

 OSPEDALE SAN RAFFAELE	<b>DRUG SUSCEPTIBILITY TESTING (DST) ON M.TUBERCULOSIS</b>	<b>IOS EBP-DMA 005</b>	
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**Destinatari: Coordinatore, Tecnici del Settore Micobatteri - EBP**

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0	Prima Emissione	21.09.2010
1	Inserito procedura per TB-eXiST software e cambio ragione sociale	03.08.2012

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## 1 SCOPE

This IOS describes methods for antimycobacterial drug susceptibility testing (DST). Antimicrobial susceptibility testing is critical in prescribing an effective drug regime for a tuberculosis patient. It is also important in the follow-up of patients who are on antimicrobial therapy but are not responding to therapy.

## 2 APPLICATION

Drug Susceptibility Testing is an integral part of the microbiological identification of *M. tuberculosis* resistances to drugs. It is applicable to the whole Advanced Diagnostics in mycobacteriology.

## 3 DEFINITIONS AND ABBREVIATIONS

BSC: Biological Safety Cabinet  
 CFU: Colony-Forming Unit  
 S (STR): Streptomycin  
 DST: Drug Susceptibility Testing  
 E (EMB): Ethambutol  
 GC: Growth Control  
 I (INH): Isoniazid  
 LJ: Löwenstein–Jensen  
 MIC: Minimal Inhibitory Concentration  
 R (RIF): Rifampicin  
 AMK: Amikacin  
 KAN: Kanamycin  
 CAP: Capreomicin  
 OFL: Ofloxacin  
 PZA: Pyrazinamide

## 4 RESPONSIBILITIES

The supervision and the correct application of the following instruction is a responsibility of the area coordinator. The execution of the test is responsibility of area technicians, master students and coordinator.

## 5 EQUIPMENT AND MATERIALS

- Autoclave
- BSC, class I or II, annually certified, with exhaust air ducted or vented to the outside
- Incubator
- Refrigerator
- BactTec<sup>TM</sup>MGIT<sup>TM</sup>960 System
- TB eXiST software
- Rack for glass tubes

- Sterile loop, 3 mm internal diameter, calibrated to 10 µl, and/or short, sterile graduated pipette or single-channel microlitre pipette with sterile tips, for delivery of 0.1-ml volumes
- Pipettes, graduated, for 0.1 ml, 0.2 ml, 1.0 ml and 10 ml
- Pipetting aids
- Vortex mixer
- Sterile glass beads, diameter 3 mm
- Sterile NaCl solution 0.9% or sterile water
- McFarland turbidity standard no. 1
- Separate waste containers (autoclavable) for pipettes and disposables
- Drug-containing media
- No drug-containing media
- BactTec<sup>TM</sup>MGIT<sup>TM</sup>960 SIRE Kit
- BD BactTec<sup>TM</sup>MGIT<sup>TM</sup>960 SIRE Supplement
- BactTec<sup>TM</sup>MGIT<sup>TM</sup>960 PZA Kit
- BactTec<sup>TM</sup>MGIT<sup>TM</sup>960 PZA Supplement
- BBL<sup>TM</sup>MGIT<sup>TM</sup> 7 ml Mycobacteria Growth Indicator Tube.
- Drug Stock solutions (Ref: IOS DMA 006)

## 6 PROCEDURES

### 6.1 Proportional Method on Loewenstein-Jensen

The proportion method (Canetti et al., modified) determines the percentage of growth (number of colonies) of a defined inoculum on drug-free control media versus growth on culture media containing the critical concentration of an anti-TB drug.

#### 6.1.1 Samples and Internal Quality Control

A pure culture of tubercle bacilli (test strain) in the active phase of growth (2–4 weeks) is the ideal sample to test; in the absence of sufficient growth (<50 colonies), DST cannot be safely interpreted. A culture of *M. tuberculosis* strain H37Rv, freshly subcultured, as control strain is needed for testing each batch of new reconstituted drugs.

#### 6.1.2 Reagents

The critical drug concentration, as well as the critical proportion of resistant colonies, has been evaluated from clinical data. While Canetti et al. originally based their method, for first-line drugs, on three concentrations per drug, they later proposed more economical versions based on only one or two concentrations. Today, a single concentration is most commonly used.

Drug	Concentration (µg/ml)
Streptomycin	4.0
Isoniazide	0.2
Rifampicin	40.0
Ethambutol	2.0
Amikacin	40.0
Kanamycin	30.0
Ofloxacin	2.0
Capreomycin	40.0

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### 6.1.3 Instructions

- 1) Put 6-7 sterile beads in a 50 ml conical tube
- 2) Add 5 ml of sterile water
- 3) Take young colonies (2-3 weeks) from a LJ positive culture and make a suspension of a 1 McF
- 4) Shake the suspension 20-30 minutes
- 5) In the meantime, prepare all vials necessary for dilutions. For each DST prepare:
  - a. 1 tube with 9 ml sterile water (A)
  - b. 1 tube with 4.5 ml of sterile water (B)
  - c. 1 tube with 4.5 ml of sterile water (C)
  - d. 1 tube with 19.8 ml of sterile water (D)
- 6) Take the bacterial suspensions from the shaker and let them stand 20-30 minutes to sediment
- 7) If necessary, adjust the suspension turbidity to 1 McF
- 8) Add 1 ml of the bacterial suspension to the tube A and resuspend well. (Suspension 1:10)
- 9) Add 0.5 ml of the suspension A to the tube B and resuspend well. (Suspension 1:100)
- 10) Add 0.5 ml of the suspension B to the tube C and resuspend well (Suspension 1:1000;  $10^{-3}$ )
- 11) From suspension C ( $10^{-3}$ ), inoculate 200  $\mu$ l on LJ without drug (GC  $10^{-3}$ ) and on LJ with drug
- 12) From the suspension  $10^{-3}$ , take 0.2 ml and add it to tube D in order to obtain a suspension at  $10^{-5}$
- 13) Inoculate 200  $\mu$ l of the suspension  $10^{-5}$  on the second growth control tube (GC  $10^{-5}$ )
- 14) Leave slightly unscrewed the caps of the tubes and put them in the incubator in a slant position.
- 15) Incubate at  $36 \pm 1$  °C
- 16) After 48-72 hours close the caps and let them in slant position till the end of the first week of incubation. After that, it's possible to let them upright.

### 6.1.4 Checking of LJ Media

Each DST consists of 6 tubes when performing first line drugs only (GC $10^{-3}$ ;GC $10^{-5}$ ; STR; INH; RIF; EMB), 6 tube when performing second line drugs alone (GC $10^{-3}$ ;GC $10^{-5}$ ; AMK; KAN; OFL or LEV;CAP),10 tubes when performed at the same time first and second line drugs (GC $10^{-3}$ ;GC $10^{-5}$ ; STR; INH; RIF; EMB; AMK; KAN; OFL or LEV; CAP) by using the same controls. Upon request other drugs may be tested: D-cycloserine, Ethionamide, *para*-aminosalicylic acid (PAS). Pyrazinamide cannot be tested in solid LJ media. LJ slants must be checked every day for

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the whole first week in order to detect presence of contaminants and for DST interpretation after 4 and 6 weeks of incubation.

### 6.1.5 Interpretation of Results

Number of colonies on the LJ with antibiotics/ Number of colonies on the control LJ

- Critical proportion 1%
- If the value is  $\geq 1\%$  : the strain is RESISTANT to the drug tested
- If the value  $< 1\%$  : the strain is SUSCEPTIBLE to the drug tested

## 6.2 BacTEC™ MGIT™ 960 System

The BACTEC MGIT 960 System is a 4-13 day qualitative test. The test is based on growth of the *M. tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (Growth Control). The BBL™ MGIT™ 7 ml Mycobacteria Growth Indicator Tube is a tube containing a modified Middlebrook 7H9 Broth which supports the growth and detection of mycobacteria. The MGIT tube contains a fluorescent compound embedded in silicone on the bottom of 16 x 100 mm round bottom tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. The initial concentration of dissolved oxygen quenches the emission from the compound, and the little fluorescence can be detected. Later, actively growing and respiring microorganisms consume the oxygen that allows the compound to fluoresce. The BACTEC MGIT 960 instrument continually monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the Growth Control tube is used by the instrument to determine susceptibility results. The BACTEC MGIT 960 instrument automatically interprets these results, and reports a susceptible or resistant result.

### 6.2.1 Reagents (see IOS EBP-DMA 006)

### 6.2.2 Instructions

- Preparation of the inoculum from Solid Media

1. Add 4 ml of Middlebrook 7H9 Broth to a 16,5 x 128 mm sterile tube with cap containing 8-10 glass beads.
2. Scrape with a sterile loop as many colonies as possible from the solid media.
3. Vortex the suspension for 2-3 min to break up the larger clumps. The suspension should exceed a 1.0 McFarland standard in turbidity.
4. Let the suspension sit for 20 min without disturbing.
5. Transfer the supernatant fluid to a second 16.5 x 128 mm sterile tube with cap. NOTE: The organism suspension should be greater than a 0.5 McFarland Standard at this step.
6. Adjust the suspension to a 0.5 McFarland turbidity standard. Do not adjust below a 0.5 McFarland Standard.
7. Dilute 1 ml of the adjusted suspension in 4 ml of sterile saline (1:5 dilution). Use this tube for inoculating drug-containing tubes. Further dilution 1:100 in sterile water is necessary to inoculate the Growth Control tube.

- Preparation of the inoculum from a Positive BACTEC MGIT 7 ml tube

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1. The day a MGIT tube is positive by the instrument is considered **Day 0**.
2. The tube should be kept incubated for at least one more day (**Day 1**) before being used for the susceptibility testing (may be incubated in a separate incubator at 36°C + 1°C).
3. A positive tube may be used for drug susceptibility testing up to and including the fifth day (**Day 5**) after it becomes instrument positive. A tube that has been positive for more than 5 days should be subcultured in a fresh MGIT tube supplemented with 0.5 ml MGIT 960 Growth Supplement and should be tested in a MGIT 960 instrument until it is positive.
4. If growth in a tube is on **Day 1** or **Day 2**, mix well (vortex) to break up clumps. Leave the tube undisturbed for about 5-10 minutes to let big clumps settle on the bottom. Use the supernatant undiluted for inoculation of the drug set.
5. If growth is on **Day 3, 4, or 5**, mix well to break up the clumps. Let the large clumps settle for 5-10 minutes and then dilute 1.0 ml of the positive broth with 4.0 ml of sterile saline. This will be a 1:5 dilution. Use this well mixed diluted culture for inoculation.

- Inoculation and incubation

1. Label an appropriate number of MGIT tubes for each test culture. Label one for GC (growth control, without drug), and one for each drug.
2. Aseptically add 0.8 ml of BACTEC 960 SIRE Supplement to each of the MGIT tubes.
3. Aseptically add 0.1 ml of properly reconstituted drug in the labeled tube. Similarly, add other drugs in the other labeled tubes. It is important to add the correct amount of drug to each tube. If possible, use a well calibrated micropipette for each addition. Use a separate pipette or micropipette tip for each drug. Do not add any drug to the GC tube.
4. Aseptically add 0.5 ml of the well-mixed culture suspension (inoculum) into each of the drug containing tubes using a pipette. Do not add to the control.
5. For the control, first dilute the test culture suspension 1:100 by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline or sterile deionized water. Mix well by inverting the tube 5-6 times. Use this diluted suspension to add 0.5 ml into the growth control tube.
6. Tighten the caps and mix the inoculated broth well by gently inverting the tube several times.
7. Susceptibility test "Set Carriers" are provided in different numbers of drug combinations. Place labeled tubes in the correct sequence in the set carrier (GC, STR, INH, RIF, EMB).
8. Enter the susceptibility set carrier into the BACTEC MGIT 960 instrument using the susceptibility test set entry feature. Ensure that the order of the tubes in the AST Set Carrier conforms to Set Carrier definitions. For example, GC, STR, INH, RIF, EMB for the SIRE standard testing.
9. In case of second line drugs testing using the Set Carrier, marked tubes may be put in any order, as long as they are correctly labeled on the tube. When printing the MGIT Report be aware that:
  - on the report the drugs are indicated as STR,INH, RIF, EMB, even though they do

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not match with the actual drugs. Erase the name of the drugs printed and replace them with the actual name of the drug corresponding to the position. The interpretation does not change.

### 6.2.3 BD EpiCenter™ TB-eXiST (Extended Individual Susceptibility Testing)

The software TB-eXiST allow:

- 1) First and second line drug testing and screening of drugs under development even at more than one concentrations
- 2) DST of *M. tuberculosis complex* and NTM, including :
  - o Breakpoint testing
  - o Extended test protocol length
  - o Visualization of growth curves per drug or per specimen
- 3) Long term storage, statistical analysis and LIS connection

#### • TB eXiST Login Workflow Description

- 1) In the Rapid Login screen, click the specimen registration icon, enter the Patient Name (or sample ID) and the Accession number (can be equal to Patient Name).
- 2) In the Available Tests frame, order the Growth Control, scan the tube sequence number, select the isolate number if available and click OK. (Only one control can be associated to an Accession number for TBeXiST tests).
- 3) Repeat step 2 ordering the drugs at the concentrations to be included in the DST.
- 4) Save the test order; this automatically prints the barcode for the TB eXiST DST.
- 5) Verify that all requested TBeX tests are associated with a tube sequence number.
- 6) Affix the barcode label onto the corresponding tube at the same height as the sequence number barcode (do not place across the sequence number barcode).
- 7) Fill in the form: EBP-DMA 005 TB eXist Inserimento Campioni Rev 1
- 8) As all tubes belonging to the same TB eXiST set must have the same start-of-test date and time (=first reading), they must all be loaded simultaneously in the same drawer of the MGIT™ 960 instrument.

- a) Open a drawer and press the “Tube Entry” soft key
- b) Scan the tube sequence number and the Accession number (barcode label)
- c) Insert the tube in the station with the green indicator
- d) Repeat instrument steps b and c without closing the drawer for all remaining tubes of the same DST.



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An Accession number associated to TB eXiST tests can not be modified at any time. Once inserted into the instrument, a tube can not be moved to another position in the same drawer or another drawer. BACTEC® MGIT™ 960 automatically sends the loaded test information to EpiCenter™. It is strongly recommended not to open any drawer during the reading window [hour - 2 minutes (:58) till hour + 5 minutes (:05)] so no tube readings are missed.

- TB eXiST Test follow-up and result interpretation

Accessed in Report-Report Filtro, via: TBeXiST Foglio di lavoro click “Anteprima di stampa” and enter the Patient Name (or sample ID) (The selection criteria available for the Worklist Report are: Beginning of Date & Time range; End of Date & Time Range, Patient ID or Name; Accession Number; Antimicrobial (Name); Organism (Name); Minimum and/or Maximum number of days in protocol; Minimum and/or Maximum GU value for the Growth Control)

According to BD’s recommendations for SIRE AST in MGIT™ 960, R/S interpretation should be done at the moment the Growth Control first passes the GU = 400 threshold. This does not necessarily coincide with the moment the TB eXiST worklist is ordered.

Once the Growth Control has reached GU ≥ 400, “Threshold 400 Reached” must be selected for the TBeXiST Growth Control Virtual Test of the isolate under consideration.

When the growth unit (GU) in growth control reaches 400, the GU values of the drug vial should be evaluated:

**S** = Susceptible – the GU of the drug tube is less than 100.

**R** = Resistant – the GU of the drug tube is 100 or more.

**I** = Intermediate (so far not used to interpret drugs resistance patterns)

When antimicrobial/concentration tests are interpreted, Test Status “Complete-S”, “Complete-I” or “Complete-R” must be selected for the TBeXiST Antimicrobial/Concentration under consideration. This action includes the result in the TB eXiST Interpretation Report. Once interpreted, the test should be finalized by on each test screen and clicking “Finalized”. By that, the test is considered closed by the system.

The TB eXiST software merely reports the S / I / R interpretation made by the User and makes no claims as to the use of these interpretations in a clinical context.

- Removal of completed TB eXiST tubes from the instrument

Remove TB eXiST - tubes from the instrument, either as:



Positive (e.g. Growth Control, resistant antimicrobial/concentration tubes and other extended cases),

or



Ongoing (e.g. sensitive antimicrobial/concentration tubes and other extended cases).

### 6.3 Pyrazinamide (PZA) Susceptibility Testing

The BACTEC MGIT 960 PZA susceptibility test is a qualitative procedure to test susceptibility of *M. tuberculosis* complex against PZA. Results are obtained within 4 to 21 days. The MGIT 960 medium is a modified 7H9 broth with a reduced pH of 5.9. The detection of growth is achieved by the oxygen sensor at the bottom of the tube in the same way as the one in the regular MGIT tube, UQ 002/4



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and the principle of the detection of resistance is the same as for SIRE drug susceptibility testing.

- Preparation of the inoculums

**Day 0** – the day a MGIT tube is positive by the instrument. Re-incubate.

**Day 1 or 2** – one or two days after instrument positive. Use undiluted for the susceptibility testing inoculation.

**Day 3, 4 or 5** – mix well and dilute 1:5 by adding 1.0 ml of positive broth in 4.0 ml of sterile saline. Mix well. Use this for the susceptibility testing inoculation.

**Day 6** and onward – subculture in a fresh MGIT tube.

- Preparation from growth on a solid medium

Follow the same procedure as described for the SIRE testing.

For susceptibility test inoculation, dilute this suspension 1:5 by adding 1.0 ml of the suspension to 4.0 ml of sterile saline or sterile deionized water. Use this diluted suspension for setting up the susceptibility.

1. Label two MGIT PZA tubes, one as GC (growth control) and one as PZA (drug containing). Using a pipette, aseptically add 0.8 ml of PZA supplement to each of the two tubes.

2. Aseptically add 0.1 ml (100 µL) of the reconstituted drug into the PZA tube. If possible, use a micropipette. Try to be as accurate as possible in adding the drug. This will give you 100 µg PZA per ml of the medium. Do not add drug to the GC tube.

3. Inoculate 0.5 ml of the culture suspension to the PZA tube using a sterile pipette.

4. For growth control inoculation, first dilute the inoculum 1:10 by adding 0.5 ml of the culture suspension (the one used for the drug tube) to 4.5 ml of sterile saline. Mix well by tightening the cap and inverting at least 5-6 times. Add 0.5 ml of this diluted suspension into the tube labeled GC.

**Note:** For PZA susceptibility test, the inoculum for the control is diluted 1:10 and not 1:100 as in SIRE AST).

5. Tighten the caps and gently invert both the MGIT tubes several times to mix.

6. Place them in a two AST Set Carriers with the sequence of first GC and the PZA.

7. Enter the PZA set into the instrument using AST set entry feature. Make sure the GC is placed first, and PZA second, in the AST Set Carrier. Select PZA as the drug in 2nd tube AST set carrier definition when performing the AST set entry.

## **7 RECORDING AND REPORTING**

- **Proportion method**

Slants should be read after 4 weeks of incubation for as a provisional result and after 6 weeks of incubation for definitive interpretation. If there is no growth on drug-free media after 6 weeks, the test cannot be interpreted and should be repeated.

For all four drugs INH, RMP, STR and EMB the critical proportion is 1%. For test interpretation refer to the Paragraph 6.1.5

The first reading of drug susceptibility test results is done after 4 weeks of incubation. At that time

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all strains showing drug resistance can be reported as drug-resistant. Because some (especially multidrug-resistant) strains grow very slowly, a further 2 weeks of incubation are needed before reporting susceptibility.

“Borderline cases” (with about 1% growth on drug-containing medium) should be reported as resistant (under reservation) and retested. Every new batch of drug-containing media prepared for DST must be quality-controlled. For each drug, a slant of the critical concentration is tested as well as the specially prepared media with lower drug concentrations. A suspension of a freshly subcultured *M. tuberculosis* H37Rv strain is used.

#### • **BacTEC™ MGIT™ 960 System**

The instrument monitors the entered susceptibility test set. Once the test is complete (within 4 to 21 days), the instrument will indicate that the results are ready. Scan the susceptibility Set Carrier and print the report. The instrument printout indicates susceptibility results for each drug. Results are qualitative: Susceptible (**S**), Resistant (**R**) or indeterminate (**X**).

The instrument interprets results at the time when the growth unit (GU) in growth control reaches 400 (within 4-13 days). At this point, the GU values of the drug vial are evaluated.

**S** = Susceptible – the GU of the drug tube is less than 100.

**R** = Resistant – the GU of the drug tube is 100 or more.

**X** = Error – indeterminate results when certain conditions occur which may affect the test, such as GU of the control reaches >400 in less than 4 days. In such situations, the test should be repeated with pure, actively growing culture which is confirmed to be *M.tuberculosis complex*.

#### • **BD EpiCenter™ TB-eXiST**

See: TB eXiST Test follow-up and result interpretation

#### • **Recording**

Results have to be reported into the specific databases of each Country or, in case of DST as part of external services for other Italian institutions, they have to be reported into the specific laboratory register and database.

Paper copies of the reports have to be archived: BacTEC™ MGIT™ 960 Reports have to be stored into the “MGIT” folder and Paper reports of Italian outpatients have to be archived into the “Referti” folder.

One Report .pdf copy has to be stored electronically into the specific folder and faxed (after phone contacts) to the recipient in charge of the patients. Once the Report is finished, the database “Pazienti Esterni” has to be completed.

## **8 RELATED DOCUMENTS**

1. Isenberg HD (Editor). Clinical Microbiology Procedure Handbook, Vol.1. American Society for Microbiology (Publisher), Washington, D.C.
2. Kent PT, Kubica GP. Public Health Microbiology, a Guide for the Level III Laboratory. Centers for Disease Control, Division of Laboratory Training and Consultation. Atlanta, GA, US Department of Health and Human Services, US Government Printing Office, 1985.
3. Middlebrook O, Cohn ALL. Bacteriology of tuberculosis: Laboratory Methods. Am J Pub Health. 1958;48:844-853.
4. Roberts GD, Goodman NL, Heifets L, et al. Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acidfast smear- positive specimens. J Clin Microbiol. 1983;18:689-696.

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5. Centers for Disease Control and Prevention. National Institutes of Health. Biosafety in microbiological and biomedical laboratories. HHS Publication No. (CDC) 99-8395. US Government Printing Office. Washington, 1999. Also available at [www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm](http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm).

6. TDR TB Website : <http://www.who.int/tdr/diseases/tb/default.htm>

7. CLSI Website : <http://www.nccls.org/>