing culture medium, which must be labeled with the sample number, isolation or control strain to be investigated:

  3 tubes with LJ medium containing nitrate,
  without anti tuberculosis drug (control),
  1 tube with LJ medium containing nitrate and I,
  1 tube with LJ medium containing nitrate and R.

Order the tubes by placing in a rack, in series, each dilution to be planted and the tubes with medium corresponding to that dilution. Leave a free spot at the beginning to systematically move each tube after planting, so that omissions or double seeding in the same tube are avoided.

- Griess Reaction Reagents 1 (50% hydrochloric acid); 2 (0.2% sulfanilamide) and 3 (n-1-naphthylethylenediamine dihydrochloride 0.1%).

Additional Materials

- A tray or plate with a glass dipstick or other support, or rack that allows the tubes to be incubated (with the top of the tube raised approximately 15 °).

Procedure

Inoculation

- From each sample, isolate or control strain to investigate, inoculate,
  0.2 ml of the master bacillary suspension in the tube with I and in the tube with R,
  0.2 ml of the 1:10 dilution, prepared from the master bacillary suspension, in each of the 3 control tubes.

- Take the first tube, gently distribute the inoculated suspension over the entire surface of the medium, check the cap tightly without tightening it, immediately tilt it in a tray or on a rack raising the top of the tube approximately 15°. Ensure that the tube does not rotate,

- Complete the previous operation with all the tubes seeded,

- Incubate at 37 ° C.

Revealed

The days after the start of the incubation in which the test is revealed varies depending on whether the direct (from the patient sample) or indirect (from the isolation) method was performed as follows.

Days of Incubation

	1st revealed	2nd revealed	3rd revealed
Direct Testing	14	21	28
Indirect Testing	7	10	14

Prepare immediately before revealing a solution with one volume of reagent 1, two volumes of reagent 2 and two volumes of reagent 3. Calculate the final volume to prepare of this solution according to the number of tubes that need to be.

First revealed
For each test performed

- Add 0.5 ml of the developer mixture to one of the three control tubes,

- If the control tube shows pink, red or fuchsia, reveal the corresponding I and R tubes, read and interpret the reaction and discard all material,

- If there is no pink, red or fuchsia appearance, discard the control tube to which the developer solution was added and incubate the rest of the undisclosed tubes.

Second Revealed
For each test whose control was not positive on the day of the first development and kept in incubation

- Add 0.5 ml of the developer mixture to the second of the three control tubes,

- If the control tube shows pink, red or fuchsia, reveal the corresponding I and R tubes, read and interpret the reaction and discard all material,

- If there is no pink, red or fuchsia appearance, discard the control tube to which the developer solution was added and incubate the rest of the undisclosed tubes.

Third revealed
For each test whose control was neither positive in the first nor in the second revealed and kept in incubation

- Add 0.5 ml of the developer mixture to the third and last of the three control tubes,

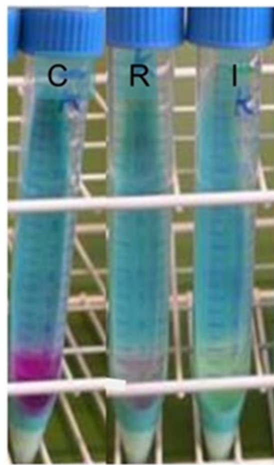
- If the control tube shows pink, red or fuchsia, reveal the corresponding I and R tubes, read and interpret the reaction and discard all material,

- If no pink, red or fuchsia appearance is displayed, the test is not interpretable

Reading and interpretation of results

- Compare the color of the tube with I and the tube with R tube with the color developed in the control tube

Control
Negative
Isolation R-sensitive and I resistant



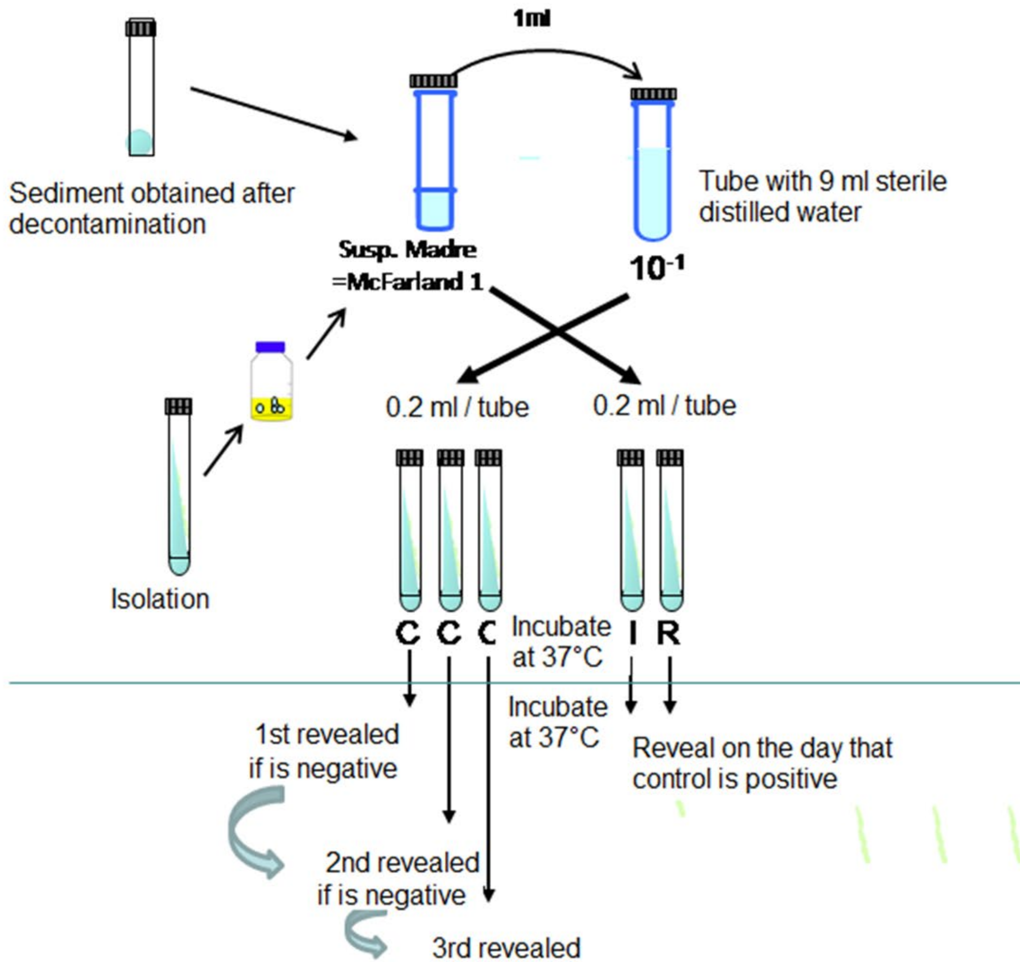
Control
Negative
Isolation sensitive to R and I

Positive
Color intensity greater than or equal to that of the con-trol tube.
It is inferred that the strain is resistant to the drug being examined.

Negative
No color or intensity of color lower than the control tube.
It is inferred that the isola-tion is sensitive to the drug being examined.

Go back to test any isolation with doubtful or non-interpretable result, preferably by a conventional.

DIRECT AND INDIRECT DST BY THE NITRATE REDUCTASE METHOD (GRIESS)



	Days of Incubation		
	1st revealed	2nd revealed	3rd revealed
Direct Testing	14	21	28
Indirect Testing	7	10	14

DST IN LIQUID MEDIUM

MGIT Commercial method

Principle

The MGIT growth indicator tube contains Middlebrook 7H9 broth and a silicone, located at its base. The silicone contains a compound with an oxygen-linked fluorochrome, and thus bonded it is inactivated. When a microorganism, breathing and multiplying, consumes oxygen, the fluorochrome is activated, and the fluorescence can be evidenced with UV light. This fluorescence is an indication of the development of the microorganism that can be detected very quickly. The inoculum of the TB bacillus standardized for ST is detected in most cases in a period of 3-14 days after inoculation.

The medium is supplied by the industry in tubes containing 7 ml for automated reading, and 4 ml for visual reading. To investigate the activity of the Z, 7 ml tubes acidified to pH 5.9 are used.

Standardized ST is indirect. To do this, add the critical concentration of each drug to be tested in a MGIT tube. To the series of tubes with drugs, a MGIT tube without drug (control) is added. To investigate the activity of Z another set of two MGIT tubes pH 5.9 should be prepared, one control and the other with the drug.

If the bacillus is sensitive, the drug will inhibit growth and fluorescence in the corresponding tube. On the contrary, if the bacillus is resistant it will develop in the tube with drug and therefore also there will be fluorescence. In both cases, the development of the bacillus and fluorescence should be manifested in the control tube.

To reproduce the principle of the proportions method, the control is seeded with an inoculum diluted 100 times, relative to that which is seeded in drug tubes (except for Z). If the fluorescence in the tube with a drug approaches or exceeds that of the control tube, it is inferred that more than 1% of resistant clones have grown and then the equipment classifies the isolation of the bacillus as resistant to the drug in question Fluorescence can be visualized by focusing UV light on each tube manually seeded, or can be monitored with equipment called MGIT 960 or 320. These numbers indicate the number of tubes that each of them can incubate. Every 60 minutes, the team stimulates each tube with UV, makes readings and registers them as growth units (GU).

In order to interpret the results, the tubes of a ST must be grouped and correctly ordered in the same carrier (AST carrier as the manufacturer in English).

There is a standard basic software (BD EpiCenter™) that interrupts and interprets the ST results when the fluorescence of the control tube exceeds 400 GU, or invalidates ST when that does not happen between 4 and 14 days of incubation (between 4 and 21 days in the case of Z). When the control exceeds 400 GU, within the aforementioned period, the equipment analyzes the growth registered in each tube with drug. The isolation is classified as sensitive to a drug if the reading in the tube containing it is less than 100 GU; otherwise it is classified as resistant. The software shows growth curves constructed with readings recorded by the equipment during the entire time the ST is incubated. The observation of the curves allows detecting and verifying results that are not normal or common. The EpiCenter software allows the entry of ST to SIRE and Z, for other series of drugs it is necessary to enter them as "undefined" or use a contraption.

There is another special software (BD EpiCenter ™ TB-eXiST) that is more complicated to use but allows the operator to design the ST with the drugs you want, as needed, prolong the incubation and make your own interpretation of the results.

The automated system is provided with SOP made by the manufacturer very detailed to perform the ST and operate the equipment. The procedures in force at the time of writing of this Manual are described here, but may vary if the manufacturer makes changes to the system.

Limitations

ST with visual reading has been standardized to test only I and R.

When automated reading is used (MGIT960 or 320), automatic interruption of the test is disadvantageous for disgonic isolates or that have a low percentage of resistant clones and may require longer incubation. This has been demonstrated for isolates with borderline resistance to R. This type of resistance is detected by molecular testing and has been associated with failure of treatment with first-line drugs.

Disorders with borderline strains have also been identified against E, and the system appears to produce false resistant results for Z.

When it is suspected that this type of isolate is present, and in the face of a disagreement with molecular methods results, it is advisable to repeat the test by the molecular method and / or in solid medium (which allows to prolong the incubation and to quantify the proportion of the bacilli population that is resistant), and to identify mutations by sequencing if this tool were available.

Precautions

Any germ coexisting with *M. tuberculosis* that has gone unnoticed or may be added during processing generates erroneous and probably serious results for the patient. It must be ensured that the isolation of *M. tuberculosis* under investigation is the only germ inoculated in the ST, verifying the purity of the culture. In addition, it must be operated under strict asepsis conditions, with good practices to avoid contamination with environmental germs and also cross contamination between different strains that are handled in a working day. The liquid medium facilitates the development of contaminants.

Usually when developing a common germ, the automatic reading equipment when detecting development before 4 days of incubation invalidates the ST. An early signal can also be generated by some environmental mycobacteria, especially if it is fast growing. The detection of environmental mycobacteria presents a challenge that requires the intervention of experienced personnel, even more so if it coexists with *M. tuberculosis.* It is a rare situation, but it can happen, especially with material from very immunosuppressed patients.

Extreme biosafety is required because aerosols with liquid media are more likely to be generated than with solid media.

The mono-resistances to E, R and Z are very rare. A result of this type must be confirmed.

Preparation of the inoculum

Biological material to be investigated

Young and pure isolates or cultures, identified as *M. tuberculosis,* obtained in liquid or solid media.

To verify purity:

- Microscopically examine an extension prepared with isolation and stained using the Ziehl-Neelsen technique (see part 1 of this Manual, Sputum). Verify that only AFB is observed

- If, despite microscopic examination, suspicion of contamination persists, splint with agar a culture sample on plates with blood agar (or chocolate agar or brain-heart), incubate at 37°C for 48 hours and verify the absence of common germs. If contamination is detected, process another isolation from the same patient if possible, and if no other isolation is available, proceed to decontaminate (see part 2 of this Manual, culture).

Additional Materials

For each isolation to investigate

- 1 glass tube with sterile screw cap, containing 4.5 ml of sterile distilled water

- 1 glass tube with sterile screw cap, containing 9.9 ml sterile distilled water

Label with the number of each material to investigate.

Procedure

Isolation of M. tuberculosis obtained in liquid medium in equipment with automated Reading

- Incubate the culture between 1 and 5 days after the positive signal is detected,

  If the culture is more than 5 days after the positive signal is detected, peal in another MGIT tube and incubate until it is again positive

Note: This is the manufacturer's recommendation. Some reference laboratories in Latin America have experienced that valid, faster and less costly results are obtained by matching the turbidity of the tube that is more than 5 days positive with that of a Mc Farland pattern 1, and the procedure is immediately followed.

- Shake the culture with vortex,

- Let stand 15 to 30 minutes to decant the lumps and avoid dispersion of aerosols,

Suspensions prepared with other types of insulation should be standardized using a turbidity pattern.

Additional Materials

- Mc Farland Turbidity Pattern 0.5

Note: The manufacturer recommends the Mc Farland 0.5 turbidity pattern. Some reference laboratories in Latin America have experienced that the use of the Mc Farland 1 turbidity pattern is more adequate to reduce the frequency of invalid results because of poor development in the control tube.

Isolation of M. tuberculosis obtained in liquid medium and detected visually

- Let the tube or bottle containing the insulation settle for 15 to 30 minutes,

- Remove with a pipette approximately 5 ml of the suspension (without touching the sediment) and transferring it to the tube or bottle with glass beads. Discard the pipette,

- Ensure that the lid of the tube or bottle is closed and vortexed until the suspension is homogeneous, without lumps,

- Leave to rest 15 to 30 minutes, to decant lumps that could persist, and decrease the risk of dispersing aerosols,

- Check that there are no lumps in the supernatant; if detected and can not be disassembled by repeating the two previous steps, to prepare the suspension again from the beginning,
- Gently pipette the supernatant (without touching the lumps that may have settled in the bottom of the tube or bottle) and transfer it to a tube with screw cap. Discard the pipette,
- Add sterile distilled water, little by little, until the opacity equals the naked eye with the turbidity pattern,
- Allow standing for at least 15 minutes before proceeding with dilutions.

Isolation of M. tuberculosis obtained in solid medium (LJ, Ogawa or agar)

Additional Materials

- Mc Farland Turbidity Pattern 0.5

Note: The manufacturer recommends the Mc Farland 0.5 turbidity pattern. Some reference laboratories in Latin America have experienced that the use of the Mc Farland 1 turbidity pattern is more adequate to reduce the frequency of invalid results due to poor development in the control tube.

By culture to be processed

1 bottle or tube of glass very resistant, or of very transparent plastic, with capacity for 15-20 ml, with screw cap, containing 5-10 glass beads, sterile, labeled with the number of each insulation to investiga-te.

Procedure

- Take with a handle bacillary mass, trying to take material from all the colonies or the whole, avoiding drag medium culture. If the insulation has poor development, take bacillary mass from all available tubes,
- Place the material in the tube or jar with glass beads. Discard the handle,
- Ensure that the lid of the tube or bottle is closed and vortex until completely disassembled, checking that there are no lumps,
- Add 1 ml sterile distilled water,
- Ensure the closure of the tube or bottle cap and vortex until homogenized,
- Let stand 15 to 30 minutes to sediment lumps and reduce the risk of dispersing aerosols,
- Check that there are no lumps in the supernatant; if detected and can not be disassembled by repeating the two previous steps, to prepare the suspension again from the beginning,
- Pipette the supernatant gently (without touching the lumps that may have decanted) and transfer it to a tube with a screw cap. Discard the pipette,
- Add sterile distilled water, little by little, until the opacity is equal, at first glance, to that of the turbidity pattern,
- Allow settling for at least 15 minutes before proceeding with dilutions.

Dilution of bacillary suspensions

Next it is denominated suspension A to be inoculated in the tube with drug. Depending on the source material, to prepare the suspension A should be or not to do some dilution, as detailed in the following table:

Isolation obtained in			Suspension A (To inoculate in the tube with drugs)
MGIT 960 or 320	Days elapsed since the positive sign of the isolation	1 o 2	Culture without dilution
		3 a 5	Culture diluted 1: 5
Suspension of other turbid isolation matched to patron Mc Farland			Suspension main diluted 1: 5

Procedure

Additional Materials

By ST to perform

- 1 tube with screw cap containing 4 ml of saline solution or sterile distilled water with the label 1: 5 and the number of isolation to be processed,

(not required for cultures obtained from automated MGIT reading equipment processed 1 or 2 days after the positive signal was detected)

- 1 tube with 9.9 ml of sterile saline or sterile distilled water with the label 1:100 and the number of isolation to be processed,
- in the case of ST to Z, add 1 tube with 4.5 ml of saline solution or sterile distilled water with the label 1:10 and the number of isolation to be processed,
- 1 calibrated single-micropipette with dispensing volume ranging from 100 µl to 1000 µl,
- tips suitable for the micropipette.

When performing the following steps, do not open a tube without having previously closed the previous one.

Dilution 1: 5 to prepare solution A
should not be performed if a culture obtained in MGIT equipment is processed after only 1 or 2 days after the positive signal.

- Take 1 ml of the culture medium supernatant in the liquid medium or the opacity slurry equal to the turbidity standard, micropipette, as appropriate, avoiding moving the sediment and transferring it to the tube with 4 ml of sterile distilled water. Discard the tip,
- Vortex,
- Leave to rest 15 to 30 minutes to decant eventual lumps and avoid the dispersion of aerosols

Dilution 1:100 to inoculate the control tube

- Micropipette 0.1 ml of suspension A and transfer to a tube with 9.9 ml of sterile distilled water,
- Vortex shake

Dilution 1:10 (to inoculate the control tube from ST to Z, if it is performed)

- Take 0.5 ml of suspension A with a micropipette and transfer to a tube with 4.5 ml of sterile distilled water,
- Vortex shake

Inoculation and incubation

Additional Materials

For the method in system MGIT960 or 320, for each insulation to investigate

- 1 *carrier* tube (AST carrier provided by the manufacturer) with adequate capacity to contain the control tube and tubes with the drugs to be tested in the ST (except the Z),
- 1 tube holder (AST *carrier* provided by the manufacturer) to contain the control tube with acidified pH and the tube with Z (in case this drug is included in the ST)

Observations
When the available carrier does not have sufficient capacity to test all the drugs that make up the ST, a second carrier with its own control should be prepared, as if it were another ST. For example, if you need to test 5 drugs and only carriers with 5 tube capacity are available, a control tube and 4 drugs will be placed in one carrier, another control tube and the remaining drug in another carrier. So, in this situation, two control tubes must be prepared.

When the available carrier exceeds the required capacity, MGIT white tubes (not inoculated, without drug) may be used to fill the voids.

Medium and Reagents

For each isolate or control strain to be investigated,

- 1 MGIT drug-free tube with OADC enrichment, labeled “control” and the number of the isolation to be investigated,
- Multiply this number by the number of carriers that need to be used, if more than one is necessary.
- 1 MGIT tube with OADC enrichment and each of the drugs to be tested (except Z), labeled with the name of the drug it contains and the number of isolation to be investigated,

For the MGIT 960 or 320 equipment MGIT tubes are used with 7 ml of medium, to perform the ST to I and R with visual reading the tubes with 4 ml of medium.

In the case of ST to Z, add for each test

- 1 MGIT tube with BACTEC MGIT 960 Z supplement without drug, labeled “control” and the number of the insulation to be investigated,
- 1 MGIT tube with BACTEC MGIT 960 Z supplement and with Z added, labeled with the drug name (Z) and corresponding insulation.

MGIT tubes, supplement and drugs must belong to lots with controlled quality.

The label can be written or adhered with a label on the sidewall of each tube of the series, being careful not to obstruct its Barcode.

Order in series on a rack each ST.

Place suspension A and continue the tubes with drugs to be tested.

Place the dilution 1: 100 of the suspension A and then the control tube of the ST (or as many carriers to use in the ST if more than one was necessary).

Place the 1:10 dilution of the suspension A and then the control tube pH 5.9 from the ST to Z (in case the drug is tested).

Leave a place at the beginning of each series to systematically displace each tube after the sowing is completed, so as to avoid omissions or repetitions.

Procedure

For each isolation to be investigated,

- Add 0.5 ml of suspension A to each of the tubes containing drug, including Z in the case where it is tested. Do not inoculate the control tube with this suspension,

- Inoculate 0.5 ml of the 1:100 dilution in the control tube of the ST (or in all control tubes if more than one carrier is used), except in the control tube of Z,

- in the case of ST to Z, inoculate 0.5 ml of the 1:10 dilution in the MGIT control tube pH 5.9,

- Fit the tube lids well, and mix the contents of each tube, inverting it 3 or 4 times,

- If you are performing the test with manual reading, incubate the tubes in a rack in an oven at 37°C,

- When using MGIT 960 or 320 equipment, place the tubes of a ST (without Z) in the appropriate *(carrier)*, or in more than one carrier if necessary, as indicated above.

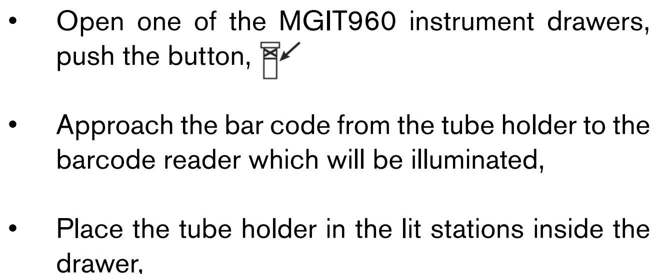
If the ST to Z had been planted, place the two acidified tubes inoculated (control and with Z) into another separate *carrier.*

Follow the manufacturer's instructions to introduce the tubes into the equipment, which, in summary, comprise the following steps:

Place in the tube holder first, to the left the control and then the drugs ordered always in the same way, systematically.

For example:
IR control (in a tube holder with capacity for 3 tubes)
SIRE control and control KACLfx (or Mxf) (in two tube holder with capacity for 5 tubes each)
Control Z (in tube holder with capacity for 2 tubes)

- Open one of the MGIT960 instrument drawers, push the button,



- Approach the bar code from the tube holder to the barcode reader which will be illuminated,

- Place the tube holder in the lit stations inside the drawer,

- Enter the corresponding ST data in the software,

With the BD EpiCenter software, it is possible to enter the ST to IR, SIRE and Z but it does not offer other possibilities. In the case that a tube holder is inserted with other drugs (for example KACLfx), enter it as if it were SIRE having noted appropriately in the records of the laboratory that, in that case, the PS has been made with KACLfx.

- Press the OK button,
- Close the drawer,
- Relate the test to patient identification using the BD EpiCenter software as follows:

- Click the second toolbar icon and the "Quick Entry" tab.

  Enter the identification number of the insulation in the fields ID and Access No,

  Enter the surname and patient's name in the corresponding field,

  Complete the rest of the information.

- Select the "Test" tab and the "Orphan" tab, below and to the left of the screen.

  Select the tube holder code of the ST that is being entered

  Assign the corresponding test number (ST)

  Use the space assigned to test number 2 or subsequent, if previous ST(s) had already been made for that patient,

  If you want to register a ST with different drugs than the equipment offers, you can select the test number greater than 10, and record in a separate listing the sequence of drugs that have been planted in that ST,

- Press OK and save.

Use in a template designed for this purpose if you do not have the software).

Reading and recording results

Manual Reading

Additional equipment and materials

- Portable lamp-flashlight that emits UV light
- 1 un inoculated MGIT tube (negative control)

Procedure
Caution: Do not impact UV light on the eyes

No darkroom is required

- Inspect every two days by affecting UV light in each tube,
- Check for orange fluorescence at the bottom of each tube. Compare with what is displayed when illuminating a negative control (MGIT without inoculating) to facilitate reading,
- When fluorescence appears on the positive control tube (seeded with the 1:100 dilution of Suspension A), incubate the test for 2 additional days at 37 °C,
- After completing the 2 additional days, compare the fluorescence of the tubes with drugs with that corresponding to the positive control tube,
- For each drug tested, interpret and record the result as indicated below.

Drug tube	Interpretation
Without fluorescence	Susceptible
with fluorescence of equal or greater intensity that the control tube	Resistant

If the control tube (without drug) does not emit fluorescence finalized the third week of incubation, it is not possible to interpret the result. Repeat the test.

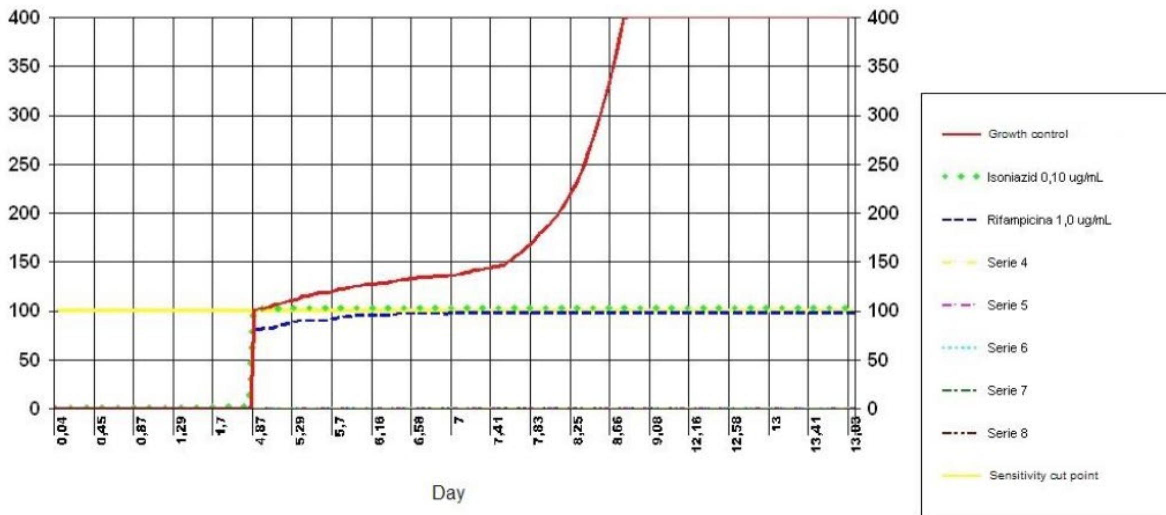
In case of doubt regarding the fluorescence corresponding to a tube containing drug, re-incubate the ST for up to 2 more days. If the question persists, repeat the test.

Automated reading (with MGIT 960 or 320 instruments)

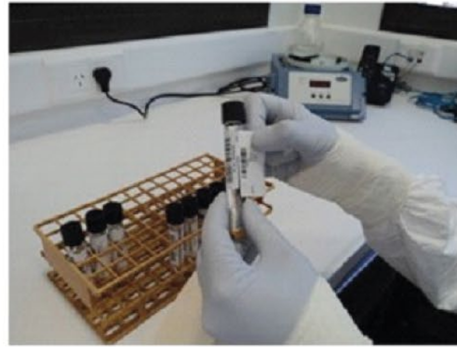
- Control daily the equipment that identifies the finished ST, reports the results automatically, and the growth curves produced,
- Once the ST is finished, manually shake the control tubes and verify the presence of the characteristic lumps of M. tuberculosis

Precautions for Interpreting Results

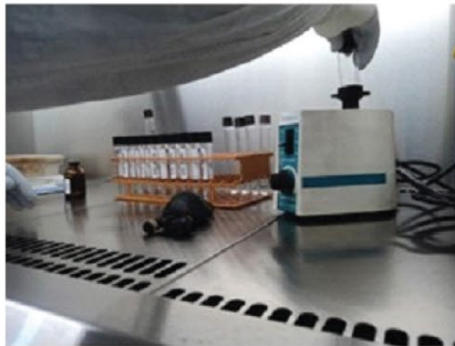
Find	Conduct to follow
Growth of very rapid control and / or presence of a homogenous bacterial suspension and / or polyresistance to drugs	Discard the presence of common germs by extending the suspected tubes Ziehl Neelsen staining and microscopic examination In case of persistent doubts, striate a sample of the broth in culture medium suitable for the growth of common germs. Discard the presence of an environmental mycobacteria verifying the speed of development of the first insulation and morphology of colonies in solid medium Identify by molecular test designed to identify environmental mycobacteria (including LPA) any isolated colony with morphology not compatible with *M. tuberculosis* If it is not available, perform the PNB or PAS test If the presence of non-acid-resistant germs is confirmed or of some environmental mycobacteria in the presence or absence of *M. tuberculosis* annul the results of ST and repeat with pure isolation of *M, tuberculosis* If the control growth was very fast and the purity of the culture of *M. tuberculosis* repeat the ST because there may have been excess inoculum
Monoresistance to E	It is infrequent, to repeat the test if possible with an alternative method
Monoresistance to Z	Z resistance not associated with RR is infrequent, repeat the test, if possible with an alternative method.
Growth curves that in drug tubes approach or maintain close to 100GU and do not exceed 400GU	May reflect borderline resistance Repeat the test with the drugs with which this finding was detected, if possible also with an alternative method
Error	Consult the equipment manual for possible cause of error solve the problem as indicated by the manufacturer before repeating the ST



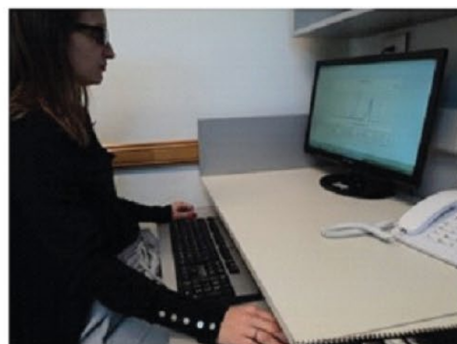
Susceptibility test in MGIT 320/960



Printing labels and tubing labeling



Preparation and inoculation ofthe bacterial suspension



Review of development records and curves within and outside the biological risk containment area

MOLECULAR SUSCEPTIBILITY TESTS

Commercial methods
Xpert® MTB/RIF

The system is being perfected technically at the time of writing this Guide. The following descriptions, instructions and recommendations are based on the evidence known to date.

Principle

This system allows the identification of the presence of the *M. tuberculosis* complex in samples of clinical origin and the mutations that most frequently originate its RR in a few hours. Integrates and automates sample processing including DNA extraction (purification and concentration of genomic material from captured and sonicated cells), nucleic acid amplification and detection of target sequences. The reagents *(primers, probes, enzymes, salts, buffers, surfactants)* are provided lyophilized into the compartments of a closed cartridge. The cartridge should be discarded after use, without being opened at any stage of the process, which minimizes the risk of cross-contamination.

The sample is initially treated with a decontaminating solution composed of sodium hydroxide and isopropanol, which minimizes the biological risk. At the same time, it renders the sample unusable for cultivation.

The first type of cartridge developed (Xpert® MTB/RIF) contains primers that amplify a region of the rpoB gene that contains 81 bp where the most frequent mutations that produce RR and a specific region of the *M. tuberculosis complex* occur. Each probe reproduces a fraction of the target sequence investigated, as it is in the bacillus that has not mutated, that is to say that it is sensitive to R. Then, 5 probes marked with fluorochrome of different color are used. When hybridized, the probes emit the fluorescent signal that is sensed by a sensor and quantified by software. The software represents in a curve the quantification of the signal in real time. For each probe, the number of amplification cycles above which the reaction is classified as positive (Ct) is standardized. If the 5 probes hybridize within that time (i.e. without delay) the presence of the R-sensitive (wildtype) *M. tuberculosis complex* is identified. If any of the probes do not hybridize or do so late in relation to that time limit, a mutation is identified in the gene region investigated and therefore the presence of *M. tuberculosis* RR is inferred. If the reaction is totally negative, it is interpreted that the presence of the *M. tuberculosis complex* has not been detected.

To increase the sensitivity of the system, the Xpert® MTB / RIF Ultra cartridge was developed with primers that, in addition to the rpo*B* gene, amplify two sequences found in multiple copies in the genome of the *M. tuberculosis complex* (IS6110 and IS1081). In addition, its PCR chamber admits higher volume (50 µl in Ultra vs. 25 µl in Xpert MTB / RIF). On the other hand, in order to differentiate mutations and to more accurately distinguish between silent mutations and those that actually confer RR, the modified software analyzes the melting temperature (or Tm) corresponding to 4 probes that overlap covering the white sequence of the rpo*B* gene. A change in the Tm pattern corresponding to the sensitive strains allows identifying the mutants.

The test is completed after about 2 hours with the Xpert MTB/RIF cartridge and 1 hour and 15 minutes with the Xpert Ultra cartridge.

The Xpert detects some mutations that generate borderline resistance and can cause false sensitive results in the MGIT 960/320 system.

The system uses:

- A control of sample processing and the presence of inhibitors (SPC by the acronym of its name in English sample *processing control*); for this it verifies the amplification of DNA of non-infectious spores of an environmental germ, *Bacillus globigii,* contained in the cartridges.

- A probe control which measures, prior to the initiation of PCR, the fluorescence signal to verify rehydration of reagents, filling of the PCR tube, probes integrity and stability of fluorescent dyes the acronym of its name in English *probe check control).*

For processing, each cartridge is inserted into a module of the equipment. There are standard equipment with 1 to 16 modules, and large equipment that can use up to 80 modules. It is a multiple platform that allows investigating, with different cartridges, not only TB but also other infectious diseases and risk factors.

The system comprises a set of modules to house the cartridges, a personal computer, software that must be kept up-to-date with the latest version to investigate TB, a scanner to capture the bar code of each cartridge, and detailed SOP for sample processing and operation of the equipment.

The procedures in force at the time of writing of this Guide are described here, but may vary if the manufacturer makes changes to the system.

Limitations

The evidence about the accuracy achieved with the Xpert Ultra is incipient at the time of writing this Guide.

There is insufficient evidence to recommend the use of this method with feces, blood or urine.

The pleural fluid is not the best sample to diagnose pleural TB, and therefore the probability of being positive for Xpert is lower in relation to other extrapulmonary samples, pleural biopsy is preferable.

Bloody or xanthochromic samples of cerebrospinal fluid may produce false negative results.

With both cartridges, it is possible to achieve greater sensitivity than the AFB to detect TB, but lower than the culture with samples with negative AFB. The Xpert® MTB/RIF Ultra cartridge is more sensitive than Xpert MTB / RIF, especially with samples containing few bacillus, but at the same time it is less specific.

With both cartridges, and most notably with the Ultra, the specificity is lower among patients with a history of previous TB because it can detect bacilli remnants after chemotherapy, inactive. The timeframe during which false positive results can be detected after the end of a TB episode has not been determined. For this reason, the system should not be used to evaluate the efficacy of treatment in a patient undergoing chemotherapy, although it may be used during treatment to detect the occurrence of RR.

For these reasons, the investigation of the samples should be completed with culture, especially to detect TB with paucibacillary samples, to give greater

support to the diagnosis of previously treated patients, and also to allow ST to be performed on drugs other than R. volume of the sample is sufficient, it can be divided to process one part by Xpert and another by culture. If this is not enough, at least two samples of the patient should be obtained whenever possible, one to be processed by Xpert and the other to be cultured. In the case of cerebrospinal fluid with low volume, Xpert processing is preferred given the urgency of the diagnosis.

The procedures in force at the time of writing of this Guide are described here, but may vary if the manufacturer makes changes to the system.

Materials provided by the manufacturer

- Sample reagent (SR by the acronym of its name in English sample reagent),),
- Sterile, plastic, disposable, graduated pipettes for the transfer of the sample,
- Cartridges containing the amplification and detection reagents.

Biological material to be investigated

Any of the following materials may be investigated

- At least 1 ml of a sputum sample, which does not contain any visible remnants of food,
- At least 0.5 ml of the sediment from a sample decontaminated and centrifuged using the culture procedure, suspended in phosphate buffer pH 6.8 in a graduated tube with screw cap (see part 2 of this Manual, Culture),
- The total of a sample of cerebrospinal fluid whose volume is between 0,1 ml and 5 ml, in a graduated tube with screw cap,
- The total of another semifluid sample with a volume between 1 and 5 ml, in a graduated tube with a screw cap,
- The precipitate resulting from the centrifugation at 3000 g for 15 minutes of a sample of cerebrospinal fluid or other semifluid samples with a volume greater than 5 ml in a graduated tube with a screw cap,
- At least 0.7 ml of the maceration of a sample of tissue taken with biopsy sterility, transferred to a tube with graduated screw cap, avoiding to take remains that have not been able to disintegrate (see part 2 of this Manual, Culture)

Procedure

Precautions

- To take advantage of the speed of the method with all its potential, to process the sample on the same day it is collected,
- Maintain sputum samples preferably in refrigeration (2-8 °C) before being processed,
- Exceptionally, if strictly necessary, sputum samples may be kept up to 3 days at a maximum temperature of 35 °C, or for 4-10 days in refrigeration,
- Maintain in refrigeration (2-8 °C) precipitates of decontaminated or centrifuged samples before being processed on the same day,
- Samples that do not reach the required minimum volume can produce falsely negative results,
- Due to the minimum biological risk involved in the application of this method, it may be carried out with the biosafety conditions required for AFB (see part 1 of this Manual, sputum smear microscopy), except to process samples that must be previously centrifuged or tissues that must be macerated. In these latter cases, the processing must be performed within BSC and with safe centrifuges (see part 2 of this Manual, Culture),
- In the case where the method is applied for sputum in laboratories of the level of primary health care, with personnel not trained to transfer solutions and samples, it must be trained in the procedures necessary to avoid cross-contamination until the time to close the cartridge,

* do not touch with the sleeve of the packaging wrappers with samples
* use of gentle maneuvers to avoid splashes and sprays
* do not touch mouths or walls of flasks or tubes containing each sample
* Adequate proper disposal of each pipette after use
* close each bottle with screw cap immediately after operating with the)

- Do not open the Xpert cartridge until the sample is added,

- Do not use cartridges that are wet or damaged, dropped or agitated after the sample has been added,

- Do not process samples after 30 minutes of being transferred into the cartridge.

The maximum number of samples that can be processed at one time is equivalent to the number of modules that will be available.

• Observe the volume of the sample,

• Dump into the package or tube containing each sample reagent (SR) in the following amounts,

  twice the volume of the sputum sample

  twice the volume of the precipitate from a decontaminated sample

  twice the volume of maceration of a fabric

  sufficient volume to complete 2 ml in the case of cerebrospinal fluid with volume less than 0.5 ml

  volume sufficient to complete 2 ml in the case of a precipitate from a centrifuged simple

• Close and shake vigorously 10 to 20 times each bottle or tube,

• Keep at room temperature for 15 minutes in total. During this time, at 5 to 10 minutes, shake again, vigorously, 10 to 20 times,

• Verify that the sample is liquid, with no lumps visible, particularly in the sputum,

• Remove the cartridge from its packaging, adhere the barcode provided, and write or affix a label on the side of each cartridge with the number of each material to be investigated, taking care not to obstruct the bar code,

• With the pipette provided by the manufacturer, aspirate the sample already treated up to the mark (2 ml),

• Open the lid of the cartridge and discharge the sample inside the compartment, smoothly and slowly avoiding the formation of aerosols,

• Close the cartridge cover firmly,

• Insert the cartridge into the machine,

• Check that the computer and computer are turned on,

• Enter the software, select “Create test” in the screen,

• When the corresponding instruction appears, scan the barcode of the cartridge whose data will be entered automatically into the corresponding fields (including batch and expiration date),

• In the corresponding field, enter (or scan if barcode labels were used) the number of the sample being processed,

• Select “Start the test” and enter the operator password,

• Open the module door showing a flashing light, insert the cartridge, and close it,

• When the test is finished, the light goes out. Wait for the system to release the module opening,

open it and remove the cartridge

- The remnants of the processed samples can be kept in refrigeration for up to 12 hours, in the event that the test is required.

Reading and interpretation of results

It is performed automatically by the equipment and shown on the screen. The equipment proceeds for the interpretation considering the following results of the controls and of the sample.

Internal controls

Control of sample processing and the presence of inhibitors (SPC)

must be positive in a negative sample, otherwise the result is classified as "invalid"

may be positive or negative in a sample in which the *M. tuberculosis complex* is detected

control of probes (PCC)

for the test to be valid, it must produce signal before the Ct standardized by the manufacturer.

Results						Interpretation
of the sample investigated				Of the internal controls		
MTB			Resistance to rifampicin	SPC	PCC	
DETECTED						DNA detected
						of *M. tuberculosis* complex
	High	(Ct <16)				Classifies the concentration of complex DNA
	Medium	(Ct 16-22)				*M. tuberculosis* detected in sample
	Low	(Ct 22-28)				according to the values of Ct
	Very Low	(Ct >28)				that is in inverse relation
	Traces	(a)				with that concentration
			DETECTED			A mutation was detected
						in the region of the ropb gene investigated
			NO DETECTED			No mutation detected
						in the region of the ropb gene investigated
			INDETERMI NED			The DNA concentration of the *M. tuberculosis* complex
						was very low and its resistance could not be determined
						the signal of this control
				Not applica ble		not required
						to interpret the results
					passed	meets acceptance criteria
NO DETECTED						No *M. tuberculosis* complex DNA was detected

Results						Interpretation
of the sample investigated				Of the internal controls		
MTB			Resistance to rifampicin	SPC	PCC	
				passed	passed	meet the acceptance criteria
			NO DETECTED			the investigated R target was not detected
INVALID						Could not determine if present
						the DNA of the *M. tuberculosis* complex
				Failure		the sample was not processed properly
						or there is any inhibitor in the sample
ERROR					Failure	one or more probes do not meet
						with the criterion for acceptance
WITHOUT RESULT						one or more probes do not meet
				Withou t result	Failure	with the criterion for acceptance
						the reaction tube was not filled correctly
						there may have been a fault in some module
				Withou t result	Failure	the system component
						Not enough information collected
						to produce a result
				Withou t result	Not applicable	the test may have been stopped
						when it was in process

(a) The "trace" result is used only for the Ultra cartridge. It indicates that traces of *M. tuberculosis complex* DNA have been detected, and no result is given about RR

It is difficult to interpret since this result concentrates the false positives, even more when it comes to patients previously treated by TB.

In this case investigate a new sample of the patient by Xpert and arrange to cultivate another sample. In the case that with the second sample it is also not possible to obtain a definitive result on the RR by Xpert, or RR is detected, the realization of the phenotypic ST will be arranged. For patients with no prior history of TB, when Xpert *M. tuberculosis* DNA is detected in at least two samples, the basis for confirming the diagnosis of TB is stronger. For patients with a history of previous TB, the possibility that inactive bacillus have been detected and decisions based on clinical and other bacteriological findings should be kept under consideration.

- When a result regarding rifampicin is not expected, considering the epidemiological situation or history of the patient, it is recommended to repeat the test with another sample of the patient to rule out errors. Thus, the test should be repeated in the following cases:

  - A RR result obtained with a sample from a patient who does not have resistance risk factors identified and who lives in a geographical area or area with a low prevalence of resistance to anti tuberculosis drugs,

  - A R-sensitive result corresponding to a patient categorized as failure, or a case contact with TB RR or MDR.

- To interpret discordance with conventional ST, consider the following:

  - The Xpert detects some mutations that generate borderline resistance that, according to some evidence, is associated with treatment failure, and can cause false-sensitive results by the MGIT 960/320 system,

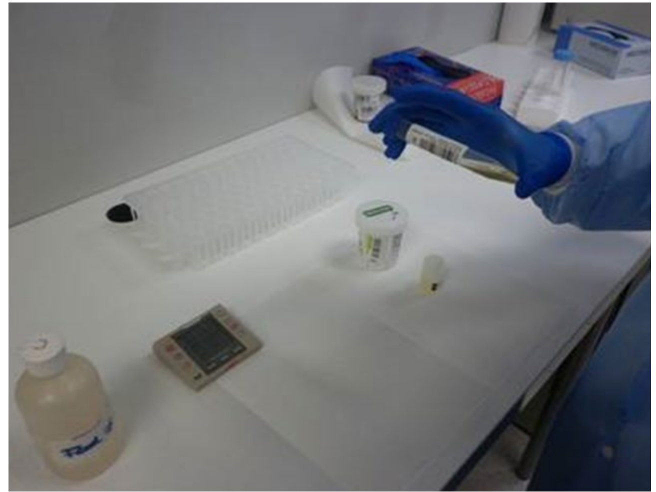
  - The system does not detect rare mutations that generate resistance and are outside the explored gene region,

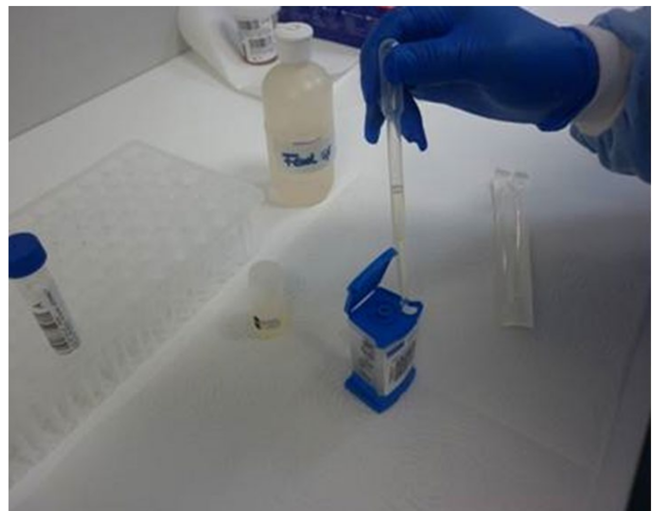
  - Molecular methods, exceptionally, can detect "silent" mutations (which do not generate resistan-ce) that can lead to false-resistant results.

  To determine whether this type of mutation has occurred, sequencing is necessary

- If the software reports "invalid", "error" or "no result", repeat the test. It can be retried with the remainder of the same sample, if it had remained, and request a new sample if the result is kept indefinite.

Obviously, in case it is confirmed that a component of the equipment is damaged, it will need to be replaced or repaired.





LiPA

Principle

Multi-brand systems developed using this technology employ multiplex PCR and reverse hybridization.

Amplify segments of the genes that produce the most frequent mutations that cause resistance to R (INNO-LiPA Rif TB, Genotype MTBDR plus and NTM+MDRTB Detection kit 2, NIPRO) and I (Genotype MTBDR plus and NTM+MDRTB Detection kit 2, Nipro). They also amplify a specific segment of the *M. tuberculosis complex;* the Nipro system also allows differentiating *M. avium, M. intracellulare* and *M. kansasii.*

On the other hand, the Genotype TBMDRsl v2 system amplifies regions of the genes where mutations that produce resistance to FQN and injectables occur.

The biotinylated amplicons that are obtained are deposited on strips having fixed probes, arranged in parallel lines. These probes recognize the genetic segments investigated for the bacillus without mutations, and the most frequent mutations that appear in the strains resistant to the investigated drugs.

After hybridization, alkaline phosphatase-labeled streptoavidin is added. Streptoavidin binds specifically to the biotin trapped in the hybrids attached to the strips (if they were formed). Then, the dye-bound phosphatase substrate (BCIP / NBT, 5-bromo-4-chloro-3'-indolyphosphate and nitro-blue tetrazolium) is added. The phosphatase reacts with this substrate, resulting in a purple-brown precipitate that, finally, allows detecting the hybridization visually. The absence of color indicates that initial hybridization has not occurred.

The lack of hybridization of any of the probes representing the non-mutated gene regions is indicative of a mutation in the bacilli contained in the patient sample being investigated. This mutation can additionally be identified by the system or not. Identification of the mutation has clinical advantages as it can guide the level of resistance developed by the strain and may allow prediction of some cross-resistance (eg a mutation in the inhA gene, predicts low resistance level to I and cross-resistance with ethionamide).

The method is implemented with reagents and SOP provided by manufacturers.

Limitations

The system requires a relatively high amount of white DNA so it can detect the bacillus and its mutations. The percentage of tests with indeterminate results can be very high with paucibacillary samples. For this reason it is advisable to carry out samples with positive smear microscopy and positive cultures.

The teams investigate the most frequent mutations that generate resistance, but not all that can occur. In general, they have a greater sensitivity to detect resistance to R than to the rest of the drugs.

False-resistant results with injectables are more common.

The Genotype TBMDR sl v2 system investigates mutations that can generate cross-resistance between FQN and between injectables used for the treatment of TB. It does not determine the susceptibility to each drug individually.

Precautions

DNA extraction can generate aerosols.

The extraction of DNA from positive cultures generates a high level of risk, must be done within the BRCA and within a BSC.

The extracted material should not be removed from the BRCA until after it has been heated and inactivated. If there is a pass through, the inactivated material must cross it to continue its processing by molecular techniques.

The processes that follow the extraction and inactivation do not generate biological risk, but high risk of cross contamination with amplicons. Therefore, they

must be performed outside the BRCA and respecting a unidirectional flow of samples, processes and personnel involved. Each of the stages mentioned below must be performed in separate environments of the bacteriology laboratory and with each other; there should be no physical communication between those areas nor the air circulating between them.

Preparation of the amplification mixture
DNA loading
Amplification
Hybridization and development

The preparation of the amplification mixture should be separated from the TB laboratory and the area where the LiPA test is revealed.

The amplification and hybridization / development could be performed in a single environment, although it is convenient to separate them so as not to contamina-te the thermal cyclers with amplicons.

No material should be moved to the place where previous steps have been taken. Tubes with amplicons should never be introduced into any area other than amplification / development.

Clothing, personal protection items, instruments and laboratory equipment must be unique to each area.

Work areas, equipment and all surfaces that can be manipulated (including doors, telephones, refrigerator and freezer handles, etc.) should be regularly cleaned and decontaminated using appropriate practices and cleaning products to remove amplicons.

Personnel who perform post-extraction tasks must be highly trained in performing molecular biology procedures and in interpreting banding patterns.

Additional equipment and materials

	Area				
Additional Equipment and Materials	DNA extraction	Preparation of amplification mix	DNA loading	Amplification	Hybridization and development
Microcentrifuge with motor and lid	X				
Water bath or heater with blocks	X				
Sonicator	X				
water bath		X			
Thermocycler				X	
Maria bath with shaking or incubator					X
Equipment for aspiration of liquid (*)					X
Micropipettes	X	X	X		X
tips for micropipettes free of DNAsas and RNAsas	X	X	X		X
2ml sterile tubes		X			
microtubes 0.2 ml for sterile PCR					
Pin					X
Pencil					X
Materials provided by the manufacturer					
Mixing for amplification		X			
Denaturation solution					X
Strips with immobilized probes					X
Hybridization Solution					X
Washing solutions					X
Trays with gutters					X
Format for bonding and reading the hybrid strips					X

(*) can be assembled in the laboratory, for example with a Pasteur pipette connected to a vacuum pump

Equipment provided by the manufacturers of the LiPAs or generic molecular testing equipment available in the laboratory may be used. The capacity of the equipment must be adequate to the workload. DNA extraction and reading of the hybridization bands can be automated with additional equipment, which is convenient for laboratories with high workload.

Biological material to be investigated

- 1 ml of the sediment of a sample, decontaminated, neutralized with phosphate buffer pH 6.8 and centrifuged using the culture procedure, re suspended in phosphate buffer pH 6.8 in a microtube (see part 2 of this Manual, Culture),
- isolates of *M. tuberculosis* obtained by culture.
- 1 ml of liquid medium,
- 1ml of a suspension in sterile distilled water, prepared with the bacillary mass taken with handle of the entire surface of solid medium.

Procedure

For each commercial equipment, the SOP provided by its manufacturer must be followed.

The procedures for the Genotype MTB DR plus v2.0 system are described below, allowing the simultaneous detection of resistance to R and I, as it is the system with which the most experience in the Region has been developed up to the time of writing this Guide Procedures may vary if the manufacturer makes changes to the system

DNA extraction

- Concentrate the bacteria of the material to be investigated by centrifugation at 10,000 - 13,000 g for 15 minutes, in microcentrifuge,
- Discard the supernatant in a closed bottle,
- Re-suspend the precipitate in 100 microlitres of sterile distilled water of DNAsas,
- Incubate in a water bath or block heater for 20 minutes at 95 ° C. Use polypropylene racks, or polystyrene floating parts (with different names in different countries such as Styrofoam, Styrofoam, Styrofoam, Styrofoam, Aniseed Foam, etc.) where drills of adequate size are made to hold microtubes firmly,
- Treat for 15 minutes on a sonicator,
- Centrifuge for 5 minutes at maximum speed,
- Transfer the supernatant to another microtube,

Note: It is possible to perform the extraction of DNA using commercial equipment that allows to dispense with the use of sonicador.

Preparation of the amplification mixture

- For each material to be amplified, including the negative control, mix on the day of use.
  10 microliters of solution A
  35 microliters of solution B.
- Aliquot 45 microliters of the mixture into microtubes for PCR.

DNA loading and amplification

- Label the PCR microtubes containing 45 microliters of the amplification mixture with the number of each of the materials to be investigated, plus a negative control,
- If a sample of a positive culture with micropipette is investigated, add 5 microlitres of DNA solution to the corresponding PCR tube,
- If a decontaminated, micropipetted sample is investigated, add 10 microlitres of DNA solution to the corresponding PCR tube,
- For the negative control (or contamination) add 5 microliters of water, instead of the DNA, to the corresponding PCR tube,
- Locate the microtubes in the thermal cycler,
- Select the program indicated by the manufacturer,

The programs are standardized for the DNA polymerase used by the manufacturer. If another is used, the time of the first step may have to be modified, as advised for the enzyme used.

	culture samples	direct sample of the patient
15 min 95°C	1 cycle	1 cycle
30 seg 95°C 2 min 65°C	10 cycle	20 cycle
25 seg 95°C 40 seg 50°C 40 seg 70°C	20 cycle	30 cycle
8 mm 70°C	1 cycle	1 cycle

- Once the amplification is completed, systematically order the samples on a rack

Hybridization

- Preheat the shaking water bath or incubator to 45 °C ± 1 °C,
- Heat HYB and STR solutions to 37-45 °C before use and mixing. The reagents should be free of precipitate,
- Heating the remaining reagents at room temperature, with the exception of CON-C and SUB-C,
- Using a suitable tube, dilute Concentrated Conjugate (CON-C, orange) and Concentrated Substrate (SUB-C, yellow) 1: 100 with their respective buffers,
  CON-C with CON-D
  SUB-C with SUB-D

  For each strip add 10 microliters of concentrate to 1 ml of the respective buffer. Prepare only the total amount needed for the reactions to be performed on the day, immediately before use.

  Mix well and equilibrate to room temperature.
- Dispense 20 microliters of Denaturation Solution (DEN, blue) into one corner of each of the tray channels, one for each sample investigated plus one for the negative control,
- Maintaining the order of the rack, add 20 microliters of each amplified sample in consecutive channels, pipetting up and down to mix well with the denaturation solution, but avoid generating aerosols,
- Incubate at room temperature for 5 minutes,
- In the meantime, using gloves and a clamp, remove the strips from your tubes. Identify them by typing under the colored mark, the numbers of each amplified sample and the negative control, keeping the order of the rack,
- Carefully add to each channel 1 ml of pre-heated Hybridization (HYB, green) buffer. Gently agitate the tray until the solution has a homogeneous color. Do this very carefully so you do not transfer the solution to the nearby gutters,
- With the clamp, place each strip in its corresponding groove, with the probes facing up (the colored marker facing up). The strips should be completely submerged. Using tweezers, you can turn the strips that may have rotated during your dive,
- Carefully clean the clamps after each use to avoid contamination,
- Place the tray in the shaking water bath or on the platform / incubator for 30 minutes at 45 ° C. Adjust the stirring frequency to achieve a complete and constant mixture of the solution. For proper heat transfer, the tray must be submerged in the water, at least 1/3 of its height,
- Remove from the bath and completely aspirate the Hybridization buffer,
- Add 1 ml of Astringent Wash Solution (STR, red) to each strip,
- Incubate for 15 minutes at 45 °C in a stirred bath or incubator

From this step, onwards, work at room temperature

- Completely remove the Astringent Wash Solution,
- Discard it in a container. Finish removing all remaining liquid by tilting the tray gently onto absorbent paper. Repeat this in all other washing steps.
- Rinse once each strip with 1 ml Rinse Solution (RIN) for 1 minute, on the shaking platform of the incubator. Delete the RIN,
- Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on the platform with shaking,
- Remove the solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN), and again for 1 minute with approximately 1 ml of distilled water (a washing bottle may be used). Working on the platform with shaking the incubator, discarding the solution each time,
- Ensure the removal of any remaining water after the previous wash,
- Add 1 ml of diluted substrate (see above) to each strip and incubate without shaking, protecting them from light,
- Depending on the conditions [i.e. the ambient temperature], the incubation time of the substrate may vary between 3 and 20 minutes. If the incubation times of the substrate are very prolonged, the background staining can be increased which, in turn, could hinder the interpretation of the results,
- Stop the reaction as soon as the bands are visible, briefly washing twice with distilled water,
- Using tweezers, remove the strips from the tray and dry them between two layers of absorbent paper.

Reading and Interpretation of results

- Align and glue strips on forms provided by the manufacturer. Protect them from light,
- Scan or photograph the image of the strips to save the record of the results for an extended period of time. The lines fade with the passage of months,
- Use the instrument for the evaluation provided by the manufacturer. To verify the reactions corresponding to each locus, to prior align the instrument with the corresponding control band,
  Conjugate Control (CC)
  Amplification Control [AC]
  M. tuberculosis complex [TUS]
  rpoB Locus Control
  katG Locus ControL
  inhA Locus Control

Verification of controls

CC
a line should appear in this zone, indicates the activity of the bound conjugate and the reaction with the substrate.

AC
If this band appears, errors in extraction and amplification may be excluded, in addition to amplification inhibitors.

When DNA is detected in the sample tested (positive result), the AC signal may be weak and even disappear due to competition with the sample DNA during amplification. In this case, the amplification reaction was performed correctly and the assay should not be repeated.

The absence of the AC band in the event of a negative test indicates errors during amplification or the presence of inhibitors in the sample. In this case, the test is invalid and the test must be repeated with the corresponding sample.

TUB
If this band does not appear, the bacterium investigated does not belong to the *M. tuberculosis complex* and cannot be evaluated by this system.

Control of the locus (*rpoB, katG, and inhA*)
They detect a region of each gene and should always be positive, when the TUB band has indicated the presence of the *M. tuberculosis complex.*

Negative control
The occurrence of TUB bands and control of the locus *rpoB, katG*, and *inhA* on the strip used for the negative control indicates cross contamination. This invalidates all the results obtained until it is verified that this type of contamination has been removed.

Verification of mutations
Wild type probes.

Only bands whose intensity is equal to or greater than the AC band will be considered as positive. As an exception, it has been recommended that the *rpoB* band WT8 should be considered positive even though it is weaker than the AC if, simultaneously, the band corresponding to the mutation *rpoB* MUT3 does not appear.

When all wild type probes of a gene are positive, no mutations are detected in the regions examined.

The absence of signal in at least one of the wild type probes indicates the presence of mutation and it is inferred that the strain investigated is resistant to the respective antibiotic.

One or more mutations detected with the *katG* probes allows (n) inferring high level resistance to I, whereas the (s) detected with the *inhA* probes infer low level resistance to I.

Probes that identify the most frequent specific mutations

Only those with intensity equal to or greater than the AC should be considered.

Compared with other samples, the positive signals from the *rpoB*MUT2A and MUT2B mutation probes may show a weaker signal.

When the band that identifies one of the most frequent mutations appears, the corresponding probe of the wild type region must not have hybridized, that is, it should appear without a signal, unless there is hetero resistance or mixture of strains in the material investigated.

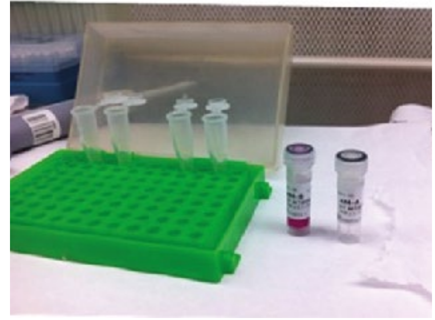
The result is indeterminate when the controls indicate that the test is valid, but bands that indicate the presence or absence of mutations can not be interpreted, for example, as being weak or totally absent.

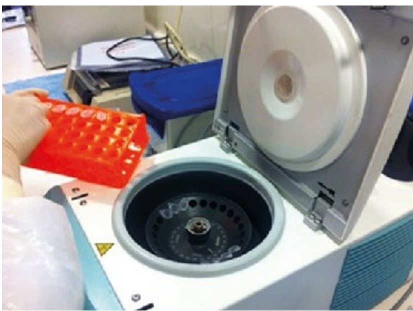
Material with invalid or undetermined results must be retested.

To interpret discordance with conventional ST, consider the following:

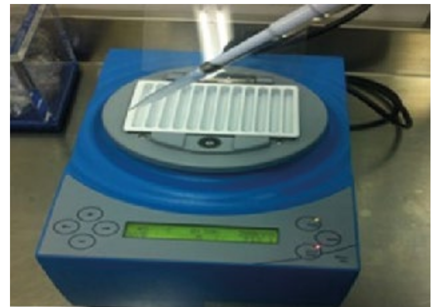
- The system does not detect the less frequent mutations that generate resistance and are outside the explored genetic regions,
- Exceptionally, molecular methods can detect "silent" mutations (which do not generate resistance) that can lead to false-resistant results,
- A sample may contain a hetero resistant strain. In this case both a mutated sequence and an Wildtype sequence can be detected. The phenotypic method can detect it or not, depending on the proportion of clones with mutated and non-mutated sequences in the sample investigated,
- A sample may contain more than one M. tuberculosis strain (both present in the patient's lesion or as a result of laboratory contamination). If one of them is mutated and the other is not, the mutation can be detected by the system, while all the wild type bands appear. If the resistance is phenotypically or not, it depends on the proportion of the mutated and non-mutated strain that is in the sample investigated,
- Some mutations can have paradoxical effects

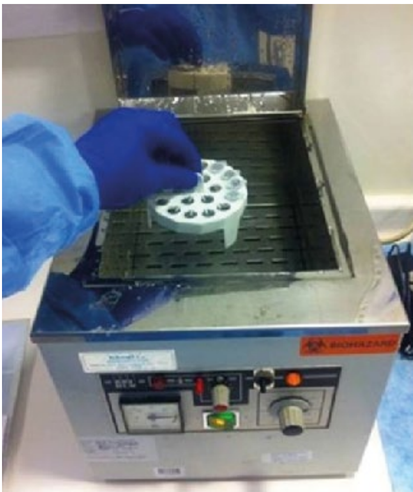
DNA extraction and inactivation



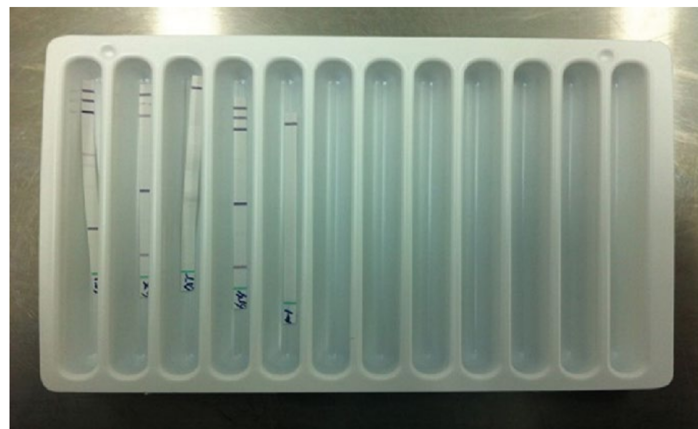


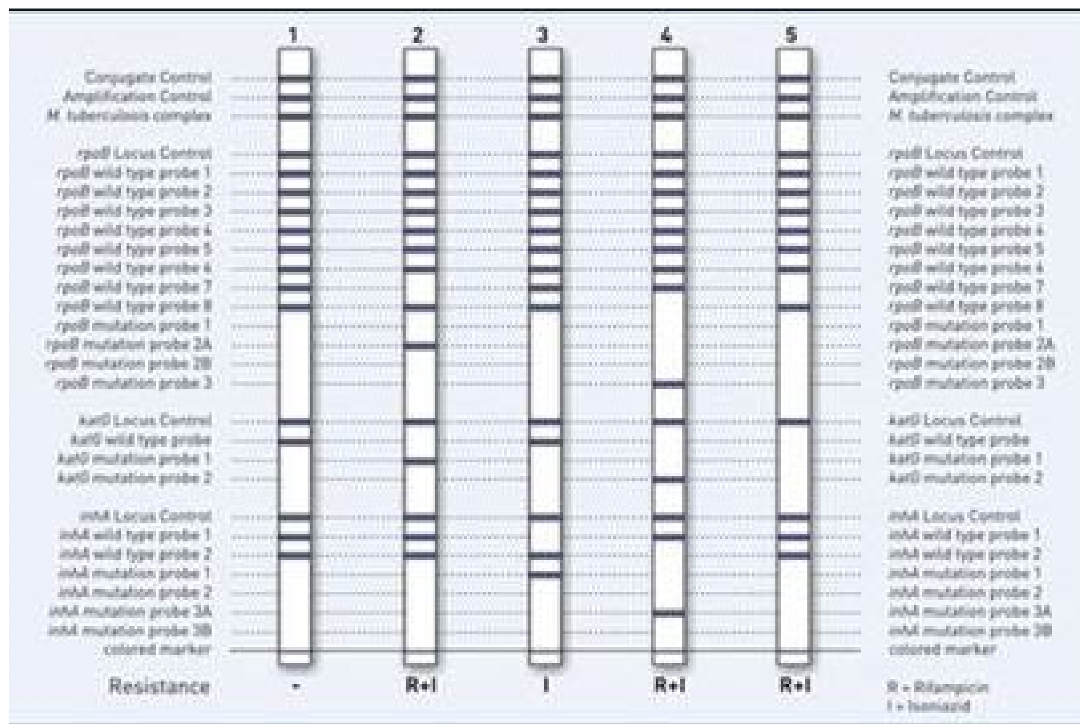
Preparation of the amplification mixture





Hybridization




1
2
3
4
5
Conjugate Control
Amplification Control
M. tuberculosis complex
rpoB Locus Control
rpoB wild type probe 1
rpoB wild type probe 2
rpoB wild type probe 3
rpoB wild type probe 4
rpoB wild type probe 5
rpoB wild type probe 6
rpoB wild type probe 7
rpoB wild type probe 8
rpoB mutation probe 1
rpoB mutation probe 2A
rpoB mutation probe 2B
rpoB mutation probe 3
katG Locus Control
katG wild type probe
katG mutation probe 1
katG mutation probe 2
inhA Locus Control
inhA wild type probe 1
inhA wild type probe 2
inhA mutation probe 1
inhA mutation probe 2
inhA mutation probe 3A
inhA mutation probe 3B
colored marker
Resistance
-
R+I
I
R+I
R+I
R = Rifampicin
I = Isoniazid

CONSERVATION OF ISOLATIONS

The reference laboratory needs to store long-term strains of Mycobacteria in conditions that allow preservation of its viability and characteristics, to have reference material, to support epidemiological surveillance and for research. Preservation can be done for a specific period or for as long as possible.

It can preserve isolates of clinical origin selected for their characteristics of interest, or that have been included in a particular study. In addition, you must keep reference strains among which you may be receiving from your Supranational Reference Laboratory with accurate resistance profiles. Several aliquots of the reference strains used in the laboratory routine are required, as well as those required by laboratories in the network that do not have (ultra) freezing equipment.

All preserved isolation must be coded so that, through the code, it is possible to trace in the registers their morphological, bacteriological and molecular characteristics, as well as their origin and the available information of the cases from which they come. The use of bar code facilitates the process. In addition, a register is necessary to quickly identify the position in a box and in the deep freezer where each isolation has been located. This will allow them to be removed quickly whenever needed without thawing or temperature increase to the rest of the collection isolates. The record shall also include the date of freezing and the medium in which the isolation has been preserved, as well as the dates of defrosting and the results of any verification of purity or characteristics

Principle

Serialized subculture of an isolation can cause genetic mutations and, therefore, alter their microbiological characteristics.

Cultures in egg-based media can be kept at a maximum temperature of 20 °C for up to a year, but the tubes used in the routine take up a lot of space. It is not advisable to preserve without freezing the cultures in liquid medium, for more than 1-2 months, because the medium deteriorates more quickly and becomes more easily contaminated, in addition clusters of bacteria are formed that make its concentration is not predictable.

The viability of *M. tuberculosis* declines much more rapidly at room temperature than at -18 °C, and more rapidly at this latter temperature than at -70 °C: It is therefore advisable to use the lowest temperature possible for preservation. In addition, it is convenient to prepare suspensions of highly concentrated bacteria in the preservation medium, to compensate for the loss of viability.

Repeated thawing and freezing decreases the viability of bacteria, in addition to increasing the risk of contamination.

The viability of environmental mycobacteria is longer and has a lower requirement than that of *M. tuberculosis.*

Biological material

- Isolates of mycobacteria of clinical origin, in solid or liquid medium

- Reference strains in liquid medium or in suspension prepared from solid medium, with turbidity equal to or greater than the Mc Farland 1 standard *(see the section on the Solid Medium Susceptibility Tests in this Guide for the preparation of this suspension).*

The cultures must be young, (in the active phase of multiplication), pure (without contamination or mixing of different mycobacteria) and abundant. Cultures in liquid medium must have turbidity equal to or greater than the standard Mc Farland 1.

Medium

- Middlebrook 7H9 medium enriched with ADC or skim milk 10%, autoclaved at 110 °C for 10

Additional material

For isolation that can be thawed only occasionally
For each isolation to be preserved.

- One 2 ml cryotube
  with external thread and cover with silicone or o-ring protection,
  with persistent humidity label and low temperature indicating the isolation code to be preserved and the date of the peal.

  Depending on the importance of isolation and storage capacity, it may be decided to preserve 2 or more cryotubes per isolation, in which case the required amount will be adjusted.

For a reference strain commonly used in the laboratory
For every 10 aliquots of the strain

- One tube of 25 ml
  labeled with strain code

- Ten cryotubes of 2 ml
  with external thread and cover with silicone or o-ring protection,
  with persistent moisture label and low temperature indicating the code of the strain to be preserved and the date of the peal.

  The total number of cryotubes to be prepared must be adjusted to the needs of the laboratory

Procedure

Work in the BRCA, within the BSC, strictly respecting procedures to preserve biosafety and sterility.

For isolation that can be thawed only occasionally

- Dispense 1.5 ml of Middlebrook 7H9 medium or skim milk into the 2ml cryotube
- Take with a handle the development of the solid medium by scraping the entire surface of the medium, or pipette or micropipette or 0.2 ml of the culture in liquid medium and transfer to the cryovial
- Ensure the closure of the cryovial cover,

For a reference strain

- Dispense 15 ml of Middlebrook 7H9 medium or skim milk into the 20 ml tube,
- Dispense 2 ml of the bacillary suspension or culture into liquid medium into the tube. Ensure the closure of the tube cap,
- Vortex homogenization,
- Distribute 1.5 ml of this suspension into each of the 10 empty cryotubes.

For all cryotubes prepared

- Locate in a box identified with a serial number,
- Place in freezer at -18 ° C for several years, or deep freezer at -70 ° C for decades, located in an BRCA,

  If more than one cryotube has been prepared by isolating a reference strain or another valuable strain, it is convenient to distribute them in different cold equipment if more than one (ultra) freezer is available, in case any of them are damaged.

INTERNAL QUALITY CONTROL

Responsibilities

The organization of the laboratory and adequate distribution of tasks is essential to prevent errors and avoidable delays.

Each of the following responsibilities should be clearly assigned to a highly trained and experienced professional (s) and / or technician (s):

- Preparation of drug culture medium,
- Indication of the series of studies to be performed based on the history and characteristics of each patient, following algorithms,
- Execution of each procedure, registration of dates and results of ST (including quality controls),
- Reading and interpretation of microbiological results,
- Control and final interpretation of the results, considering the antecedents and characteristics of the patient, preparation and signing of the report,
- Supervision, authorization or indication of special procedures, SOP changes, implementation of corrective measures.

The supervisor is responsible for conducting quality management as an overcoming and motivating process, and documentation of the outcome of that process. You should verify the proper availability of:

- manuals, technical guides and SOP,
- working algorithms,
- human resources, equipment and supplies,

For all aspects evaluated, indicators and targets should be established to assess the existing quality and its gradual increase.

The limits of acceptability must also be defined. The supervisor conducting quality management must distinguish errors.

- not acceptable and which must determine the suspension of the reports or of a certain activity until it is documented that the required quality level has been recovered (i.e. unacceptable results of a ST type or for a given antibiotic),

- which can be corrected while continuing the activity (i.e. avoidable delays).

The errors identified guide not only corrective measures but also the design of necessary re-training programs.

Standard Operating Procedures

As a preventive method of errors, it is necessary to standardize each procedure that is performed, both to operate in the laboratory and to apply the microbiological methods implemented and to control their quality. The main ones are:

- Input of biological material,

- Entry / exit of personnel to the laboratory and to the BRCA,

- Algorithms to be applied according to the antecedents and characteristics of each case
- Methods for processing biological material,

- Recycling of material,

- Waste treatment,

- Internal and external quality control,

- Operation and maintenance of equipment,

- Preparation of reports,

- System of registration and archiving of documents,

- Inventory of inputs and equipment,

Each institution can have its own standards and format for the development of SOPs, which should be used by the laboratory. SOPs should reflect exactly what is done in the work routine. The following items present the basic information that usually contain the SOPs corresponding to laboratory techniques.

Purpose and scope
Definitions and abbreviations
Staff Qualification
- Medical fitness
- Education and training

Procedures
- Beginning
- Biosafety conditions
- Biological material to be processed
- Necessary equipment and materials
- Reagents and solutions
- Detailed procedure instructions
- Reading, recording and verification of results
- QA

Other procedures and related documents

Bibliography

Changes, the foundation of every change

In the institutions there are also SOPs of transversal activities, common to all or several working groups (for general support services, maintenance and operation of equipment, administrative, etc.), which also comprise the body of instructions to be followed by the laboratory.

Critical Control Points

Application of current algorithms

The supervisor should check that the most appropriate method or combination of methods is applied according to the characteristics and background of each case investigated by ST.

When the workload is very high, some cases can be selected to perform this control. It is informative to

retrospectively trace the antecedents and procedures applied to cases that present a special challenge (with a history of anti tuberculosis treatment, that have been resistant to several drugs, that have MGIT growth curves that express doubtful results, etc.).

The percentage of cases from which biological material has been received may be calculated from time to time without information on its classification and treatment. It is not related to the quality of laboratory work. However, if it is high, the laboratory should contact the TB Control Program to trigger corrective measures in the health system. Otherwise, it is not possible to optimize the application of available methods or to maintain continuous surveillance of resistance to anti tuberculosis drugs.

Technical Procedures and Records

Phenotypic susceptibility tests

In the routine, operators must:

- Control each batch of culture medium used for ST,
- Maintain a systematic record of the results of this control, which should be reviewed by the supervisor.

ST must control each batch of solid medium that is prepared or commercially purchased with a strain of *M. tuberculosis* sensitive to all drugs and resistant to each of the antibiotics in the batch.

In the case of liquid medium, the control should be performed every working day, because the antibiotic medium is prepared in sufficient quantity only for that day. In laboratories with high workload this is not possible. Minimally each batch of MGIT medium put into use should be monitored and then at least one monthly check should be performed if that lot is used for several months.

It is possible to use strains that are resistant to more than one antibiotic to simplify the procedure, but MDR or XDR M isolates should not be used to reduce the biological risk. The strains used for external quality control, provided by the Supranational Reference Laboratories, are useful for this purpose.

If an incorrect result is detected with the control strain (s), the ST performed with that batch of medium must be canceled and repeated with a new batch. An error could be detected only with some drug (s), and then the results for that drug (s) will be voided. It may also happen that the only error that is detected with a batch of medium is that of false sensitive. In this case the “resistant” results can be reported, but all STs with “sensitive” result must be repeated. And conversely if only false resistant results are detected.

Eventually, a batch of media could successfully pass quality control with reference strains, but subsequently produce a suspicious series of resistant results with either drug, due to deterioration. The supervisor must be alert to this type of alarm

Given the evidence of poor quality of the environment used for the ST, which is exceptional, the following points should be verified to identify the cause:

- Quality of the medium without antibiotics (see the Quality Control Chapter of part 2 of this Manual dedicated to culture),
- Preservation of antibiotics at the temperature indicated by the manufacturer within desiccators and with desiccant salts,
- Accuracy in weighing and dilution of each antibiotic, aliquoted, labeled and preservation of stock solutions in deep freezer,
- Discard the remainder of any vial with stock solution that is thawed,
- Accuracy in the dilution of the stock solution and volume added the culture medium,
- Refrigeration of the medium with drug during its storage,
- Cleaning and proper functioning of refrigerators and deep freezers.

Errors with a tendency give indications about the priority points to investigate to identify the causes.

Examples:

False-sensitive results with the reference strain may

be caused by:

- Excess of drug in the culture medium

- Systematic error of a laboratory worker when reading, validating or interpreting the results.

False-resistant results with the reference strain may be caused by:

- poor conservation of antibiotics or culture medium,

- defect of the drug in the culture medium,

- systematic error of a laboratory worker when reading, validating or interpreting the results.

The most erratic errors can be caused by imprecise procedures in the preparation of the culture medium or the realization of the ST.

On the other hand, from the records, the supervisor can verify the quality of:

- The inoculum
  You can keep a record of the percentage of ST with result: contaminated, underdeveloped or excessive, with poor dilutions, etc.

- Identification of *M. tuberculosis,* at least for all cases where resistance to anti-tuberculosis drugs has been detected.

  The results of the identification test for each of these cases and their corresponding controls will be monitored.

The supervisor should also check the quality of the results records. If the workload is very high, you can take a random sample of ST, on days also randomly selected, to verify your records.

In case of using solid medium, in those days, the ST in incubation can be visualized and those already finished to verify if the record of intermediate and final results adequately reflects the following items:

- Exact number or estimate of the number of colonies developed, although the isolation is sensitive,

- Abnormalities in the morphology or rate of development or distribution (non-homogeneous) of the colonies,

- Presence of liquid remaining and oversold

In the case where ST is performed in a liquid medium, the ST can be selected and the results and growth curves stored by the automated reading equipment with the final results contained in the database laboratory.

The quality of temperature records for refrigerators and incubators should also be checked.

Molecular Susceptibility Tests

The supervisor may

- To visualize on selected days at random the procedures to verify compliance with SOP,

- systematically review the results of the controls (positive, negative, contamination) for each series of samples or isolates investigated by each operator,

- verify the accuracy of transcription of results to the registration system, at least on randomly selected days,

- periodically monitor and graph the percentage of invalid, non-interpretable results and errors; verifying if they are within the range expected for each method. The frequency with which this control is performed will depend on the number of tests performed by the laboratory.

Cross contamination

The supervisor shall

- Display, on randomly selected days, compliance with good practices to minimize the risk of bacilli transfer
  (see page 49 of part 2 of this Manual devoted to culture),

- Maintain alert to results of ST performed consecutively, or on the same day of work, which show an equal and uncommon resistance profile. In that case, it will be verified if the patient's background justifies that profile. Otherwise, you must arrange for the ST to be repeated from the primoculture to rule out possible cross-contamination.

In laboratories with a very high workload and having implemented molecular epidemiology techniques, it is convenient to genotyping a monthly sample of the isolates investigated, and all isolates with a coincident infrequent susceptibility profile, to check whether there is an identity between them. If an identity is detected that is not consistent with the epidemiological information of the cases involved, the determinations should be repeated, preferably with a new isolation of each patient. It is also necessary to visualize and correct the procedures.

Delay

With a suitable frequency, depending on the workload of the laboratory, evaluate:

- Delay between the reception of the material and planting of the ST,

  At least 95% of the samples received for molecular testing should be investigated on the day of receipt or the later.

  At least 95% of the isolates that require ST in culture medium should be planted within 48 hours of receiving or developed in the laboratory.

- Delay in the delivery of results,

  At least 95% of the results must have been delivered within the following deadlines, from the day of their ST
  42 days for ST performed in LJ,
  23 days for ST performed on Middlebrook agar,
  16 days for ST performed at MGIT,
  2 days for samples investigated by Xpert,
  3 days for samples or isolations investigated by LiPA

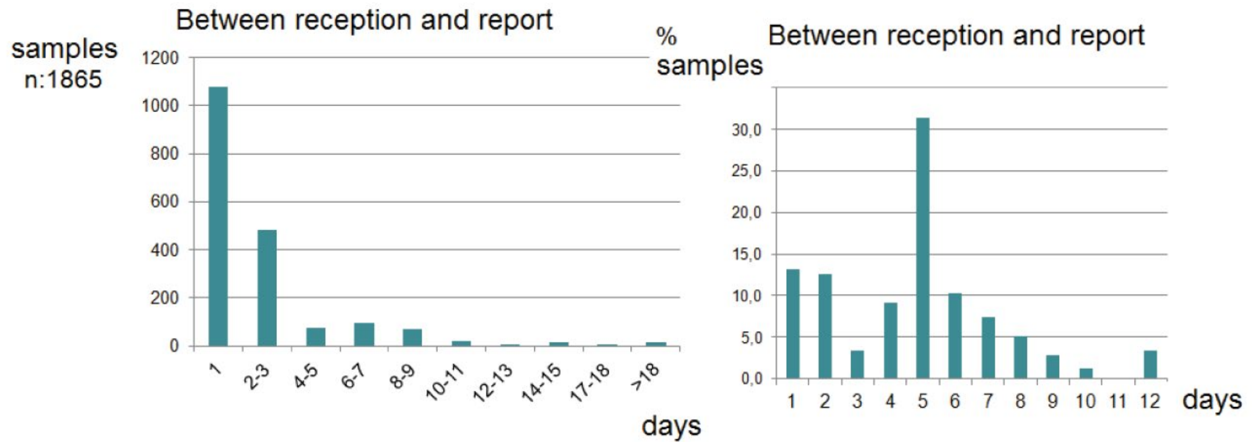
At least 80% of the R and I resistant results detected in culture medium must have been reported within the
23 days for ST in LJ,
16 days for ST on Middlebrook agar,
7 days for MGIT.

Ideally, the delay between the date of sample collection and the availability of ST results (according to the method) should also be analyzed to identify and contribute to correcting excessive times that are the responsibility of the health team or the transport system of samples. If excessive time is evident, the analysis can be stratified (by geographic area, laboratory that refers the material, etc.) to identify where corrections are necessary.

The delay analysis should also consider the one corresponding to failure detection and ST repetition.

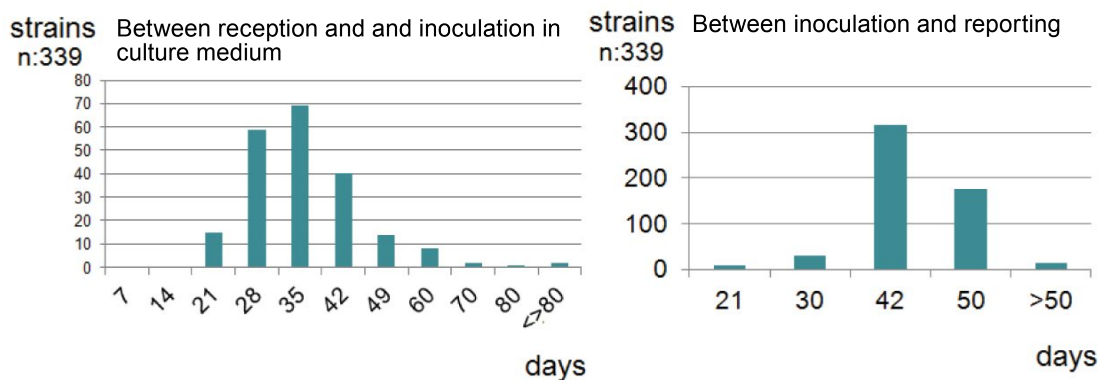
It is convenient to graph these delays to facilitate their monitoring.

Example 1:



The number of samples distributed according to the number of days that have been delayed in reaching the laboratory since they were taken is shown on the left, and to the right the percentage of these samples distributed according to the number of days elapsed since the reception of the material in the laboratory and the report of results produced by the Xpert system. A very high percentage of samples are received the day after or up to 3 days after sampling. This might be acceptable if samples are concentrated from several centers more or less removed from the laboratory. But then, only 29% of the results were reported within the acceptable time (on the day of arrival of the sample or the next), and this is not an acceptable situation.

Example 2



In this other example, a completely unacceptable delay to plant the isolates that this laboratory receives to carry out the proportions method is evident. In addition, while most of the results are reported within acceptable timeframes for this method, it is necessary to verify why it has taken 50 days or more from the sowing to report a not inconsiderable percentage of results.

After this analysis it is necessary to discern if the delay is avoidable or is a consequence of work overload and / or limitation of human resources or equipment, to propose the solution.

Databases designed with commercial software (Excel, Access or other), or specially developed for the laboratory, simplify and streamline this type of analysis.

Results report

The supervisor and / or professional responsible for signing the report shall verify:

- The correct transcription of results from the records to the format used to report them

,
- the results of the quality controls of the medium, reagents and corresponding procedures,
- the constancy in the format of the method employed, investigated drugs and, in the case of phenotypic ST, the concentrations tested,
- consistency of results and clarity of observations,

  recent samples using different methods (in the same laboratory or in different laboratories of the network)

  previous samples.

  In case of inconsistencies (eg resistance to I detected by molecular method and not detected in the culture medium or resistance to a drug detected with a previous sample of the patient that has disappeared), it must be reported that inconsistent results have been obtained with that drug and arrange for the repetition of the test (s) starting from the first isolation, if possible, and / or from another sample of the patient.

  If, after discarding possible errors, the difference between the results of different methods can be explained by limitations of any of them, the report should explain it, in language comprehensible to the health personnel, to guide the interpretation. For ex. if a mutation that determines borderline resistance is detected by the molecular method, but the ST in MGIT 960/320 is sensitive, it will be explained that it is very possible that the isolation is resistant to R and that the culture system has not detected resistant clones of late development. Or if no resistance to I was detected by a molecular method but by ST in culture medium, it will be explained that the isolation is most likely resistant to I by some mutation not investigated by the molecular method used.

- Mention of pending studies to complete the sensitivity profile or corroborate certain results (if they are being performed);

This is a critical professional responsibility for the quality of work. Incorrect, inconsistent or confusing reports are very serious because they may impair the clinical handling of the case.

It is worth mentioning that there is a Network of Supranational Laboratories which, under the umbrella of the World Health Organization, normalizes and coordinates globally the external evaluation of ST and the surveillance of resistance to anti-tuberculosis drugs. The Supranational Laboratories also provide support with biological reference material, and technical advice for the implementation of ST methods in the NRL and, later, its expansion in the network of network laboratories.

When a country has established the corresponding formal links, a Supranational Laboratory supervises the NRL, at least once a year. The NRL should then repeat this control in all laboratories of the National Network that perform ST and accept to participate in the quality control program (see Manual of Procedures for

External Quality Evaluation of bacteriological methods applied to the diagnosis and control of treatment of the tuberculosis).

This type of control is intended to detect serious, persistent and generally systematic errors. But it does not have sufficient power to identify other errors that may appear in the routine, so it does not replace internal quality control.

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APPENDIX I

MINIMUM BIOSAFETY REQUIREMENTS

The second chapter of this Guide (Organization of the supply of the susceptibility test in the laboratory network) has presented the level of risk and minimum biosafety requirements defined by the World Health Organization in relation to each bacteriological and molecular method.

Biosafety measures for laboratories with low and moderate levels of biological risk have been described in detail in Appendix 1 of Parts 1 and 2 of this Manual on BK and Culture respectively, which should be reviewed before reading this chapter. Only additional requirements and measures recommended for laboratories operating at high biological risk, i.e. those that manipulate isolates (positive cultures) of *M. tuberculosis* to identify or investigate their susceptibility to drugs, are considered here. These laboratories generally concentrate isolates that are resistant to antituberculosis drugs, which further increases the level of risk.

Staff Aptitude

Document for each person working on the BRCA (including the one hired for cleaning or ancillary work):

- Medical aptitude (upon admission and after any accident) and regular medical monitoring,
- Knowledge of the risk to which it is exposed,
- Knowledge of the elements of personal protection to be used in the BRCA, adjusting respirators, positioning / removing masks (or activating / deactivating more complex respirators if any) and glove placement / removal,
- Training to operate the equipment to be used,
- Ability to perform safe maneuvers to avoid exposure and minimize biological risk,
- Knowledge and application of SOP,
- Knowledge and ability to execute the contingency plan in case of accident / incident.

The most experienced staff should conduct the training. Until assuming all the tasks for which each person was hired, he should perform tasks with an increasing degree of biological risk. At each stage, the performance is adequate. Knowledge should be refreshed and staff re-evaluated, at least annually.

If staff cannot operate safely or react serenely and efficiently to an accident / incident, they should be assigned to another type of task. In the same way, staff with a disease or other transient or permanent condition that increases the risk of TB (HIV infection, diabetes, pregnancy, etc.) should be carried out. In all cases, current regulations and medical opinion will be considered.

Even if experienced, a person should not work alone in the BRCA, so that he can be assisted where necessary. Support staff may be outside the BRCA provided they can observe the BRCA through a glass panel. An alternative is the installation of security cameras inside the BRCA that allow to monitor the procedures that are performed there from outside.

You should maintain a fluid relationship with a doctor who specializes in the treatment of drug-resistant TB, and the contact telephone number must be displayed to immediately require your assistance in the event of an accident. This precaution is advisable since work insurers do not always have specialist doctors who can react immediately in these cases.

Infrastructure, facilities and equipment

The location of the different tasks, according to the level of risk they generate, the requirements of the different areas and the equipment should follow the guidelines detailed in part 2 of this Manual, dedicated to culture. In this case, the recommendations contained in the "Biosafety scaling in laboratories with high biological risk" must be applied.

The opening and processing of positive cultures of M. tuberculosis should be carried out in an BRCA that minimally has the following facilities and equipment:

- Entry through an anteroom that separates the laboratory where it operates with the greatest biological risk of the transit of people and other areas of the institution,
- A system that directs air from functionally clean to dirty areas and sterilizes it through a HEPA filter, before being expelled to the outside,
- BSC (s) which must be connected to a battery or UPS in case of power failure,
- Autoclave within the BRCA or in a surrounding area.

A small laboratory can serve as an anteroom or it can be installed with a secure closure, to the ceiling, and an additional door. The antenna maintains the air pressure difference. If there is no air injection system inside the BRCA, the air must enter from "clean" functional areas (where no positive crops are operated), to "dirty" (BRCA), through grids located in the part bottom of the anteroom doors. It is desirable that these grills have pre-filters to ensure that clean air enters. It is also advantageous that the two doors of the anteroom have automatic closing and an interlock system so that only one door is opened at a time.

The anteroom also serves as a lobby for staff to cover work clothes with which they circulate outside the BRCA, with clothing to be worn only in the BRCA. Upon graduation, those clothes will be discarded or placed in the anteroom. Therefore it should have amenities for separating clean clothes from those used in the risk area (coat racks, shelves) and also benches are useful for dressing comfortably. UV lamps (tubes) can be installed so that it is possible to radiate with them, as closely as possible, the surface of clothing and footwear to be reused. They must be triggered after the staff is removed.

Lamps must be very clean and periodically renewed to maintain their germicidal activity. This is a precaution for elements that have not been contaminated, at least cautiously. All contaminated material must be replaced and autoclaved immediately.

BRCA air must be expelled away from people traffic and air intakes, ensuring 6-12 changes per hour. Depending on the size of the laboratory and the number of BSCs in operation, air addressing and renewal can be achieved simply by installing a duct connected to each BSC that expels the air to the outside.

To preliminarily estimate whether extraction with ducts is sufficient, someone can calculate the volume of the laboratory

and the volume of air ejected per hour by the BSC. A CSC of 150 cm wide expels about 500 m^3 / hour. It is calculated by applying the formula:

Ejected air = air inlet area x velocity x air time in second
Example

> BSC with a window that measures 1.50 mx 0.2 m and circulates the air at 0.5 m / sec
> 1 hour = 3600 seconds
>
> Dimensions of the laboratory 5 m x 10 m x 2.5 m high = 125 m^3
>
> Air ejected by BSC per hour = 1.50 m x 0.2 m x 0.5 m / sec x 3600 sec = 540 m^3/h
>
> To completely change the laboratory air 6 times in an hour, it is necessary to expel
> 125 m^3 x 6 = 750 m^3, and to renovate it 12 times in one hour 125 m^3 x 12 = 1500 m^3.
>
> In this laboratory, therefore, two BSCs with the duct connected to the outside should be minimally operated, or a BSC and an additional HEPA filter extractor to expel similar amounts of air.
>
> It is advisable to have the assistance of a professional air-handling specialist to make accurate measurements and calculations.

The “thimble” type duct, designed for Class II BSCs type A2, over the “rigid” is preferred because it prevents adjustments to be made to the BSC due to air fluctuations. In addition, in case of electrical interruption or imbalance, the air that can return to the laboratory, by decreasing the pressure, does not invade the HEPA filter of the BSC. The duct in a thimble has a bell shape and is placed on the cabin, leaving an opening of about 5 cm where the ambient air is sucked when the BSC is working. It must have a removable or openable part to allow for controls and measurements during the certification process. It is also advisable, or necessary in the case where the duct is of great length, the placement of a fan in the external end of the duct. Qualified personnel must perform all the installation.

Whatever the air handling system, frequent monitoring of its proper functioning is required. The simplest checks of the air direction can be made with smoke tests. It is advantageous to have air flow meters.

The same anteroom by which one enters can carry out the exit of the personnel. There are more complex laboratories with double circulation that have a different exit room than the entrance hall.

To maintain air insulation and directionality, windows must be sealed. It is desirable that there be a panel with transparent glass that allows to visualize, from outside, the operations within the BRCA. One of these openings can serve as an emergency door (breaking the glass if necessary).

The regular source of electrical energy from a laboratory of this type must be reliable. Even so, they must be installed UPS must ensure at least 15 minutes of operation autonomy of each BSC. Thus, in the event of a power interruption of the regular source, the operator may close the tubes or flasks opened, discard the contaminated material and disinfect the surfaces, before the operation of the BSC.

Floors, walls, doors, frames, lighting and exposed structures must be covered with chemical disinfection resistant material. Ideally, they should be continuous, without joints. Do not adhere posters or notes on surfaces that prevent their cleaning and disinfection. Nor should there be plants or ornaments of any type.

Also, there should be at least one lavatory in each laboratory, with disinfectant soap and paper towels, preferably near the exit door. Whenever possible, it is convenient to install faucets that can be operated with the elbow or, if maintenance is assured, using automatic sensors.

Incubators (including automated reading equipment), refrigerators, and freezers that contain (potentially) infectious biological material should be located within the BRCA.

Intercoms or telephones are required to allow staff working within the BRCA to communicate with the

outside. Staff cannot enter / exit personal mobile telephony equipment.

Appropriate cabinets and shelves are also necessary for orderly and comfortable storage of the material to be used within the daily or weekly BRCA, in sufficient (but not excessive) quantity. There should be no boxes on the floor, countertops or equipment because they do not allow cleaning and disinfection of surfaces, subtract working space and generate risk. The storage of larger quantities of supplies should be organized outside the BRCA.

Appendix II presents the general characteristics of the equipment required and the additional equipment recommended whenever it is possible to access them.

Elements of personal protection

Institutions with advanced quality management processes require documenting the delivery and availability of protection elements for each person performing in the laboratory.

Clothing

The laboratory staff must have working clothes that allow them to remove the street clothes and circulate through the general areas of the laboratory. The personnel that work within the BRCA must have additional clothing for that area, which minimally consists of:

- footwear or covers disposable footwear (if no overalls are used),
- trousers and jackets or overalls,
- cloth or disposable cap (if you have it coveralls, optional is not used),
- air filter units or masks / covers to ensure the filtration of *M. tuberculosis* (optional),
- gloves.

This clothing must replace or totally protect the work clothes with which the personnel circulates in other areas of the laboratory. We can be used outside the BRCA. They must be in sufficient quantity, located in the anteroom, and also inside the laboratory in order to be able to quickly replenish it in case of an incident / accident.

The dress must be completely closed, particularly at the front. The sleeves should be long and with elasticized cuffs. It can be made with reusable fabric, or disposable material (high density fibers). In any case, the material must be sufficiently resistant to traction, tearing and splash penetration of any kind. Although reusable, the coat or overalls should be immediately replaced in the event of an accident with (potentially) infectious material.

The reusable footwear must be closed, of waterproof and disinfectable material. Alternatively, wearable shoes can be worn to protect footwear with which it is circulated in other areas of the laboratory). Rain boots are a good choice.

For simplicity, it is advisable to wear overalls that have a cap and cover shoes.

Respiratory protection

They provide respiratory protection in addition to that provided by the certified air conditioner and BSC, but in no way replace it. They are of optional use although they are highly recommended, especially when operating with liquid media and / or when working frequently with drug resistant culture.

There are more or less complex respirators and filter units, even with motorized air injection systems. For the case of the TB BRCA, the N95 masks or mask (US standard) or EN149.2001 (European Union Standard) are perfectly acceptable. They must be in sufficient quantity in the anteroom and within the BRCA for eventual replacements in case of deterioration.

Equipment is marketed to check the good fit of each model of mask for each operator, using sensor substances, whose use is recommended when the biological risk is very high.

Personnel should be trained in the use of respiratory protectors and the manufacturer's instructions for use and operation of respirators should be followed.

To put on a new disposable mask:

- Place the cap first (if used),
- Take one hand with the mask on the outside, leaving the elastic adjustment straps free,
- Position the mask covering the nose and mouth,
- Take the upper elastic strip and fit it on the head, over the ears,
- Take the lower elastic strip, slide it around the head to fit the neck,
- Adjust the metal piece that is located on the nose, pressing with the fingers from the nose to the ears,
- Check the fit of the mask.

To remove the mask:

- Remove gloves first (see instructions below) and wash hands,
- Remove the mask then taking only the elastic strips, without touching the outside of the mask that is potentially contaminated, or the internal to not contaminate it,
- Discard in a autoclavable bag.

It is advisable to use each mask only once. However, if this is not possible, and if no incident / accident occurred at the time of use, each worker can reuse his mask, while remaining functional, i.e. until he detects that the adjustment is not perfect, that the strips elastic have relaxed or breathing is not comfortable. Institutions can regulate reuse limited to a limited number of working days, taking into account the manufacturer's indications and the level of risk.

Normally a mask should not be contaminated with *M. tuberculosis* if it was used in a laboratory with routing and renewal of air, conducting the risk operations within a well-functioning BSC, without the occurrence of aerosol or splash releases outside the BSC and without the mask being intentionally or inadvertently touched with the gloves with which infectious material was manipulated. However, in the case where it is reused for more than one working day, it is necessary to provide additional training to personnel to avoid contamination by improper maneuvers, and provision should be made for additional pairs of gloves to follow the following instructions.

To remove the mask **after the first use**

- Save the mask inside a paper bag labeled with the name of the worker to whom it belongs.
- Place only one mask inside each bag and condition it so that the mask is not crushed.
- The name of each operator can also be written on the elastic bands of the mask, before being used for the first time, for safety.

to put on the mask to re-use

- Place the cap first,
- Put on a pair of gloves,
- Remove the mask from the bag,
- Put on and adjust the mask following the steps mentioned above for a new mask.
- Discard the gloves in a autoclavable bag,
- Put on another pair of gloves before entering the BRCA.

The mask should be replaced immediately if it is damaged, if its adjustment is not perfect, if it prevents comfortable breathing or if it has been contaminated by aerosols or splashes due to an accident / incident or nasal secretion.

When disposing of the mask, also discard the bag.

Gloves

They must be available in the anteroom and inside the BRCA, in an adequate quantity even for spare parts in case of unforeseen events. The provision must consider the sizes necessary for each operator to work comfortably.

They may be latex, vinyl or nitrile. They must cover the cuff of the jacket or overalls that are used. They cannot be reused.

Personnel should be instructed to wear gloves.

Before entry, in the anteroom:

- If masks are reused, put on a pair of gloves to place and discard later (see instructions above)
- Put on double pair of gloves, covering the cuffs of the dress before entering the BRCA.

Within the BRCA:

- Discard the outer pair of gloves when finished operating with infectious material inside the BSC,
- Put on a new pair of outer gloves and remove it to finish the work inside the BRCA.

At the exit, in the anteroom, remove the remaining pair of gloves:

- Take the cuff of a glove and slide it out by inverting it (the inner part wraps the outer one) in such a way that possible contamination is protected,
- Holding that newly pulled glove with the other gloved hand,
- Insert the disengaged finger into the cuff of the other glove (which is still in place) without touching its exterior. As before, slide it out by inverting it and, at the same time wrapping the first glove that has been removed,
- Discard the gloves in a autoclavable bag,
- Wash hands with disinfectant soap.

Incident / Accident Kit

It must be available within the visible and identified BRCA so that responsible personnel can react immediately and rapidly in case of spillage or rupture of containers with (potentially) infectious material outside the BSC. It is composed of:

- disposable absorbent cloths,
- autoclavable or disposable forceps,
- phenol 5% (in a well-sealed bottle) or disinfectant with a similar action, bactericidal for *M. tuberculosis* in a few minutes,
- 1 box of resistant and thick, disposable gloves,
- 1 box of masks (N99 masks are preferable for this situation),
- autoclavables bags,
- box or container for sharps.

In addition, it is desirable to have aliquots of the first and second line drug stock solutions employed to make the ST (at least I, R, Mxf, aminoglycosides). If an operator accidentally produces a wound while handling *M. tuberculosis*, the affected area can be quickly washed, pressed to bleed if possible. Then, quickly resort to these suspensions, mix them according to the known or suspected sensitivity profile of the strain that was manipulating, without wasting time in dilutions or calculations, and applied immediately on the wound, and then turn to a specialist doctor.

Practices in the biological risk containment laboratory

The laboratories must have accurate SOP for entry, operation within the BRCA and exit of the same, depending on the degree of complexity of the facilities and methods implemented, and which must be strictly followed.

The following are procedures that should be minimally respected.

Entry

Access must be authorized only for trained staff

- Not enter the anteroom with any element (bijouteri, watch, mobile phone, etc.), or employed to work outside the BRCA,
- Record the name of the operator and time of entry,

- If the laboratory has a pass-through, enter the boxes with samples and insulation,

- Check the correct functioning of the air circulation system in the external indicators (if they were installed),

- Check that any door that is crossed is correctly closed.

In the anteroom:

- Inspect the integrity of the clothing and protection elements to be used,
- Wear the exclusive clothing of the BRCA following the instructions above,
  pants and jackets or overalls
  footwear or cover shoes
  cap (if the overall does not)
  air filter units or face masks
  double pair of gloves, covering the cuffs of the sleeves of the dress

Inside the laboratory

Processing of biological material:

- Apply the recommendations for biosafety listed in page 49 and Appendix I of part 2 (Culture) of this Manual, in addition to those specifically mentioned here,

- Use double pair of gloves. The outer pair must be replaced at the end of the work within the BSC and in the case where it is contaminated or broken. Discard the external torque within the BSC every time it is abandoned,

- Maintain order within the BSC,

- Separate into the BSC the “clean” material of the (potentially) infectious (for example to the left and to the right of the operator respectively),

- Limit the number of materials to be processed at a time, not to cram the BSC,

- Stay alert to resist the temptation to touch your nose, eyes or exposed skin with your hands, clothing or instruments in use. Put on a pair of sterile external gloves, or request assistance from a co-worker to do so in case it is really necessary,

- Use tips of micropipettes protected against aerosols.

Whenever posible, choose control strains pansensitive and avirulent

When it is necessary to use drug-resistant control strains, do not use M. tuberculosis MDR or XDR so as not to further increase biological risk.

Information record

- Enter in computer or tablet any data or image that is necessary to file. Use a scanner where necessary. These computers must be connected in network with external computers, to export the information, without having to transfer physical registers.

Disposal and recycling material

The regulations in force in the country and in the institution, must be respected for the disposal of pathogenic biological material and the preservation of the environment. For the material that generates biological risk, the following precautions should be minimized:

- While performing the laboratory procedures, separate the material used in unbreakable and autoclavable containers (eg. made of polypropylene or stainless steel).

  Bags and containers should have different colors, characteristics and / or labels to classify disposable material, sharps, recyclables and hazardous chemicals.

	MATERIAL	AUTOCLAVABLE CONTAINER
Disposable	Paper, Gloves	Closed bags, located inside containers.
	Handles, tips of micropipettes, plastic pipettes or swabs used to operate with crops.	Container with lid (glass jar or similar)
	Petri dishes, with the closure of their covers secured with adhesive tape	Postcards
	Plastic bottles with biological samples, with their caps closed	
	Supernatants, remaining bacterial suspensions	Bottles with lid
	Sharpening material or instruments	Disposable, puncture-proof containers
To recycle	Glass Pipettes	Pipettes with Lid
	Glass tubes, with their caps closed.	Containers with Lid
	Glass jars with water or reagent remnants, with their caps closed	

Autoclavable bags and containers must be located at each workstation. Small pipetters and containers will be inside each BSC; larger tubing or plate containers may be located on a small table next to each BSC. Larger containers for tubes or plates may be located on a small table next to each BSC. It should be noted that the lid of the tubes, flasks, plates and containers are closed before discarding and transporting the material to the autoclave:

- Do not use very large containers, do not compact the material inside them, fill them only up to two thirds, to allow the steam from the autoclave to penetrate properly,

- The material to be discarded and recycled, after the task is completed. Do not let the material accumulate for several days,

The personnel working within the BRCA is responsible for autoclaved material if the autoclave is within that area, or move if the autoclave is in an adjoining area. The transfer of the material should be done in safe, shock resistant and tightly closed container, disinfected by its exterior. The use of carts located outside the area facilitates minimizing risk of falls.

- No element used within the BRCA can be removed to be discarded without being previously autoclaved. This must be respected even if the element has not been used material (potentially) infectious. Stationery is included in this precaution. are exceptions:

  - Explosive or environmentally polluting chemical wastes should be disposed of according to the regulations of each institution, in labeled containers. These containers should be decontaminated on the outside before being removed,

- Non-infectious biological material that requires additional processing outside the BRCA (eg DNA). It must be placed inside a box whose surface can be decontaminated before exit. The egress will be done through the pass-box if it is installed.

Output

- Check the good closing of every door that crosses,
- Remove clothing and personal protective equipment,
- Dispose of disposable protective elements (cap, shoes, mask and gloves following the above instructions, and disposable overalls) within autoclavable bags,
- Conditioning the protection elements to be reused,

  Disinfect the respirators externally or place the masks in individual paper bags, following the instructions above

  Hang the cloth clothes in hooks intended for each operator

  Order the reusable footwear of each operator in appropriate lockers
- Wash hands thoroughly with disinfectant soap,
- Record the time of departure to the área.

Cleaning and maintenance

Cleaning of doors, openings, floors, ceiling, walls, external ducts, cranks and filling of towel dispensers and soap, etc. must be supervised by personnel trained to operate and use personal protective equipment, under supervision.

- Schedule cleaning at convenient times, when the activity in the BRCA is zero,
- Use disinfectable elements, which are used exclusively for that area, organize a closet to store it,
- Use disposable or autoclavable cloths and mops (in which case mops should be removable from your cane),
- Soak the sticks and mops in disinfectant detergent solution, do not dry clean,
- Place cloths and mops in autoclavable containers or bags, autoclave before recycling,
- Disinfect sticks, buckets and containers and store in BRCA organized within the cabinet.

Equipment in general, and in particular incubators, refrigerators and freezers containing (potentially) infectious biological material, must be cleaned by the trained personnel, who normally operate within the BRCA.

Where specialized personnel are required to inspect / repair equipment, disruption of tasks, disinfection of all surfaces and complete renewal of BRCA air (with the air handling system provided) shall be arranged. Technicians who enter must use the protection elements usually employed by the personnel and work under supervision.

Transport of positive cultures

Review the recommendations detailed in the Appendix of part 2 of this Manual dedicated to the culture for packaging and transportation to the reference laboratory of positive cultures.

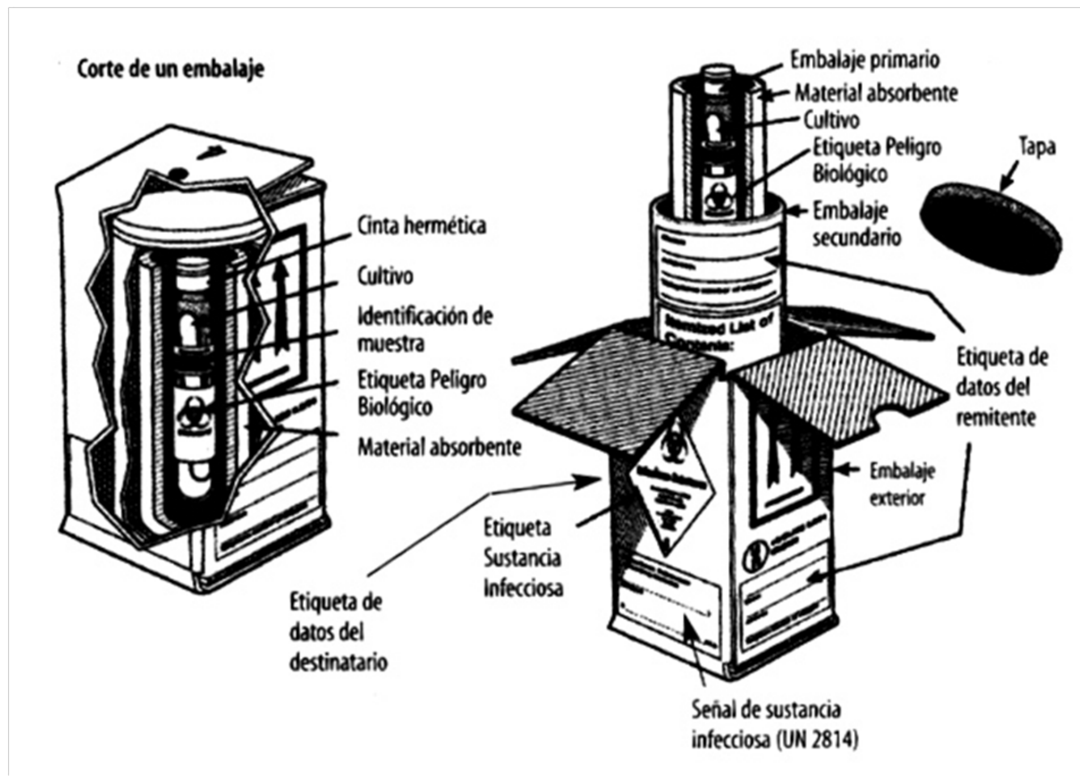
Transport of this type of material must be carried out by an authorized company for the transport of biohazard material and comply with local, national and, if applicable, international regulations.

It must be documented, prior to shipment, that the recipient laboratory meets the minimum biosecurity requirements to operate with the material being sent. International shipments should not be made until they have verified that the recipients have the required authorizations issued and until they are not sure that the parcel will be collected as soon as possible at the airport. Airline, flight number, departure times and

dates, guide number of the parcel and time and date of arrival of the flight must be communicated to the recipient.

The United Nations Committee of Experts on the Transport of Dangerous Goods regularly updates recommendations for safe transport. In relation to TB, the samples are UN 3373 and cultures of *M. tuberculosis* UN 2814, category A (infectious substances affecting humans). The International Civil Aviation Organization (ICAO) uses these recommendations as a basis for developing regulations for the safety of air transport. For its part, the International Air Transport Association (IATA) has included additional requirements.

(http://www.iata.org/ps/publications/dgr/index.htm)



Containers for transport and receipt of M. tuberculosis culture.

Anteroom separating the biological risk containment laboratory

Transparent panel that shows the activities inside the containment laboratory from an area with low risk

Pass through, for entry / exit of material that can not be autoclaved

Duct connected in thimble on Class II type A biological safety cabinet that generates negative pressure

APPENDIX II

EQUIPMENT

It is recommended to review the APPENDIX III and II of parts 1 and 2, respectively, of this Manual on Bacilloscopy and Culture, respectively. The most important equipment or characteristics there of not considered in these annexes will be considered here and are necessary or useful for the laboratory performing ST.

In general, the more complex equipment of a high biological risk laboratory requires preventive and corrective maintenance by specialized professionals. It should be programmed for each team and funded.

When it is planned to acquire the more complex equipment, of high cost, it is necessary to ensure previously:

- Space for equipment model suitable for laboratory needs on the floor or table, considering the opening of its door(s) or drawers if it has them and for the circulation of air around, without affecting the movement of personnel, access of the equipment through existing openings power supply and sockets,
- conditioning of the temperature in a stable way, according to the requirements of the equipment,
- acceptable noise level, considering the one that generates the equipment to acquire added to the existing one,
- installation of equipment with verified operation,
- training of personnel for their use, interpretation of errors or faults, cleaning and preventive maintenance that may be within their reach,
- guarantee of good operation for at least 1 year,
- updating the software to the latest available version,
- if the equipment uses non-generic reagents, produced by the same manufacturer (i.e MGIT 960/320, GeneXpert), guarantee the availability of reagent stock required by the laboratory, with an appropriate expiration date and immediate replacement in case that a lot was defective,
- purchase of optional accessories (i.e. pipe fittings suitable for laboratory routine for MGIT 320/960 equipment, electricity converters, UPS,
- the purchase of spare parts whose replacement is relatively frequent or whose lack of immediate availability can determine the stoppage of the tasks, (i.e. BSC filters, resistors for electric autoclaves, etc.),

- regular maintenance service, certification of correct operation, technical assistance and replacement of defective components immediately, in case of equipment malfunction, the laboratory could be paralyzed for lack of operation of some critical equipment (i.e. autoclave, BSC, refrigerators / freezers). When there is no technical capacity in the country for the verification and maintenance of more complex equipment (particularly BSC), it is very important to create it. Experience in the Region shows that contracting services from other countries is costly and can hardly be guaranteed with the required frequency or urgency. Even with insured maintenance, a contingency plan must be foreseen in the event that this type of equipment stops working temporarily.
- references of users (national or international) who already own the equipment,

All requirements must be explicit and clear in the documentation that is required for the purchase process.

Certain WHO prioritized systems for TB control are available at preferential prices for governmental and non-profit organizations from non-high-income countries and / or can be purchased through strategic funds. At the preferential price you have to add international and local transportation costs, insurance, customs and taxes applied by each country. It is advisable to consult and be updated with this type of facilities.

The use, calibration, control, validation and maintenance of the equipment must be registered in standardized formats, which must be archived. Records that automatically produce the most complex equipment or sensors (i.e. temperature) that can be added should also be archived. Consider that the records cannot leave the BRCA without prior autoclaving, so it is convenient to use software and databases whenever possible, and scan the written documents. This material should be reviewed by those responsible for quality, biosafety and / or maintenance.

On the other hand, the organization and establishment of regular equipment cleaning program, including the interiors of refrigerators / freezers and incubators, are necessary.

Personnel working at the BRCA must be trained to operate all equipment in that area to perform maintenance procedures that do not require specialized personnel and is responsible for cleaning any equipment used with (potentially) infectious material.

Computers, scanner

As has been mentioned repeatedly, to reduce the risk it is necessary to have one more computers and one scanner inside the BRCA, connected in network with other computer(s) and printer located outside the BRCA.

The BRCA computer(s) may have moderate capacity because it is possible to link them to a larger capacity that are outside and contain the databases and images, as well as the software with which it is operated. The number of computers should be thought based on the number of operators that perform in the BRCA, as well as the space available. Thus, laptop computers and data entry tablets may be convenient.

Biological Safety Cabinet

The appropriate model for laboratories of high biological risk is Class II type A2 because it has the ducts contaminated under negative pressure or surrounded by ducts with negative pressure. As already mentioned in Appendix I of this manual, the BSC (s) must be connected to a battery or UPS, in case of power outage and, in the event that there is no another system for directing the air for the creation of negative pressure, must have a duct (preferably thimble type) that removes the air outwards. When it is necessary to extend the outer duct so that the air is expelled away from occupied buildings or air intakes, the placement of an external fan may be necessary. In that case, you can choose a fan that can continue to operate even after the CS.

Laminar flow cabinet

In or near the reagent and media preparation area it is convenient to have a cabin that protects the sterility of the solutions or media being prepared. An existing cabin can be used that is commonly used by several laboratories. This facilitates and improves the work, in relation to the use of burner (s) on the counter, and is especially recommended for laboratories with medium or high workload. They are also particularly necessary for the preparation of highly enriched medium such as the Middlebrook series or MGIT.

They can ensure sterility through the flow of air passing through HEPA filters with >99.99% efficiency to retain particles of at least 0.1 or 0.3 µm. Filtered air is projected horizontally or vertically in the work area. It is not necessary to filter the air before ejecting it, and if they do not, they do not ensure operator protection. They should be used to work with materials that do not generate dangerous aerosols or vapors.

They must have a work surface that is wide enough to locate all the material necessary for the preparation and packaging of culture media. They have UV tubes to sterilize the work surface and stainless steel, epoxy, polyurethane and / or polypropylene surfaces that allow them to be kept very clean and disinfected. When it is necessary to purchase it, check that it has the required number of plugs for electrical connection, low noise level, and good lighting in the work area.

Pass through

It is not a requirement, but it is convenient. It is a double door cabin that is advantageous for passing material to or from the BRCA after disinfection of the outer container surface. It is mounted in the most convenient position of a wall separating the BRCA from the rest of the laboratory, preferably away from the areas where the staff operates. There is equipment with doors that are interlocked, so that only one of the doors can be opened at a time, to deposit or to remove material. They may also have audible alarms indicating the opening of one of the doors. They must be constructed of smooth and disinfectable material such as stainless steel.

For the TB laboratory a small chamber is adequate, sufficient to enter the biological material that must be processed inside the BRCA, or to extract non-infectious biological material resulting from the processes applied within that area and which cannot be autoclaved (DNA). It can eventually be used to enter small reagents or reagents that, for any reason not foreseen, are not available inside. This way, all the procedures that the personnel inside the BRCA are required to complete and search for this material are avoided.

Autoclave in biological risk containment area

It must have the appropriate size to autoclave the material that must be extracted daily from the BRCA, for disposal or recycling.

Autoclaves with a single door are suitable. If the autoclave is located inside the BRCA, it is desirable to be electric to avoid excessive heat and steam inside the working environment.

Whenever you have the necessary space and the possibility of acquiring and maintaining it regularly, it is convenient to have an autoclave with two doors, one opening towards the BRCA and another towards the material washing area. This type of equipment has electronic temperature and pressure sensors whose measurements are displayed on a control panel and reported through a printed ticket and / or transmitted to a computer using software. Normally, they are programmable so that it is possible to use different cycles of sterilization, depending on the material that has been placed inside. Camera air is removed prior to filtration. Generally, it is necessary to couple this type of autoclave with a system to purify the water it uses. They can be purchased with carriages to transport the material and to discharge it comfortably, as well as trays to leave the load inside the autoclave.

Refrigerator, freezer

Outside the BRCA, refrigerators (1-8 °C) are required to preserve culture media, drugs, solutions and reagents that require that temperature (as directed by the manufacturer). In addition, a freezer (separate or as

a refrigerator compartment) is required to ensure -18 °C or less to preserve antibiotic solutions. They should be located in a "clean" area nearby or, preferably, within the area dedicated to the preparation of culture media and reagents. Refrigerators that have transparent doors are suitable for quickly identifying the spaces where the material to be extracted or introduced, minimizing the opening time of the equipment.

Within the BRCA a refrigerator (1-8 °C) is required for media and reagents, another for potentially infectious material (samples / isolates being investigated) and in reference laboratories a freezer is required -18 °C or preferably deep freezer (-70 °C), to preserve the collection of strains / isolates in the long term.

The following are very convenient features: external temperature indicators, visual and auditory alarms indicating temperature rise, minimum ice formation, sufficient and convenient internal organization of shelves / drawers to facilitate the recording and control of stock. For laboratories with a high workload it is convenient to acquire more sophisticated equipment that allows the recording and monitoring of temperature automatically, using its own software. Alternatively it is possible to add sensors and external equipment that perform this registration.

Microbalance or analytical balance

They are suitable for weighing antibiotics and most of the reagents required for molecular testing, because they are designed to weigh small quantities, ensuring accuracy in the range of sub milligrams. In addition, it is weighed inside enclosed compartments that avoid dust, environmental particles and air currents that may interfere. It can be selected from several models according to the maximum and minimum quantity that need to be weighed in the work routine. For the needs of a TB laboratory that performs ST, it must ensure legibility and repeatability on the order of 0.01 mg or less. Accurate cleaning, leveling and calibration of a balance are keys to reliable results. The balance must be installed in a "clean" area (without biological risk), on a very firm table and away from equipment that can cause vibrations and drafts.

Micropipettes

They are necessary to measure and transfer volumes of 1 ml or less, ensuring an accuracy of 1% or more. You can choose between several models: one or more channels, manual or electronic, to measure fixed or variable volume, with different ranges and accuracy. Models should be chosen to ensure easy ejection of the plastic tip. For the BRCA they must be autoclavable, in case they are accidentally contaminated. Routinely they must be disinfected with 70% alcohol after being used, reason why also they must resist the disinfectant.

Most micropipettes use universal and generic tips and are preferable to those that use peculiar tips that may not be available in the market with a wide and sustained offer over time.

For the methods described in this Manual that are carried out in the BRCA, normally single and manual micropipettes are suitable, with dispensing of variable volume, one between 10 µl and 100 µl and another between 100 µl and 1000 µl. For the preparation of LiPA, the need is added for two micropipettes dispensing between 10 and 100 µl in the areas dedicated to the preparation of DNA amplification and loading mixture. In the area of preparation of media, for MGIT, another micropipette is required that dispenses between 100 µl and 1000 µl.

To maintain accuracy with prolonged use, the micropipettes must be cleaned following the manufacturer's instructions and calibrated periodically.

Person or cattle counter

It can be electronic or mechanical; it increases the count with each manual pulse. It facilitates the counting of colonies for the proportion method.

Magnifying glass

With 3-5x magnification, manual or with foot, optionally can have peripheral illumination. Facilitates colony count for the proportions method.

Plastic Bag Sealer

It allows the preparation of plastic bags by heat sealing, with seals, of the size that is required. It is suitable for bags that are not used in large quantities or for sizes not commercially available.

Portable ultraviolet lamp

It is necessary for manual reading of MGIT tubes. It can be of any type as long as it emits light in the UV range. In Dermatology they are called Wood Lamps

MGIT 960 ó 320

The equipment incubator and reader of cultures realized in MGIT have capacity to incubate 960 tubes in 3 drawers or 320 tubes in 1 drawer. The model to choose depends on the workload and availability of space in the BRCA.

It attaches to a UPS and a printer. As already explained, it is very convenient for a laboratory that performs ST to purchase it with EpiCenter™ software and EpiCenter™ TB-eXiST, for which operation it is necessary to connect to a computer and a barcode scanner.

Carriers (AST carriers according to the manufacturer's name) must have purchased the ST. The quantity and type must be calculated in relation to the load and the working algorithm. There are tubes with capacity for 2, 3, 4, 5 and 8 tubes.

The equipment requires to be calibrated periodically for what is necessary to acquire the necessary calibrator tubes.

GeneXpert

You can choose equipment of 1 (normal or portable version with rechargeable battery to operate for 12 hours), 2, 4 or 16 modules. It attaches to a barcode scanner and a computer. The modules are individually replaceable, if necessary.

The equipment needs to be calibrated periodically so it is necessary to purchase cartridges that make up the calibration kit and the CD with the necessary software.

Equipment for LiPA

They may be of different trademarks, including those marketed by the manufacturers of the reagents necessary for the ST to anti tuberculosis drugs described in this Manual. It is possible to use basic equipment with which every molecular biology service counts. These laboratories generally have versatile equipment for various work protocols.

In general, it is necessary to verify that the temperature range in which each equipment works includes the required by the technique described and that can process / incubate micro tubes or strips in sufficient quantity to solve the workload of the laboratory in each working day.

Laboratories in countries with very high MDR TB burden and having concentrated LiPA tests in one or a few laboratories with very high workloads may choose to automate DNA extraction, hybridization, reading and interpretation of the results.

The following are some basic equipment considerations, which may be useful when it is necessary to acquire it only for the techniques described in this manual.

PCR Cabinet

They are recommended to prepare the amplification mixture in laboratories with high workload, to reduce the risk of contamination. They are small cabins that facilitate the decontamination and inactivation of DNA through HEPA filter systems through which the air that is driven towards the work area, and radiation, UV passes. They have internal stainless steel, epoxy, polyurethane and / or polypropylene surfaces that allow cleaning and disinfection.

Microcentrifuge

Since it is used to concentrate sample sediments and bacillary suspensions, it must have a rotor with aerosol containment cap and can be transferred to the BSC for opening. It must reach 13000 g (no rpm) or more during centrifugation. Because it is used to concentrate sample sediments and bacillary suspensions, it must have a rotor with a cap for aerosol containment and can be transferred to the BSC for opening. Must reach 13000 g (no rpm) or more during centrifuge

Waterbath or heater with blocks

Two teams are needed, either a Maria bath or dry heater with blocks, one inside BRCA and another outside, in the area dedicated to the amplification of nucleic acids. They must operate in the ambient temperature range up to 100°C ± 0.1 °C and allow the placement of floating or inlet microtubes, respectively.

On the other hand, there must be additional equipment in the hybridization area where the trays containing the test strips and the amplification product should be incubated with agitation. This can be done in a shaken Mary Bath, but it is more practical and safe to do it dry, on any platform / incubator with horizontal rotating agitation, whose speed can be graded. It must operate in the ambient temperature range-55 ° C and, in this case, the uniformity and accuracy of the temperature ensuring the equipment is very critical.

Thermocycler

There is simple equipment that allows saving a certain number of programs for different amplification protocols, with a single block to place microtubes. And there are also equipment with increasing complexity to which they can save a high number of programs and work with more than one at a time, with a temperature rating (usually used to standardize methods still in development), with several types of blocks to place microtubes, in connection with other cyclers or a computer, with real-time PCR, etc. The simplest ones are adequate to implement the tests described in this Guide, the heating block should be able to hold 0.2 ml PCR microtubes in quantity suitable for the laboratory workload and to work in the range of 0 to 100°C. The critical parameters for ensuring efficient procedures are: accuracy (± 0.2 °C or less), high heating / cooling rate (5 °C / sec or more) and high temperature uniformity in the block (± 0.5 °C between different holes or less).

Vacuum Cleaner

There are small equipment consisting of a vacuum pump, collector (s) for the aspirated liquid that accept different types of pipettes and tip adapters with which it is possible to perform the aspiration, with velocity and volume of aspiration adjustable. But a piece of equipment simply assembled in the laboratory, for example with a Pasteur pipette connected to a standard vacuum pump, can be used.

APPENDIX III

GLASS, PLASTIC AND OTHER MATERIALS

It is recommended to review Appendix III of part 2 of this Manual dedicated to Culture. The material not described in that Appendix will be considered here and it is necessary or useful for the laboratory that performs ST.

In general, all the material must be made of disposable plastic material (and at the same time sterile when it is going to be used for any ST) or autoclavable. Through internal quality controls, it must be verified that the material used does not generate false results, either because it inhibits the development of *M. tuberculosis* or because it absorbs the antibiotics on the surface, decreasing its effective concentration.

In order to maximize the biosafety, the resistance of the materials must be verified, and the tightness and tightness of the closure of the lids.

McCartney Bottles

Resistant glass, with capacity up to 30 ml, with aluminum or bakelite screw cap that have Teflon discs or other material for hermetic closing. Containing the glass beads, they are useful for the homogenization of bacillary suspensions. Alternatively, tubes can be used with bakelite lid, of very transparent and resistant glass.

Glass Beads

Borosilicate is sold by weight (generally 1 kg), its diameter can be approximately 3 to 6 mm.

Petri dishes

Glass or preferably of plastic, disposable of 100 mm in diameter, divided into quadrants, for the Middlebrook 7H10 / 7H11 agar proportions method.

Desiccator

Glass or plastic, vacuum-sealed, with or without the use of grease, to preserve the antibiotics. Several ones are necessary, for the different temperatures at which the reagents are preserved, and with the adequate capacity for the work routine.

Micropipette tips

They must be suitable for the available micropipettes (check compatibility according to information normally provided by the manufacturer) and the volume to be transferred,

To operate with (potentially) infectious material and / or the employee for molecular testing, sterile tips are required, with aerosol protection. This protection can reduce useful capacity (the volume you can transfer).

It is preferable to discard the tips used. However, there are autoclavable tips that allow reuse a limited number of times. In this case, and when necessary, the aerosol barrier must be replaced manually (it can be prepared with cotton) after thorough cleaning and before sterilizing the tips.

For molecular tests, they must be free of enzymes that can cut the genetic material (DNAasas and ARNases). Autoclaving destroys these enzymes

Microtubes

They should be chosen according to the required volume (between 0.2 and 2 ml). They may have different colors to encode their contents, they may be sterile or not. They must be selected according to the application.

- Cryotubes: resistant to (ultra) frozen, with lid with external thread (to prevent contents from contaminating gloves when they are open), with hermetic seal secured by o-ring inside the lid, should facilitate permanent labeling, there are with bar codes for laboratories that have implemented this coding system,

- Tubes for microcentrifuge: they support 20.000 g, conical bottom, with pressure cap whose sealing must be very safe (otherwise they are uncovered during the centrifugation),

- Ultra-thin wall PCR tubes (to ensure rapid temperature changes in contact with the thermocycler block) with flat caps and very secure closure (otherwise they are uncovered during the thermocycler causing a high risk of cross-contamination).

Boxes and racks for microtubes

Of propylene, they may have different colors to encode their contents.

It is convenient to buy a sufficient amount of

- Polypropylene racks to accommodate 96 sterile tips can be autoclaved, in order to recycle them,

- boxes with transparent lid, that allow the safe fitting of microtubes inside, for their ordered storage in refrigeration or (ultra) freezing, allowing the quick identification of the tubes that need to be removed. There are models with gridded cover that allow to label on it the code corresponding to the tube that is located in each position,

- racks or holders for microtubes of each of the sizes that are used, stackable to allow them to be stored neatly in the smallest possible space

APPENDIX IV

PREPARATION OF REAGENTS AND MEDIUM

General Precautions

This task must be performed outside the BRCA to avoid unnecessary exposure to biological risk of the personnel performing it.

To ensure good quality, preservation and control of drug stock

- Work only with products from reliable manufacturers, which document the complete characteristics of each of them (risk that generates, biological source or method of synthesis, physical appearance, solubility, purity, storage conditions) and that provides certificate of analysis of each lot, many times accessible through Internet,

- work only with antibiotics provided for analysis and certifying their potency or the parameters needed to calculate it; not to use drugs used for the medication because they contain excipients,

- accept only the drugs in the manufacturer's original packaging, hermetically sealed (non fractionated by any supplier),

- keep all drugs in their original bottles designed to ensure the best conditions (seal tightness, light protection and / or cooling if necessary, etc.),

- keep the antibiotics at the temperature indicated by the manufacturer in desiccators containing some desiccant material such as silica gel or calcium chloride, preferably with vacuum,

- wait for all desiccators that have been refrigerated to be allowed to cool before opening them to prevent the condensation water from moistening drugs that are withdrawn from it,

- immediately discard any reagents that have altered their normal or expired characteristics,

- maintain a database identifying for each drug or reagent the number of each batch received, location in the warehouse or (ultra) refrigerator, expiration dates, reception, opening and withdrawal, as well as the minimum stock that the laboratory must maintain each according to its workload,

- label in a detailed and durable form all reagents and media prepared in the laboratory, with name, batch number date of preparation, date of opening and expiration,

- maintain a regular warehouse cleanliness and order plan, cabinets, refrigerators and freezers.

A lot of precision is needed to prepare the medium and reagents used in a laboratory that performs ST, especially those containing antibiotics and those employed for molecular testing. To ensure the highest precision possible in weighing and measuring small quantities, use:

- well-calibrated analytical or microbalance balance,

- microtubes or paper or paper containers for weighing, disposable, as small as possible in relation to the amount by weight and light, of smooth and non-absorbent surfaces, well preserved to keep them very clean, free of dust,

- pipette or micropipette, as appropriate, to allow the necessary volume of diluent to be measured, but not excessively large,

- dual-capacity pipettes,

It is advisable not to weigh amounts of antibiotics less than 100 mg.

The rule of changing the pipette or micropipette tip should be carefully adhered to after each dilution or when changing solution or solvent.

Considering all the precautions that must be taken to ensure accuracy, when the ST is decentralized it may be appropriate for the reference laboratory to be responsible for the purchase, weighing and distribution of the antibiotics, together with instructions for making the corresponding dilutions in the laboratories of the network that prepare drug culture medium.

Turbidimetric patterns

Mc Farland Solution

Materials

- One 10 ml pipette
- One 1 ml pipette
- 1 pro pipette
- One 15 ml tube with screw cap
  (Use the same type of tube that is used to prepare the master bacillary suspensions, so that the visual comparison is more accurate).

Reagents

- Aqueous solution of barium chloride (BaCl2) 1%
- Aqueous solution of sulfuric acid (H2SO4) 1%.

Procedure

- Mix the solutions inside the tube with screw cap, with different volumes according to the number of standard to be prepared:

Mc Farland No. 1

Aqueous solution of barium chloride ($BaCl_2$) 1 %	0,1 ml
Aqueous solution of sulfuric acid ($H2SO_4$) 1%	9,9 ml

Mc Farland No. 0,5

Aqueous solution of barium chloride ($BaCl_2$) 1 %	0,05 ml
aqueous solution of sulfuric acid ($H2SO_4$) 1%	9,95 ml

- Keep the tube tightly closed between 2 and 25 ° C.

Before use, allow the tube to reach room temperature and vortex.

Discard if evidence of evaporation, contamination, discoloration or evaporation is detected. Renew, at least, every six months

BCG 1 mg/ml

Equipment

- BSC or laminar flow equipment
- Pro pipette

Materials

- 1 ampoule or bottle of lyophilized BCG vaccine
- 1 sterile 1 ml pipette
- One 10 ml pipette
- Sterile distilled water
- One 15 ml tube with screw cap
  (Use the same type of tube that is used to prepare the bacillary suspensions, so that the visual comparison is more accurate).

Procedure

- Verify the amount of bacillary mass contained in the BCG vaccine vial as declared by the manufacturer on its label,

- Within a BSC or laminar flow equipment, reconstitute the contents of that vial with sufficient volume of sterile distilled water to reach a concentration of 1mg/ml,

- Transfer the suspension to the tube with screw cap,

- Keep it with its lid tightly closed, in refrigeration.

Before use, vortex
Discard any evidence of contamination, lumps or evaporation
Renew at least annually.

Culture medium with antibiotics and selective inhibitors

Principles

Stock solution

In some cases it is necessary to select carefully the formula of the antibiotic that is acquired, among those available in the market. It is necessary to use:

- dihydrostreptomycin sulfate and not streptomycin sulfate because the latter is initially more active than the former and then its activity rapidly decays upon incorporation into the LJ,
- DL-cycloserine and not D or L-cycloserine because the latter two are much less soluble.

In order to avoid weighing the antibiotic or selective inhibitor whenever a batch of medium is prepared, a concentrated solution **(stock solution)** should be kept frozen, except in cases where the concentration or activity of the drug is not stable in solution (R, cycloserine, ethionamide). The mother solution must be sufficiently concentrated to minimize the risk of inactivation of the antibiotic during freezing and, at the same time, ensure complete dissolution of the drug. Usually a concentration of 10,000 μg/ml (10 mg/ ml) or 20,000 μg/ml (20 mg / ml) is adequate.

Potency is the proportion of pure antibiotic containing the drug, expressed as a percentage, μg / mg or, in mg / g. It can vary from manufacturer to another and from batch to another. The potency can be declared directly by the manufacturer or can be calculated from the following data, also declared by the manufacturer: 1) purity of the drug, 2) water content, and 3) proportion of antibiotic containing the molecule of the drug (active fraction). The active fraction is 100% if the drug molecule contains only antibiotics. But it is less if the antibiotic is provided as salt, or if it contains water or impurities. Normally I and R are sold as anhydrous drugs and with purity ≥99%. In this case the potency is practically 100% or 100 μg / mg and no adjustments are necessary. But if adjustments with other drugs, such as aminoglycosides, capreomycin and FQN are necessary, they can be purchased in the form of salt and sometimes with a certain content of water and / or impurities.

Once the potency is known, it is possible to calculate the weight and volume of diluent needed to prepare a mother solution with a certain concentration.

Examples

		(Sequi) Dihydrostreptomycin Sulfate	(Di) Amikacin Sulfate	Levofloxacin hemihydrate
Information declared by the manufacturer	Potency (u / g)	undeclared	786	undeclared
	formula	$C_{21}H_{41}N_7O_{12}$. $1.5H_2SO_4$	$C_{22}H_{48}N_5O_{13}$. $2H_2SO_4$	$C_{18}H_{20}FN_3O_4$. 0.5 H_2O
	molecular weight	730.71	781.76	370.38
	purity	98.10%	no declared	98.00%
Calculations made in the laboratory	Water content	it does not have		0.5 H_2O
	Molecular weight of the salt	1,5[(2*1.0) + 32,06 + (4*16.0)]=147,09		------------------
	molecular weight of water	------------------		0,5(2*1,0 + 16)= 9
	molecular weight of the antibiotic free of salt and water	730,71 - 147,09 = 583,62		370,38 - 9= 361,38
	Proportion of antibiotic in the acquired drug	583,62 / 730,71=0,7987 (79,87%)		361,38 / 370,38 = 0,9570 (95,70%)
	Power (%)	79,87*98,10/100 = 78,35	78,6	95,70*98,0/100 = 93,8
	Potency (µg / mg)	783	786	938

In these examples, dihydro-Streptomycin was purchased as salt (sulfate). Subtracting the molecular weight of the sulfate containing the molecule, the molecular weight of the dihydro-S representing 79.87% of the total weight of the molecule was obtained. And that would be the potency if the drug were totally pure. But, the certificate of analysis of the batch indicates that the purity is of 98.1%, reason why that percentage should be applied on 79.87%. By doing this calculation, 78.35% of the weight of the drug purchased is dihydro-S pure or, in other words, every milligram of drug contains 783.5 µg of antibiotic. The potency is then 78.35% or 783.5 µg/mg.

In the case of Lfx, it was acquired with a formula containing half a molecule of water. By subtracting the weight of the water, the weight of the pure antibiotic, which accounts for 95.7% of the total molecular weight, can be calculated. As well, the purity of the batch is 98.0%, 93.8% of the drug purchased corresponds to Lfx. The potency is 93.8% or 938 µg / mg.

The manufacturer declares the potency the A acquired so that, in this case, it is not necessary to calculate it.

Once the potency is known, it is possible to calculate the amount of drug to be weighed to prepare the stock solution, depending on the volume of this solution that is required. Assuming that 10 ml of a stock solution having a concentration of 10 mg / ml is to be prepared, it is necessary to weigh 100 mg (100,000 µg) of the antibiotic and dissolve it in 10 ml of the diluent. 100 mg would be directly weighed in the case of an antibiotic with purity> 99%, free of salt and water. In other cases, it is necessary to calculate how much of the drug is to be taken to have 100 mg (or 100,000 µg) of antibiotic.

Continuing with the above examples, for the case of dihydro-S-sequesulfate, it has been calculated that each mg of drug purchased contains only 783.5 µg of antibiotic. Then applying the rule of proportions or rule of three:

78,5 µg of antibiotic -------- 1mg of acquired drug

100.000 µg of antibiotic ------ $x = \frac{1 * 100.000}{783,5}$ = 127,63 mg of acquired drug

That is, it is necessary to weigh 127.63 mg to have 100 mg (or 100,000 µg) of antibiotic, and dissolve them in 10 ml of its diluent.

The general formula summarizing this reasoning is

$$\text{weight (mg)} = \frac{\text{Volume mother solution (ml) * mother solution concentration (ug / ml)}}{\text{Potency (ug/mg)}}$$

Applying the formula to the 3 previous examples

	(Sequi) Dihydrostreptomycin Sulfate	(Di) Amikacin Sulfate	Levofloxacin hemihydrate
Potency (u /mg)	783,5	786	938
heavy amount (mg)	132	105	125
volume diluent (ml)	132 * 783,5 / 10 000 = 10,3	105 * 786 / 10 000 = 8,2	125 * 938 / 10 000 = 11,7

By weighing such small quantities, the error can be increased by the need to repeatedly add or withdraw drug from the container located within an analytical balance. To avoid this type of error, it is advisable to weigh an approximate amount and then calculate the volume of diluent to be added.

Following the example, about 10 ml of a stock solution of 10,000 μg / ml of dihydro-Streptomycin having a potency of 78.35% is desired. That is, 127, 63 mg should be added to 10 ml of diluent. When adding with a spatula drug on the paper or container placed in the balance, trying to reach an amount close to 100 mg, the balance indicates that what has been weighed 132 mg. Applying the rule of proportions

127.63 mg of drug ------------------- 10 ml of diluent

132 mg of drug ------------------- $x = \frac{132 * 10}{127,63} =$ 10,34 ml

That is, the 132 mg of the drug that have been weighed, should be dissolved in 10.34 ml of diluent to obtain a stock solution of 10,000 μg / ml or 10 mg / ml

The general formula for adjusting the volume of diluent according to the amount of weighed drug is

$$\text{weight diluent (mg)} = \frac{\text{weight of the drug (mg) * potency (ug / mg)}}{\text{Concentration of the stock solution (ug / ml)}}$$

Assuming that 132, 105 and 125 mg of each of the 3 drugs of the examples considered have been weighed, the following are the corresponding calculations

	(Sequi) Dihydrostreptomycin Sulfate	(Di) Amikacin Sulfate	Levofloxacin hemihydrate
Potency (u /mg)	783,5	786	938
heavy amount (mg)	132	105	125
volume diluent (ml)	132 * 783,5 / 10 000 = 10,3	105 * 786 / 10 000 = 8,2	125 * 938 / 10 000 = 11,7

That is, it is necessary to add 10.3, 8.2 and 11.7 ml of diluent to each of the weighed drugs to obtain, in all cases, a stock solution of 10,000 μg / ml or 10 mg / ml.

Diluents

The dissolution of each antibiotic begins with the recommended solvent, depending on the solubility that it has. It can be more soluble in water or in a base (NaOH) or an alcohol (such as ethanol) in an organic solvent (DMSO or dimethyl formamide).

If it is not water, the first diluent is used only to perform the initial complete dissolution, in the minimum possible amount, and then water is used for subsequent dilutions. Thus, the residue of the initial diluent is insignificant. For certain drugs that are practically insoluble in water, it is necessary to use an organic solvent to continue diluting the mother solution. In this case, it is important to ensure that the final dilution must be added in a low proportion in relation to the culture medium (≤1.5%) since the solvent can have an inhibitory effect and / or alter the permeability of the wall of the bacillus, and therefore its susceptibility to the antibiotic. It may also be necessary to incorporate a control without antibiotic in the ST in which the diluent is added in the same proportion as in the medium with drug.

Conservation

The mother solutions of the antibiotics do not need to be sterilized by filtration. If, exceptionally, repeated episodes of contamination with the same antibiotic are recorded, the corresponding mother solution should be discarded.

It is not convenient to preserve the mother solutions prepared with organic solvents or with any alcohol because the solvent can evaporate and, therefore, the concentration of the antibiotic increases and is uncertain. You can only choose to keep them when you have consistently demonstrated and documented the best conditions to do so (container with very tight seal, temperature, time).

The aqueous solutions, however, can be frozen at -18 °C or less, in aliquots. In general terms they last for one year, if the freezing has not been interrupted. Regardless of the time elapsed, they should be discarded if deterioration is detected through the quality controls that must be carried out with reference strains and with each batch of medium that is prepared.

If for any reason the mother solution cannot be stored at -18 °C or less, reliably, it should be discarded after being used to prepare a batch of medium.

The volume of each aliquot should be slightly higher than necessary to prepare a batch of medium used in the laboratory routine. Each aliquot should be thawed on the day a batch of medium is prepared, and the remainder should be discarded.

Critical concentrations

After reviewing the evidence published up to the time of writing these guidelines, the WHO recommended the use of the following concentrations of antituberculosis drugs in culture media:

Critical concentrations of drugs recommended by WHO for the susceptibility test of *Mycobacterium tuberculosis* WHO/CDS/TB/2018.5

Group	Drug	Concentration (µg/ml)			
		Löwenstein-Jensen	Middlebrook 7H10	Middlebrook 7H11	MGIT 960/320
1st line, oral	Isoniazid	0.2	0.2	0.2	0.1
	Rifampicin	40.0	1.0	1.0	1.0
	Ethambutol	2.0	5.0	7.5	5.0
	Pyrazinamide	-	-	-	100.0
Injectables	Streptomyicin	4.0	2.0	2.0	1.0
	Kanamycin	30.0	4.0	-	2.5
	Amikacin	30.0	2.0	-	1.0
	Capreomycin	40.0	4.0	-	2.5
Fluoroquinolones	Levofloxacin	2.0	1.0	-	1.0
	Moxifloxacin [a]	1.0	0.5	0.5	0.25
	Gatifloxacin	0.5	-	-	0.25
Other second-line	Ethionamide	40.0	5.0	10.0	5.0
	Protionamide	40.0	-	-	2.5
	Linezolid	-	1.0	1.0	1.0
	Clofazimine	-	-	-	1.0
	Bedaquiline	-	-	0.25	1.0
	Delamanid	-		0.016	0.06

[a] For isolations with mutations that determine a moderately elevated MIC of Mxf above the critical concentration, it may be considered to increase the daily dose of Mxf in the treatment. In this case it is recommended to test the Mfx at 1 µg/ml in MGIT or 2mg/l in 7H10.

To test 2nd line drugs WHO recommends the indirect method since the direct one has not been sufficiently validated.

It not recorded for certain drug concentration and means for insufficient evidence exists to make a recommendation or for which the results are not accurate enough.

It has been proposed that isoniazid could be maintained for the treatment of cases affected by strains of *M. tuberculosis* that are resistant to the concentration presented in the previous table, but sensitive to a higher concentration. However, the evidence was still insufficient at the time of writing these guidelines to recommend the highest concentration that best allows guiding that clinical decision.

With E, the results are not totally equivalent using different means, but there is not enough evidence to recommend changes in the concentration of any of them.

At the time of writing these guidelines, Lfx, Mxf, ethionamide, protionamide, linezolid, clofazimine, bedaquiline and delamanid were not yet included in the external quality assessment program conducted by the LSN Network. The evidence that supported the selection of the concentrations of these drugs in the culture media had been produced mostly under research conditions and in reference laboratories. The indications for performing ST may vary when the experience with them increases.

In general, the concentrations recommended are subject to periodic review in light of the evidence that

occurs. The reference laboratories must keep this information updated.

Culture Medium

The culture medium without and with drugs used in each ST must belong to the same batch (i.e. they must have been prepared at the same time). This ensures that differences in development that can be observed between the control and the medium with drug (s) is not caused by different quality of the medium.

Assuming that you want to prepare batches of the following type:

- Without drug (control), I and R to investigate cases that were sensitive to R, and eventually also to I, by rapid tests
- Without drug (control) I, R, S, E, FQN and injectable used by the country's NTP
  to investigate cases with

  resistance to R and / or I detected by rapid tests
  history of resistance to R and / or I
  exposure to TB RR or MDR (contacts)
  first-line drug intolerance

The average volume of each batch should be calculated in relation to the workload and the capacity to prepare medium, considering that it is convenient to use them within the month of preparation.

Example

Average monthly work load (including repetitions)

ST a I y R	29
ST a I, R, S, E Lvfx, Mxf , K, A, C	2
Method employed	of proportion in LJ
Volume of LJ dispensed by tube	7 ml

Required LJ volume

Batch A (I and R)		**Approximate**	**Volume**
No drug (control)	7 ml * 29 * 6 = 1218 ml	1200 ml	1200
With each drug	7 ml * 29 * 2 = 406 ml	400 ml	+(400*2)
			2000 ml
Batch B (I, R, S, E, Lvx, Mxf, K, A, C)			
No drug (control)	7 ml * 2 * 6 = 84 ml	100 ml	100
With each drug	7 ml * 2 * 2 = 28 ml	30 ml	+(30*9)
			370 ml

The volume of each tube has been multiplied by the number of ST / month, by the number of tubes used in each ST. Then, to facilitate calculations and preparation, the resulting figures have been rounded. Finally, we have added the total volume of media that needs to be prepared considering the number of drugs that make up the batch.

Thus for Batch A, 400 ml of LJ are required with each of the drugs (I and R) and 1200 ml of drug-free medium for the controls, making a total of 2000 ml.

For Batch B, a total of 370 ml (100 ml for control and 30 ml for each of the drugs) is required. These 370 ml can be separated from a lot of LJ that is prepared in the day, leaving the remnant to be packaged as a common LJ for crops or residues.

In the same way calculations can be made to prepare Middlebrook medium plates by replacing tubes by quadrants and by considering the volume dispensed in each quadrant.

In the case of laboratories with a very high workload, it may happen that there is insufficient capacity (human resources, inspissator, etc.) to prepare the total volume of medium you need on a monthly basis. In that case it will be necessary to prepare smaller batches and repeat the procedure more than once per month.

To achieve the critical concentration in the medium, it is possible to prepare stock solutions with different concentrations, as appropriate, and to add to the medium an amount of solution similar or equal to all drugs. Or standardize the concentration of the stock solutions, for example, at 10,000 µg / ml, and vary the volume of solution that is added to the medium. For convenience, in this Guide the latter alternative was adopted, except with PNB whose concentration is relatively high.

An amount of antibiotic or inhibitor solution not exceeding 1% of the volume of the medium may be added and, to homogenize such a small aggregate volume, it must be carefully and thoroughly mixed. Alternatively, a volume of antibiotic solution that exceeds 1% of the volume of the medium can be added, which facilitates homogenization, but in this case it is necessary to make corrections to reach the critical concentration, considering the total volume of half plus that of the antibiotic solution being added. For convenience, this Manual adopted the first alternative, except with PNB.

For biosafety, resistant glass tubes must be used to pack the LJ and it is preferable to use wide tubes (eg. 20 x 150 mm) because this favors a better distribution of the inoculum and the greater growth of the colonies, which, in turn, facilitates their rapid identification and counting. If tubes of another type of synthetic material are used, it is necessary to verify that it does not inhibit the development and that it allows to perfectly visualizing the developed colonies. In laboratories of level 3 safety, the use of cotton should be avoided in order to avoid blocking the BSC filters and air handling systems. This is why the use of tubes with a screw cap is indicated.

The following are critical points

- **The homogenization of the medium** after the addition of the antibiotic to ensure that all prepared tubes or plates contain the same concentration,
- for **Middlebrook agar,** the temperature **of the medium at the time of drug addition,**
- in the case of LJ, the time (**40-45 minutes) and coagulate the medium at (80 °C)**
- **preservation of the medium in refrigeration, protected from drying and light.**

Stock solution of antibiotics and selective inhibitors

To weigh drugs and calculations

Equipment

- Calibrated analytical or calibrated microbalance
- Lighter (desirable)

Materials

- Markers for fine tip glass,
- Brush,
- Alcohol 70%,
- Sterile petri dish,
- Spatula,
- Glass marker

For each antibiotic solution to be prepared:

- One 15 ml polypropylene tube, transparent, wide mouth, with lid, sterile, labeled with the name of the antibiotic or inhibitor
- For PNB, 1 paper or plastic container for weighing.

Reagents

- Each of the drugs that make up the batch of medium, with known potency

See the method described above to calculate the potency in the case where it is not declared by the manufacturer.

Procedure

- Switch on the balance 5 minutes before use, check that it is balanced before use,
- To preserve sterility, it is advisable to light and locate a cigarette lighter about 20 cm from the balance,
- Open the balance door and brush the pan and interior surfaces with a brush,
- Place the tube, gently, on the center of the saucer,
- Close the door,
- Tare the tube (the scale should dial 0.0000),
- Open the door, remove the tube gently,
- Put the spatula in alcohol, flame it in the burner, let it cool (it can be supported on a sterile Petri dish located near the burner while not in use).

Perform the following two steps near the burner:

- Open the bottle, with the spatula try to take at once an amount slightly higher than 100 mg of the drug, close the bottle,
- Open the lid of the tube and unload inside it with the spatula, close the lid,
- Open the scale door, carefully place the tube in the center of the pan and close the door,
- Wait for the balance to stabilize,
- If the weight is less than 100 mg, repeat the above 7 steps to add more antibiotic into the tube, and slightly match or exceed that amount,
- Remove the tube from the balance,
- Record the exact weight of antibiotic on the wall of the tube and on the work sheet.

Repeat the procedure with all antibiotics and / or selective inhibitors that make up the batch of medium (except the GNP for which the procedure is described below).

- For each drug that has been weighed, calculate the total volume of diluent to be added with the following formula:

$$\text{Diluent volume (ml)} = \frac{\text{weight of the drug (mg)} * \text{potency (µg/mg)}}{\text{Concentration mother solution (µg/mg)}}$$

- Record the calculated volume on the worksheet and on the wall of the bottle containing each antibiotic or selective inhibitor

For PNB:

- Repeating the procedures described above, accurately weigh 2.5 g of the drug on paper or container.

Dissolution of drugs and conservation

Equipment

- Sterility cabin (preferable) or lighter,
- Vortex type agitator,
- (Ultra) freezer -20 ° C or less,
- Pro pipette or automatic pipettor

Materials

- Sterile 1 or 2 ml pipettes
- Sterile 10 ml pipettes
- Cryotubes, with screw cap, from 2 to 5 ml for stock solutions that can be frozen for a long time choose the tube according to the volume of each aliquot to be preserved, considering the volume of medium with each drug that is prepared in the routine, according to the workload

For PNB:
- A sterile, graduated 100 ml glass erlenmeyer flask
- A 5 ml pipette

Reagents

- Transparent polypropylene tubes containing each weighted drug, labeled with its name, weight, and with the volume of diluent needed to prepare the stock solution,
- Sterile distilled water,
- diluents indicated for the drugs to be diluted as detailed in the table below,

For PNB

- HCl 1N
- phenolphthalein solution

Drugs and initial diluents

	Drug	Sal[a]	Diluent
1st line, oral	Isoniazid		Sterile distilled water
	Rifampicin[b]		Dimethylsulfoxide or Dimethylformamide
	Ethambutol	Hydrochloride	Sterile distilled water
Injectables	Dihydrostreptomycin[c]	sulfate	Sterile distilled water
	Kanamycin	sulfate	Sterile distilled water
	Amikacin	base or sulfate	Sterile distilled water
	Capreomycin		Sterile distilled water
Fluoroquinolone	Ofloxacin		Sterile distilled water NAOH 4% sterile
	Levofloxacin		Sterile distilled water NAOH 4% sterile
	Moxifloxacin[d]	Hydrochloride	Sterile distilled water NAOH 4% sterile
	Gatifloxacin		Sterile distilled water NAOH 4% sterile
Others	Ethionamide[b]		Dimetilsulfoxido o Etilenglicol
	DL Cycloserine[e]		Sterile distilled water
	PAS		Sterile distilled water
Selective inhibitors for identification	Hydrazide of thiophene-2-carboxylic acid TCH		Sterile distilled water
	P-nitrobenzoic acid (PNB)		Sterile distilled water NAOH 4% sterile HCL 1N Phenolphthalein 0.1% in ethanol (as indicator)

(a) Form generally provided by manufacturers, can be anhydrous formulas or with different H_2O content
(b) Dissolve at the time of use, do not freeze the stock solution
(c) Use dihydrostreptomycin sulfate and non-streptomycin sulfate
(d) It may not be necessary NaOH
(e) Employ DL-Cicloserine

Procedure

Work inside a sterile cabin (preferable) or by the side of the lighter taking care of asepsis

Antibiotics soluble in dimethylsulfoxide
R, ethionamide

- With a 2 ml pipette add dimethylsulfoxide inside the tube containing each drug, stirring until complete dissolution is achieved, record the volume added in the worksheet
- With a 10 ml pipette add sterile distilled water in sufficient quantity to complete the volume of calculated total diluent (to reach a concentration of 10,000 µg/ml with the amount of weighted drug); record the volume added in the worksheet
- Shake until complete homogenization.

Linezolid, clofazimine, bedaquiline, delamanid

- With a 10 ml pipette, add dimethylsulfoxide until the volume of calculated total diluent is reached (to reach a concentration of 10,000 µg/ml with the amount of weighted drug) record the volume added in the worksheet
- Shake until complete homogenization

Use dissolved stock solutions in dimethylsulfoxide on the day of preparation and discard the remaining, unless rigorously and consistently demonstrated and documented stability of the frozen stock solution under certain conditions (cryotube with very tight seal, temperature, maximum time).

Antibiotics and soluble inhibitors at basic pH (FQN, PNB)

FQN

- With a 10 ml pipette, transfer 5 ml of sterile distilled water into the tube containing each drug, record the volume added in the worksheet,
  Mfx and gatifloxacin can be dissolved only with water, without the need for the addition of NaOH. If so, skip the next step,
- With a 2 ml pipette, add 4% NaOH dropwise, stirring until the complete dissolution of the antibiotic is checked, record the added volume in the worksheet,
- In 10 ml pipette add sterile distilled water in sufficient quantity to complete the calculated total diluent volume (to reach a concentration of 10,000 µg / ml with the amount of heavy drug); record the added volume in the worksheet,
- Aliquot the stock solution in cryotubes, transferring in each the appropriate volume of stock solution, according to the volume of medium that is usually prepared in the laboratory,
- Assign a batch number and label each cryotube with the antibiotic name and batch number,
- Store the cryotubes at -20 ° C, preferably at -70 ° C to 12 months. If you are going to prepare half on the same day, reserve an aliquot without freezing for that purpose,
- Register the batch number, the volume of each cryotube, the number of aliquots and the expiration date in the worksheet.
  (see record model at the end of this chapter).

PNB:

- Transfer the weighted 2.5 g to the Erlenmeyer,
- Add 10 ml of 4% NaOH with a 10 ml pipette. If necessary add more NaOH until complete dissolution is checked,
- Add enough distilled water to make up to 80 ml,
- With 1 ml pipette, add 2 drops of the phenolphthalein solution,
- With a 5 ml pipette, add 1N HCl dropwise until the color change of phenolphthalein (approximately 3 ml is required),
- Add distilled water again to make up to a final volume of 100 ml,

- Aliquot the stock solution in cryotubes, transferring in each one the suitable volume the stock solution,

- Assign a batch number and label each cryotube with the antibiotic name and batch number,

- Store the cryotubes at -20 °C, preferably at -70 °C to 12 months. If you are going to prepare half on the same day, reserve an aliquot without freezing for that purpose,

- Register the batch number, the volume of each cryotube, the number of aliquots and the expiration date in the worksheet.
  (see record model at the end of this chapter).

Antibiotics and water-soluble inhibitors
(I, E, injectables, cycloserine, PAS, TCH)

- In 10 ml pipette add sterile distilled water in sufficient quantity to complete the calculated total diluent volume (to reach a concentration of 10,000 µg/ml with the amount of heavy drug); record the added volume in the worksheet,

- Aliquot the stock solution in cryotubes, transferring in each one the appropriate volume of the stock solution, according to the volume of medium that is usually prepared by the laboratory,

- Assign a batch number and label each cryotube with the antibiotic name and batch number,

- Store the cryotubes at -20 °C, preferably at -70 °C to 12 months. If you are going to prepare medium for the ST on the same day, reserve an aliquot without freezing for that purpose,

- Register the batch number, the volume of each cryotube, the number of aliquots and the expiration date in the worksheet.
  (see record model at the end of this chapter).

If you do not have an ultra-freezer that maintains the temperature reliably, in all cases, prepare the stock solution at the time of its use and discard the entire remnant.

Löwenstein Jensen with antibiotics or selective inhibitors

Equipment

- Sterility cabin (preferable) or Bunsen burner,
- Vortex type agitator,
- Pro pipette or automatic pipettor,
- Coagulator.

Materials

- Latex or vinyl gloves,
- Pipette for pipette disposal,
- absorbent paper and alcohol 70 ° to disinfect the cabin or surfaces of the work table,
- Markers for glass with fine point,
- Pipettes of 1 ml or micropipette to dispense 1 ml with sterile tip,
  1 pipette or tip per dilution 1:10 to be performed, see the following table

- Sterile 2 ml pipettes,
  1 for each antibiotic that integrates the batch of medium

- Transparent 15 ml tubes with 9.0 ml of sterile distilled water exactly measured,
  1 for each 1:10 dilution to be performed, see table below
  Labeled with the corresponding antibiotic name, and the dilution of the stock solution which will contain (1:10 or 1:100); add “final” to the last necessary dilution.

- Graduated glass or polypropylene flasks or stoppers, capped, sterile, labeled with the name of each antibiotic,
  Quantity: equal to the number of drugs in the batch
  Volume: at least 2 times greater than the volume of medium to prepare with each drug, to be able to homogenize it

- 15 ml wide test tubes (i.e 20x150 mm), screw cap, sterile, in sufficient quantity to package the total volume of the medium to be prepared.

- Polyethylene bags or plastic boxes clean and disinfected, with hermetic lid, and sufficient capacity to preserve the middle batch.

Reagents and culture media

- Mother solution of each antibiotic that integrates the lot of medium to be prepared,
  If a frozen aliquot is used, remove from the cold in advance, so that it takes room temperature before being used.

- Sterile erlenmeyer flask, with LJ prepared at day, filtered and un coagulated, volume necessary to prepare the complete batch of medium without and with drugs.
  See above, in this Appendix, the method for calculating the required volume
  See Appendix IV of part 2 of this Manual, dedicated to culture, the method of preparation of the LJ medium.

Process

Work in a sterile cabin (preferably) or with a lighter, with work surfaces disinfected with 70° alcohol, taking very strict care of the asepsis conditions.

Dilutions of the mother solutions

- Make the dilutions of the stock solutions of the antibiotics and inhibitors that make up the batch of medium, on the same day it is prepared, as indicated below:

Dilution and addition of the antibiotic solution to the Lowenstein Jensen medium

	Drug	Concentration of mother solution µg/ml	Dilutions to be made of the mother solution	Concentration of the last dilution µg/ml	Volume to be added of the last dilution per 100 ml of LJ ml	Final concentration of the antibiotic in LJ µg/ml
1st line orales	Isoniazide	10,000	two serials 1:10	100	0.2	0.2
	Rifampici	10,000	none		0.4	40.0
	Ethambutol	10,000	one 1:10	1,000	0.2	2.0
Injectables	Dyhidro streptomycin	10,000	one 1:10	1,000	0.4	4.0
	Kanamycin	10,000	none		0.3	30.0
	Amikacin	10,000	none		0.3	30.0
	Capreomycin	10,000	none		0.4	40.0
Fluoro quinolones	Levofloxacin	10,000	one 1:10	1,000	0.2	2.0
	Moxifloxacin	10,000	one 1:10	1,000	0.1	1.0
	Gatifloxacin	10000	two serials 1:10	100	0.5	0.5
Other second-line	Etionamide Protionamide	10,000	none		0.4	40.0
Antibiotics and selective inhibitors used for identification	D L Cycloserine	10,000	none		0.3	30.0
	PAS	10,000	two serials 1:10	100	1.0	1.0
	Hydrazide of thiofene-2-carboxylic acid (HTC)	10,000	one 1:10	1,000	0.2	2.0
	P-nitrobenzoic acid (PNB)	25,000	none		2.0	500.0

Distribution

- Homogenize the LJ by manually rotating the bottle or erlenmeyer containing it,

- Fraction the medium in each bottle or graduated erlenmeyer, labeled with the name of each drug that integrates the bat, measuring very accurately the volume of necessary medium. Close each vial or erlenmeyer immediately afterwards,

- Keep the medium remaining in its container to be packaged as an LJ without a drug.

Antibiotic added

- Take the tube with the stock solution or the "final" dilution, as appropriate, of the first antibiotic. Vortex,

- Select the bottle containing LJ labeled with the name of that antibiotic,

- Pipette or micropipette, transfer the required volume of the antibiotic solution into the bottle with LJ, close the bottle,

- Thoroughly homogenize by manually rotating and carefully mixing the medium into the package,

- Repeat the previous 4 steps with all the antibiotics that make up the lot,

- Discard all remaining stock solutions and dilutions used.

Distribution and coagulation of the medium:

- Take the LJ bottle with the first of the antibiotics that make up the batch and homogenize it again before packaging,

- Follow the instructions given in Appendix IV of part 2 of this Manual to distribute the medium in the test tubes and coagulate. Take care that the temperature does not exceed 80 °C and the coagulation time does not exceed 40-45 minutes,
- Allow to cool to room temperature,

- Label each tube with the name of the antibiotic it contains (or with a symbol or color that systematically identifies each antibiotic that is tested in the laboratory) and the batch number,

Repeat the process with the medium containing each of the antibiotics in the batch, and finally with the medium without the drug.

Separate the LJ tubes with no drugs necessary for the performance of the ST with the antibiotics that make up the lot, so that it is used exclusively for ST and not for other purposes.

Keep the entire batch at 4 °C for a maximum of 1 month in a polyethylene bag or plastic box with a lid, labeled with the name of the antibiotic or drug, batch number and expiration date.

Protect from light. Discard any evidence of dehydration or contamination.

Provide quality control of the batch of medium prepared with reference strains (see the Internal Quality Control chapter of this Manual).

Lowenstein Jensen with I and R for the nitrate reductase test

Equipment

- Sterility cabin (preferable) or lighter,
- Vortex type agitator,
- Pro pipette or automatic pipettor,
- Coagulator.

Materials

- Latex or vinyl gloves,
- Pipette for pipette disposal,
- Absorbent paper and alcohol 70 ° to disinfect the cab or work surfaces,
- Markers for glass with fine point,
- 4 pipettes of 1 ml or micropipette to dispense 1 ml with sterile tip,
- 3 sterile 2 ml pipettes,
- 2 transparent 15 ml tubes with 9,0 ml of exactly

measured sterile distilled water,
labeled with I and the dilutions of the I stock solution to be contained (1:10 and 1:100 "final").

- Three graduated flasks or erlenmeyers, of glass or polypropylene, with lid, sterile
  Volume: at least 2 times greater than the volume of medium they must contain, in order to homogenize it,
  Label one with the name "control", one with "I" and one with "R"

- 15 ml test tubes, screw cap, sterile
  in sufficient quantity to package the total volume of the batch of medium to be prepared,

- Polyethylene bags or plastic boxes clean and disinfected, with hermetic lid, and sufficient capacity to preserve the lot of medium.

Reagents and culture medium

- stock solution of I 10,000 µg/ml,
- stock solution of R 10.000 µg/ml,
  See the way to prepare above in this Appendix, under the title Stock solution of the antibiotics and selective inhibitors
  If a frozen aliquot is used, remove from the cold in advance, so that it takes room temperature before being used

- Stock solution of potassium nitrate (KNO_3) 200 g/l dilute 1 g KNO_3 per 5 ml of sterile distilled water,

- Sterile erlenmeyer flask, with LJ prepared at day, filtered and uncoagulated

  Separate the necessary volume according to the workload,
  See calculation example at the end of this item
  See Appendix IV of part 2 of this Manual, dedicated to Culture, the method of preparation of the LJ medium.

Procedure

Work in a sterility booth (preferably) or with a lighter, with work surfaces disinfected, taking very strictly aseptic conditions

Dilutions of isoniazid stock solution 10,000 µg/ml

- Vortex the stock solution,

- Place it in a rack and then place the two tubes containing 9 ml of water labeled 1:10 and 1:100 "final",

- To make the first dilution 1:10, pipette 1 ml of the stock solution and transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:10. Discard the pipette or tip used,

- Vortex the tube containing the 1:10 dilution, pipette or micropipette 1 ml and transfer to the second tube containing 9 ml of distilled water labeled 1:100 "final". Discard the pipette or tip,

- Vortex the final dilution.

Potassium nitrate added

- Add 0.5 ml of the stock solution of potassium nitrate per 100 ml of the medium into the flask containing the LJ without coagulating,

- Homogenize the LJ by manually rotating the bottle or erlenmeyer containing it,

- Fraction the medium in three graduated flasks or erlenmeyers, labeled "control", "I" and "R" accurately measuring the volume of medium transferred to each. The "control" bottle should contain three times the volume of the other two. Close each vial or erlenmeyer immediately after use.

Antibiotic added

- Vortex the final dilution of the "I" solution,

- Pipette 0.2 ml of this solution into each 100 ml of LJ with potassium nitrate contained in the bottle labeled "I". Close the bottle immediately after use,

- Vortex the "R" stock solution,
- Pipette 0.4 ml of the same per 100 ml contained in bottle "R". Close the bottle immediately after use,

- Homogenize both vials thoroughly, manually rotating and carefully mixing the medium into the container,

- Discard the remaining stock of antibiotic stock solutions and dilutions that have been used.

Distribution and coagulate of the medium

Take each bottle of LJ that integrates the batch and homogenize it again before packaging,

- Follow the instructions given in Appendix IV of part 2 of this Manual, dedicated to Culture, to distribute the medium in the test tubes and coagulate. Take care that the temperature does not exceed 80 °C and the coagulation time does not exceed 40-45 minutes,
- Allow to cool to room temperature,
- Label each tube with the name of the medium (or with a symbol or color that systematically identifies the LJ lot with control potassium nitrate, with I and with R) and the corresponding batch number,

Store at 4 °C for a maximum of 1 month in a polythene bag or plastic box with a lid labeled with the antibiotic or drug name, batch number and expiration date. Protect from light.

Discard any evidence of dehydration or contamination.

Provide quality control of the batch of medium prepared with reference strains (see the Internal Quality Control chapter of this Manual).

Example:

Average monthly work load (Including repetitions)			
Nitrate reductase tests with I and R		19	
Amount of test tubes		5	(3 controls, 1 with I, 1 with R)
Volume of LJ dispensed by tube		5 ml	
Volume of LJ needed for two	=	19*5*5 = 475 ml	

To facilitate the procedure, round the total volume of LJ medium with potassium nitrate to 500 ml. These 500 ml can be separated from a lot of LJ that is prepared in the day, leaving the remnant to be packaged as a common LJ for crops or peaches.

Add to 500 ml of LJ 2.5 ml of stock solution of potassium nitrate
Fraction in 3 bottles
 1 bottle with 300 ml, will not have drug and will be employed for the controls
 2 vials with 100 ml each
  Add one of them 0.2 ml of the last dilution of I
  Add to the other 0.4 ml of the stock solution of R

For the preparation of the reagents for the development of the nitrate reductase test (solutions 1, 2 and 3) see Appendix IV of part 2 of this Manual dedicated to culture.

For the preparation of the reagents for the development of the nitrate reductase test (solutions 1, 2 and 3) see Appendix IV of part 2 of this Manual dedicated to culture.

Agar Middlebrook 7H10 with antibiotics

Equipment

- Sterility cabin,
- Vortex type agitator,
- Water bath,
- Pro pipette or automatic pipettor.

Materials

- Latex or vinyl gloves,
- Markers for fine tip glass,
- Pipette for pipette disposal,
- absorbent paper and alcohol 70 ° to disinfect,

- Pipettes of 1 ml or micropipette that can dispense 1 ml with sterile tip
  1 pipette or tip per 1:10 dilution to be performed,

- Sterile 2 ml pipettes,
  1 for each antibiotic that integrates the batch of medium

- Transparent 15 ml tubes, screw cap, containing 9 ml sterile distilled water accurately measured,
  1 dilution per 1:10 dilution to be performed
  labeled with the appropriate antibiotic name, and the dilution of the stock solution to be contained (1:10 or 1: 100). Add the "final" label to the last necessary dilution

- Disposable Petri dishes, very transparent, sterile, divided into 4 compartments
  in sufficient quantity to package the total volume of the medium to be prepared
  Label each quadrant of each plate as appropriate: "control" (for the medium without drug) and the name of each antibiotic that integrates the medium.
  See the suggested scheme for organizing the mean distribution in the quadrants in the Middlebrook 7H10 Middle Proportions Method chapter of this Handbook

- Polyethylene bags or plastic boxes clean and disinfected, with hermetic lid, and sufficient capacity to preserve the environment.

Reagents and culture medium:

- Mother solution of each antibiotic that integrates the batch of medium to be prepared,
  If a frozen aliquot is used, remove from the cold in advance, so that it takes room temperature before being used,

- Erlenmeyer flask containing 200 ml Middlebrook 7H10 enriched with OADC, sterile, melted and kept in a water bath at 50-55 °C,
  As many bottles as necessary to prepare the complete batch of media without and with drugs.
  Label each bottle with the name of each antibiotic in the batch, and with the label "control" for the drug-free medium
  It is not convenient to work with fractions of medium with volume greater than 200 ml because the agar solidifies quickly
  See above, in this Annex, the method for calculating the volume of media required
  See Annex IV of part 2 of this Manual, dedicated to cultivation, the method of preparation of the Middlebrook 7H10 medium.

Procedure

Work in sterile cabin disinfected with alcohol 70°, taking very strictly aseptic conditions.

Dilutions of the stock solutions

- Dilutions of stock solutions of the antibiotics that make up the batch of medium, on the same day it is prepared, as indicated below.

Dilution and addition of the antibiotic solution to Middlebrook 7H10 medium

	Drug	Concentration of mother solution ug / ml	Dilutions to be made of the mother solution	Concentration of the last dilution ug / ml	Volume to be added of the last dilution per 100 ml of MiddleBrook 7H10 ml	Final concentration of the antibiotic in MiddleBrook 7H10 ug / ml
1st line, oral	Isoniazide	10.000	two serials 1:10	100	0.4	0.2
	Rifampicin	10.000	two serials 1:10	100	2.0	1.0
	Ethambutol	10.000	one 1:10	1000	1.0	5.0
Injectables	Dihydrostreptomycin	10.000	one 1:10	1000	0.4	2.0
	Kanamycin	10.000	one 1:10	1000	0.8	4.0
	Amikacin	10.000	one 1:10	1000	0.4	2.0
	Capreomycin	10.000	one 1:10	1000	0.8	4.0
Fluoroquinolones	Levofloxacin	10.000	two serials 1:10	100	2.0	1.0
	Moxifloxacin	10.000	two serials 1:10	100	1.0	0.5
Other second line	Ethionamide	10.000	one 1:10	1000	1.0	5.0
	Linezolid	10.000	two serials 1:10	100	2.0	1.0

- Vortex all stock solutions of the antibiotics that make up the batch of medium to be prepared,
- Select the mother solutions of the antibiotics that need to be diluted, place them in a rack and, after each one, locate the labeled 9 ml tube (s) required,
- To make the first dilution 1:10, pipette or micropipette 1 ml of the stock solution and transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:10. Discard the pipette or tip used,
- If necessary, make the second 1:10 dilution. Vortex the tube containing 10 ml of the 1:10 dilution, pipette or micropipette 1 ml and transfer to the second tube containing 9 ml of distilled water labeled 1: 100. Discard the pipette or tip,
- Select the mother solutions of the antibiotics that do not need to be diluted, and the dilutions marked with the "final" label, order them in another rack.

Middlebrook 7H10 with antibiotics

- Take the tube with the stock solution or the "final" dilution, as appropriate, of the first antibiotic. Vortex,
- Select the bottle containing 200 ml of Middlebrook 7H10 LJ medium labeled with the name of that antibiotic,
- With a pipette or micropipette, transfer the necessary volume of the antibiotic solution into the bottle,
- Thoroughly homogenize, carefully rotating and mixing the medium into its container, manually,
- Quickly dispense 5 ml into all quadrants of the labeled plates with the name of the antibiotic you are working with. Avoid the formation of bubbles. The agar layer should have a depth of 3-4 mm,

- Repeat the previous 5 steps with all the antibiotics that make up the batch,
- Dispense the drug-free medium ("control") into the corresponding quadrants,
- Allow to solidify at room temperature inside the sterility booth,
- Discard the remnant of all aliquots of mother solutions and their dilutions unfreezed and used in the day,
- Immediately use or keep inverted plates at 4 ° C for a maximum of 1 month in a polythene bag or plastic box with a lid labeled with the antibiotic or drug and batch number. Protect from light. Discard any evidence of dehydration or contamination.

Provide quality control of the batch of medium prepared with reference strains (see the Internal Quality Control chapter of this Guide).

Medium and reagents for the pyrazinamidase test

Equipment

- Sterility booth (preferred) or lighter,
- Autoclave,
- Water bath,
- Propipette or automatic pipettor.

Materials

- Markers for glass with fine point,
- Latex or vinyl gloves,
- Pipette for pipette disposal,
- Absorbent paper and alcohol 70 ° to disinfect the cabin or work surfaces,
- Erlenmeyer flask, graduated, with lid, autoclavable with capacity that at least doubles the volume of medium to prepare,
- Sterile 10 ml pipette,
- Glass tubes of 5 ml, wide, with screw cap
  quantity sufficient for the volume of the medium and reagent batch to be prepared.

Growing medium

Broth Dubos prepared on a commercial basis following the manufacturer's instructions without enrichment	250	ml
Pyrazinamide	25	mg
Sodium pyruvate	0.5	g
Agar	3.75	g

Adjust the quantities according to the volume of medium to be prepared in relation to the workload

- Weigh the Z, sodium pyruvate and agar and add them to Dubos broth,
- Heat in a water bath at 100° C until the agar is melted and the medium is clarified (approximately 10 minutes),
- Dispense 2 ml of medium into each tube,
- Sterilize the tubes in an autoclave at 1 atmosphere for 15 minutes,
- Allow to solidify with the tubes in vertical position,
- Store at 4 °C for a maximum of 1 month in a polyethylene bag or plastic box with a lid, labeled with the media name and batch number. Protect from light. Discard any evidence of dehydration or contamination.

Reagents

Iron and ammonium sulfate solution 1%

Iron and ammonium sulphate $SO_4Fe_2NH_4$	1	g
Distilled water	100	ml

Adjust the weight of the ammonium iron sulfate according to the quantity of water molecules containing the formula of the available drug.

- Dissolve iron and ammonium sulfate, vortex,
- Dispense 2 ml into small screw cap tubes (or a larger volume if several tests per day are performed),
- Autoclave 15 minutes at 1 atmosphere,
- Label,
- Store at 4 °C.

MGIT with antibiotics for MGIT 320 or 960 equipment

They can be prepared with the antibiotics provided by the manufacturer of the MGIT system, following their instructions, but verifying that the concentrations recommended by WHO are used. Kits can be purchased that allow testing of IR, SIRE, Z and second line drugs. Antibiotics purchased from other providers can also be used.

The preparation of tubes with the SIRE and Z kit provided by the equipment manufacturer is described by way of example. Note that the medium and enrichment required for the Z test are different.

Then, and also as an example, the option of preparing tubes for the performance of ST to second-line drugs is presented, using antibiotics from other suppliers.

Mother solution or stock of the antibiotics provided by the manufacturer

Equipment

- Sterility cabin,
- Vortex type agitator,
- Pro pipette or automatic pipettor,
- Freezer - 20 ° C or less.

Materials

- Latex or vinyl gloves,
- Pipette for discard of pipettes and / or bottle for tip discarding,
- Absorbent paper and alcohol 70 ° to disinfect the cab,
- 5 ml pipettes,
  1 per antibiotic to be prepared
- Pipettes or micropipette with sterile tip,
  which dispenses the volume that has been selected for each aliquot to be preserved according to the amount of ST performed daily by the laboratory
- Cryotubes, with screw cap, of 2 to 5 ml,
  quantity and volume that is appropriate in relation to the amount of ST performed daily in the laboratory
- Marker for fine tip glass,
- Sterile distilled water.

Reagents

- BD BACTEC ™ MGIT ™ 960 KIT SIRE consisting of:

  MGIT Isoniazid (MGIT-I) containing 33.2 µg
  MGIT Rifampicin (MGIT-R) containing 332 µg
  MGIT Streptomycin (MGIT-S): 332 µg
  MGIT Ethambutol (MGIT-E): 1660 µg

  or the IR kit composed only of I and R in the same amounts as those mentioned

- BD BACTEC ™ MGIT ™ 960 PIT KIT
  containing 20,000 µg of Z

  For safety, it is not recommended to use syringes to make the dilutions. That is why it is convenient to remove the seal from the bottles and then, to dissolve the drug, carefully remove your rubber stopper.

Procedure

Working inside a sterility cabin, with disinfected surfaces, strictly taking care of asepsis

- Pipette 4 ml of sterile distilled water into the bottle containing I. Discard the pipette,

- Pipette 4 ml of sterile distilled water into the bottle containing R. Discard the pipette,

- With pipette, add 4 ml of sterile distilled water into the bottle containing S. Discard the pipette,

- Pipette 4 ml of sterile distilled water into the bottle containing E. Discard the pipette,

- With pipette, add 2.5 ml of sterile distilled water into the bottle containing Z. Discard the pipette,

- Mix the contents of each vial with vortex stirrer and verify that the drugs are completely dissolved,

- With pipette or micropipette, distribute each stock solution to cryotubes, transferring in each one the appropriate volume according to the amount of ST that are performed daily in the laboratory,

- Record the batch number of each stock solution, volume, number of prepared aliquots, concentration (8.3 μg/ml for I and 83 μg/ml for R and S and 415 μg/ml for E and 8000 μg/ml for Z), date of dissolution and expiration date,

- Label each cryotube with the name of the antibiotic and batch number. Record the expiration date on the container where they are placed,

- Store the cryotubes at -20 °C. preferably at -70 °C to 6 months or until the expiry date of the antibiotic if earlier. If you are going to prepare medium for the ST on the same day, reserve an aliquot without freezing for that purpose.

MGIT with SIRE

Equipment

- Sterility cabin,
- Vortex type agitator,
- Micropipettes to dispense from 100 to 1000 μl.

Materials

- Latex or vinyl gloves,
- Bottle for tip discarding,
- Absorbent paper and alcohol 70 ° to disinfect the cab,
- Micropipettor tips,
- Clean and disinfected polypropylene racks.

Reagents, culture medium and supplement

Select the aliquots of the drugs that integrate the ST to be performed in the day and leave them at room temperature.

- An aliquot of stock solution of S (83 μg/ml),
- An aliquot of stock solution of I (8.3 μg/ml),
- An aliquot of stock solution of R (83 μg/ml),
- An aliquot of stock solution of E (415 μg/ml),
- OADC Supplement,
  (oleic acid 0.6 g/l, bovine albumin 50 g/l, dextrose 20 g/l and catalase 0.03 g/l)
  After opening, close and protect with parafilm any remaining OADC that is saved for later working days. It gets very easily contaminated

- BD BBL ™ MGIT ™ MGIT tube with 7 ml of medium,
  Amount necessary for the performance of the ST scheduled for that day.

Procedure

- Order in racks and label the number of tubes necessary for the ST programmed for the day, having a first “control” tube (without drug) and then one with the label of each drug to be tested,
- With Pipette or micropipette add 0.8 ml of OADC in each MGIT tube,

- With micropipette, add 100 μl of the S solution to each tube labeled “S”

- With micropipette, add 100 μl of the I solution to each tube labeled “I”,

- With micropipette, add 100 µl of the R solution to each tube labeled "R",
- With micropipette, add 100 µl of E solution to each tube labeled "E"

- Discard any remaining stock aliquots of stock solutions of the thawed antibiotics used on that day.

MGIT with Z

Equipment

- Sterility cabin,
- Vortex type agitator,
- Micropipettes to dispense from 100 to 1000 µl.

Materials

- Latex or vinyl gloves,
- Bottle for tip discarding,
- Absorbent paper and alcohol 70 ° to disinfect the cab,
- Micropipettor tips,
- Clean and disinfected polypropylene racks.

Reagents and culture medium and supplement

- An aliquot of stock solution of Z (8000 µg/ml),

- Supplement BACTEC MGIT 960 Z, (0.1 g/l oleic acid, 50 g/l bovine albumin, 20 g/l dextrose and 0.03 g/l catalase, 1.1 g/l polyoxyethylene stearate)

- BACTEC MGIT 960 Z with 7 ml of medium, amount needed for the ST scheduled for that day.

Procedure

- Sort in racks and label the necessary series of tubes by having a first "control" tube (without drug) and then one with the Z label for each ST to be made,

- Pipette or micropipette add 0.8 ml of BACTEC MGIT 960 Z Supplement in each MGIT 960 Z tube,

- With micropipette, add 100 µl of the stock solution of Z to each tube labeled "Z"
- Discard any remnants of the aliquot of Z stock solution thawed and used in the day.

Preparation of MGIT with drugs provided by the manufacturer for automated reading equipment MGIT 320/960

	Drug provided by the manufacturer in the bottle (ug)	Distilled water to be added (ml)	Concentration of the mother solution (ug / ml)	Volume to add mother solution in each tube with 7ml of MGIT + 0.8ml supplement OADC (ml)	Final concentration of the antibiotic in the medium (a) (ug / ml)
isoniazid	33,2	4	8,3	0,1	0,1
rifampicin	332	4	83	0,1	1,0
ethambutol	1660	4	415	0,1	5,0
streptomycin	332	4	83	0,1	1,0
pyrazinamide	20000	2,5	8000	0,1 (b)	96,4 (c)

a. Whereas 0.5 ml of the inoculum was added.
b. use the medium and OADC supplement of the Z kit to achieve a pH of 5.9
c. however, the BD company states that the concentration is 100 µg/ ml in its documentation

MGIT with second-line drugs using antibiotics from other suppliers

Equipment

- Sterility cabin,
- Vortex type agitator,
- Micropipettes for dispensing from 100 to 1000 µl,
- Propipettes or automatic pipettor.

Materials

- Latex or vinyl gloves,
- Bottle for tip discarding,
  Absorbent paper and alcohol 70 to disinfect the cab,
- 1 ml pipettes,
- 5 ml pipettes,
- 10 ml pipettes,

- Transparent 15 ml tubes with 9.0 ml of sterile distilled water exactly measured, in quantities necessary for all 1:10 dilutions to be labeled with the appropriate antibiotic and dilution of the stock solution to be contained (1:10 or 1:100); add "final" to the last necessary dilution.

- 10 ml clear sterile tubes one for each drug, labeled with the name of each water-soluble antibiotic and the last dilution that corresponds to it; mark as "final", e.g. Label for K and C: "1:48 final".

- 5 ml clear sterile tubes with 900 µl of exactly measured dimethyl sulfoxide in necessary quantity for all the 1:10 dilutions to be made with the drugs not in water (clofazimine, bedaquine and / or delamanid) labeled with the name of the corresponding antibiotic, and the dilution of the stock solution that will contain (1:10 or 1:100 or 1:1000)

- - 5 ml clear sterile tubes. One for each drug not soluble in water with the name of the antibiotic and the last dilution that corresponds to it; mark as "final" Ex. Label for clofazimine: "1:240 final"

- Sterile distilled water

- Dimethylformamide (if clofazimine, bedaquiline and / or delamanid are tested)

- Tips of micropipettes

- Clean and disinfected polypropylene racks

Reagents, culture medium and supplement

- An aliquot of each stock solution of each drug that integrates the lot (10,000 µg/ml)

  See above in that Annex, under the heading "Stock solution of antibiotics and selective inhibitors", how to prepare these solutions.

  If a frozen aliquot is used, remove from the cold in advance, so that it takes room temperature before use.

- OADC Supplement
  (oleic acid 0.6 g/l, bovine albumin 50 g/l, dextrose 20 g/l and catalase 0.03 g/l)
- BD BBL ™ MGIT ™ MGIT tube with 7 ml of medium
  quantity needed for the realization of the ST scheduled for that day

Procedure

Work in a sterility cabin with work surfaces disinfected with 70° alcohol, taking very strict care of the asepsis conditions.
Dilutions of antibiotic stock solutions

- Dilute the stock solutions of the antibiotics that make up the batch of medium, on the same day it is prepared, as follows:

Dilution and addition of the antibiotic solution to the MGIT

	Concentration mother solution µg/ml	Dilutions to be made of the mother solution		Concentration of the last dilution µg/ml	Volume to be added of the last dilution for each tube 7 ml of MGIT + 0,8 ml of suplement OADC ml	Final concentration of the antibiotic in the medium (*) µg/ml
Kanamycin	10,000	one 1:10	one 1:4,8	208	0.1	2.5
Amikacin	10,000	two serials 1:10	one 1:1,2	83	0.1	1.0
Capreomycin	10,000	one 1:10	one 1:4,8	208	0.1	2.5
Levofloxacin	10,000	two serials 1:10	one 1:1,2	83	0.1	1.0
Moxifloxacin	10,000	two serials 1:10	one 1:4,8	21	0.1	0.25
Gatifloxacin	10,000	two serials 1:10	one 1:4,8	21	0.1	0.25
Ethionamide	10,000	one 1:10	one 1:2,4	417	0.1	5.0
Protionamide	10,000	one 1:10	one 1:4,8	208	0.1	2.5
Linezolid	10,000	two serials 1:10	one 1:1,2	83	0.1	1.0
Clofazimine	10,000	two serials 1:10	one 1:1,2	83	0.1	1.0
Bedaquiline	10,000	two serials 1:10	one 1:1,2	83	0.1	1.0
Delamanid	10,000	three serials 1:10	one 1:2	5	0.1	0.06

(*) considering that 0,5 ml of inoculum should be added

- Shake in vortex all the mother solutions of the antibiotics that make up the batch of medium to be prepared
- Place in a rack the drugs that are going to be diluted in water and after each place the 9 ml tube or tubes that are required (n) to perform the 1:10 dilutions, and an additional tube to perform final dilution
- Place in another rack the drugs that are going to be diluted in dimethylsulfoxide and then place the 5 ml labeled tube (s) that is required to perform the 1:10 dilutions, and an additional tube to perform final dilution
- Label each tube ordered in series with the name of the antibiotic and dilution it will contain. Mark the last dilution as “final”

K, C

- To perform the 1:10 dilution, take 1 ml of the mother solution by a pipette or micropipette and transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:10. Discard the pipette or tip used
- Shake with vortex
- Transfer 1 ml of the 1:10 dilution of the antibiotic to the empty tube labeled “1:48 final”
- Add 3.8 ml of sterile distilled water to this last tube.
- Shake with vortex

A, Lvx, linezolid

- To perform the first 1:10 dilution, take 1 ml of the mother solution by a pipette or micropipette and transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:10. Discard the pipette or tip used
- Shake with vortex
- Perform the second 1:10 dilution, taking 1 ml of the 1:10 dilution by a pipette or micropipette, transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:100. Discard the pipette or tip used
- Shake with vortex
- Perform the third dilution, taking 1 ml of the dilution 1:100 by a pipette o micropipette and transfer it to the adjacent empty tube labeled “1:120” final “Discard the pipette or tip used
- Add 0.2 ml sterile distilled water to the tube labeled “final”
- Shake with vortex

Mfx

- To perform the first 1:10 dilution, take 1 ml of the mother solution by a pipette or micropipette and transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:10. Discard the pipette or tip used
- Shake with vortex
- Perform the second 1:10 dilution, taking 1 ml of the 1:10 dilution by a pipette or micropipette, transfer it to the adjacent tube containing 9 ml of distilled water labeled 1: 100. Discard the pipette or tip used
- Perform the last dilution, taking 1 ml of the 1:100 dilution by a pipette o micropipette and transfer it to the adjacent tube, empty labeled “1:480 final”. Discard the pipette or tip used
- Add 3.8 ml sterile distilled water to the tube labeled “final”
- Shake with vortex

Ethionamide

- To perform the 1:10 dilution, take 1 ml of the mother solution by a pipette or micropipette and transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:10. Discard the pipette or tip used
- Shake with vortex
- Transfer 1 ml of the 1:10 dilution of the antibiotic to the empty tube labeled "1:24 final"
- Add 1.4 ml sterile distilled water to this last tube
- Shake with vortex

Protionamide

- To perform the 1:10 dilution, take 1 ml of the mother solution by a pipette or micropipette and transfer it to the adjacent tube containing 9 ml of distilled water labeled 1:10. Discard the pipette or tip used
- Shake with vortex
- Transfer 1 ml of the 1:10 dilution of the antibiotic to the empty tube labeled "1:48 final"
- Add 3.8 ml of sterile distilled water to this last tube
- Shake with vortex

Bedaquiline

- To perform the first 1:10 dilution, take 100 μl of the mother solution by a micropipette and transfer it to the adjacent tube containing 900 μl of dimethyl-sulfoxide labeled 1:10. Discard the tip used
- Shake with vortex
- Perform the second 1:10 dilution taking 100 μl of the 1:10 dilution by pipette or micropipette and transfer it to the adjacent tube containing 900 μl of dimethylsulfoxide labeled 1:100. Discard the tip used
- Perform the last dilution taking 500 μl of the dilution 1:100 by pipette or micropipette and transfer it to the adjacent tube empty and labeled "1:120 final" Discard the pipette or tip used
- Add 100 μl of dimethylsulfoxide to the tube labeled "final"
- Shake with vortex

Delamanid

- To perform the first 1:10 dilution, take 100 μl of the stock solution by a micropipette and transfer it to the adjacent tube containing 900 μl of dimethyl-sulfoxide labeled 1:10. Discard the tip used
- Shake with vortex
- Perform the second 1:10 dilution, taking 100 μl of the 1:10 dilution by a pipette or micropipette and transfer it to the adjacent tube containing 900 μl of dimethylsulfoxide labeled 1:100. Discard the tip used
- Perform the third 1:10 dilution, taking 100 μl of the 1:100 dilution by a pipette or micropipette and transfer it to the adjacent tube containing 900 μl of dimethylsulfoxide labeled 1:1000. Discard the tip used
- Perform the last dilution, taking 500 μl of the dilution 1:100 by a pipette or micropipette and transfer it to the adjacent tube empty and labeled "1:2000 final" Discard the pipette or tip used
- Add 500 μl of dimethylsulfoxide to the tube labeled "final"
- Shake with vortex

Note: in laboratories with a very high workload, the indicated

quantities must be multiplied to make the final dilution by a factor, in order to obtain the volume needed to prepare all the MGIT tubes with drugs that must be prepared in each working day.

MGIT with drugs

- Order in racks and label the number of tubes needed for scheduled ST for the day, providing a first tube "control" (without drugs) and then one with the label of each drug to be tested
- Add 0.8 ml of OADC in each MGIT tube by a micropipette.
- Shake with vortex the "final" dilution of the first drug and add 100 µl of that solution to each tube labeled with the name of that drug by a micropipe-tte
- Proceed in the same way with each of the drugs to be tested
- Shake with vortex all MGIT tubes prepared for ST
- Discard the remaining aliquots of mother solutions or thawed stock and their dilutions.

MGIT with antibiotics for visual reading with UV lamp

Equipment

- Sterility cabin,
- Vortex type agitator,
- Micropipettes for dispensing from 100 to 1000 µl,
- Propipett or automatic pipettor.

Materials

- Latex or vinyl gloves,
- Bottle for tip discarding,
- Absorbent paper and alcohol 70 ° to disinfect the cab,
- 1 ml pipettes,
- Tubes with 9.0 ml of sterile distilled water exactly measured,
  in quantities necessary for all 1:10 dilutions to be performed
  labeled with the name of the antibiotic and the dilutions they contain
  Label for I: 1:10, 1:100 and 1:1000
  Label for R: 1:10, 1: 100
- Two sterile 10 ml tubes,
  labeled with the name of the antibiotic and the last dilution that corresponds to it, to mark as "final"
  Label for I: "1:2000 final"
  Label for R: "1:200 final"
- Sterile distilled water,
- Micropipettor tips,
- Clean and disinfected polypropylene racks.

Reagents, culture medium and supplement

- An aliquot of stock solution of I 10,000 µg/ml,
- One aliquot of stock solution of R 10,000 µg/ml),
  See above in that Annex under the title "Stock solution of antibiotics and selective inhibitors" how to prepare these solutions
  If a frozen aliquot is used, remove from the cold in advance, so that it takes room temperature before being used
- OADC supplement for sensitivity test
  (oleic acid 0.6 g/l, bovine albumin 50 g/l, dextrose 20 g/l and catalase 0.03 g/l)
- MGIT ™ BD BBL tube with 4 ml of medium,
  quantity needed for the realization of the PS scheduled for that day

Procedure

Work in sterility booth with work surfaces disinfected with alcohol 70°, taking very strictly aseptic conditions.

Dilutions of antibiotic mother solutions

- Dilutions of stock solutions of the antibiotics that make up the batch of medium, on the same day it is prepared, as indicated below.

Dilution and addition of antibiotic solution to MGIT for visual reading

	Concentration solution mother (ug / ml)	Dilutions to be made of the mother solution		Concentration of the final dilution (µg / ml)	Volume of final dilution in each tube with 4 ml of MGIT + 0.5 ml of OADC (ml)	Final concentration of the antibiotic in the medium(µg / ml)
isoniazid	10.000	three serial 1:10	one 1:2	5	0,1	0.1
rifampicin	10.000	two serial 1:10	one 1:2	50	0,1	1.0

- Locate the stock solutions of the antibiotics in a rack and after each, locate the labeled 9 ml tubes required to make the 1:10 dilutions, and at the end an empty tube to make the final dilution,

I 5 µg/ml

- Shake with Vortex the solution mother of I,
- To make the first dilution 1:10, pipette or micropipette 1 ml of the stock solution and transfer it to the adjacent tube containing 9 ml of water labeled 1:10. Discard the pipette or tip used,
- Shake with vortex,
- Carry out the second dilution taking with pipette or micropipette 1 ml of the 1:10 dilution and transfer it to the contiguous tube labeled "1:100". Discard the pipette or tip used,
- Shake with vortex,
- Perform the third dilution taking with pipette micropipette 1 ml of the 1:100 dilution, transfer to the adjacent tube with 9 ml of water, labeled "1:1000". Discard the pipette or tip used,
- Shake with vortex,
- With micropipette or pipette, transfer 1 ml of 1:1000 dilution to the last empty tube, labeled "I 1:2000 final". Discard the pipette or tip used,
- Add to the last tube 1 ml of sterile distilled water,
- Shake with vortex.

R 50 µg/ml

- Shake with Vortex the solution mother of R.
- To make the first dilution 1:10, pipette or micropipette 1 ml of the stock solution and transfer it to the adjacent tube containing 9 ml of distilled water labeled "1:10". Discard the pipette or tip used,
- Shake with vortex,
- Pipette or micropipette 1 ml of the 1:10 dilution into the contiguous tube containing 9 ml of distilled water labeled "1:100". Discard the pipette or tip used,
- Shake with vortex,
- With a micropipettor or pipette, transfer 1 ml of the 1:100 dilution to the last empty tube labeled "1: 200 final". Discard the pipette or tip used,
- Add to the last tube 1 ml of sterile distilled water,
- Shake with vortex.

MGIT with I and R

- Sort in racks and label the number of tubes needed for the ST programmed for the day, having a first "control" tube (without drug) and then one with the label "I" and another with the label "R"
- With micropipette add 0.5 ml of OADC in each MGIT tube,
- Vortex the final "I" dilution with micropipette, add 100 µl of this solution to each tube labeled "I"
- Vortex the "final" dilution of R and with micropipette, add 100 µl of that solution to each tube labeled "R",
- Vortex all MGIT tubes prepared for the ST.

Registration Templates

Registry ... preparation of stock solutions of antibiotics

Date
Operator

							Diluent aggregates				Frozen Aliquots			
Antibiotic	Brand	Batch N°	Potency (u/mg)	Weighted Quantity (mg)	Concentration solution stock (u / ml)	Total volume of diluent calculated (*) (ml)	DMS	NaOH 0,1N (ml)	Distilled water (ml)	Total volume of diluents (ml)	Batch N°	Volume of each aliquot (ml)	Number of aliquots	Observations (**)

DMS:
Dimethylsulfoxide
(**) Anomalies and
consequent actions

Registy .... .LJ with antibiotics

Date
Operator

Batch of medium N ° | **Saline solution N °** | **Eggs reception date** | **Malachite Green Batch N °**

	Volume of LJ (ml)	Aggregate antibiotic solution			Coagulation		Observations (**)	QA				Use	
		Mother Batch N °	Dilution (*)	Volume (ml)	Temperature (°C)	Time / minutes		Date	Register N°	Approved Yes / No	Observations	Start date	End date
I													
R													
E													
S													
K													
A													
C													
Without antibiotic													

(*) SM (Mother Solution) or 1:10 or 1: 100
(**) Anomalies and consequent actions

Registry .... Middlebrook 7H10 with antibiotics

Date
Operator

Batch of medium N ° **Glycerol Batch N °** **Number of plates**

	Volume of 7H10 (ml)	Aggregate antibiotic solution			Observations (**)	Quality Assurance				Use	
		mother batch N °	Dilution (*)	Volume (ml)		Date	Register N°	Approved Yes / No	Observations (**)	Start date	End date
I											
R											
E											
S											
K											
A											
C											
Lvx											
Mxf											
Without antibiotic											

(*) SM (Mother Solution) or 1:10 or 1: 100
(**) Anomalies and consequent actions

Registry .... Reception and use of MGIT system inputs

Reagent	Reception					Quality Assurance					Use	
	Date	Quantity	Batch	Observations (*)	Responsible	Date	Register N°	Approved Yes / No	Observations (*)	Responsible	Start date	ENd Date
MGIT Tubes												
Kit IR												
Kit SIRE												

(*) Anomalies and consequent actions

Registry …………………………… MGIT preparation with antibiotics

Date
Operator

Batch MGIT N°

Batch
OADC N°

		Aggregate antibiotic solution			Amount of tubes prepared	Observations	QA				Date of use
	Stock Solution Batch N°	Dilutions carried out (if applicable)		Volume tube (ul)			Date	Register N°	Approved Yes / No	Observations (*)	
		01:10	Final								
I											
R											
E											
S											
K											
A											
C											
Lvx											
Mxf											
Without antibiotic											

(*) Anomalies and consequent actions

Registry.......................... .. Receipt and use of molecular system inputs

	Reception					Errors registered with daily checks					Use	
	Date	Quantity	Batch	Observations (*)	Responsible	Date	Register N°	Type of error	Observations (*)	Responsible	Start date	End Date
GeneXpert MTB / Rif Cartridges												
Kit LPA												

(*) Anomalies and consequent actions

ORGANISMO ANDINO DE SALUD
CONVENIO HIPOLITO UNANUE