

 OSPEDALE SAN RAFFAELE	PREPARATION OF DRUGS FOR DST TESTING	IOS EBP-DMA 006	
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Destinatari: Coordinatore, Tecnici del Settore Micobatteri - EBP

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Rev.	Descrizione modifiche	Data
0	Prima emissione	01/10/2010
1	Cambio ragione sociale	07/01/2013

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1. SCOPE

The present instruction describes procedures to reconstitute and to obtain antimicrobials stock solutions to be used for antimicrobials susceptibility testing.

2. APPLICATION

The present instruction is applicable within the Advanced Mycobacterial Diagnosis (DMA) area. It is directly linked to IOS EBP-DMA 005 for drug susceptibility testing (DST) on first and second line antitubercle drugs.

3. DEFINITIONS AND ABBREVIATIONS

MGIT	Mycobacterial Growth Indicator Tube
DST	Drug Susceptibility Testing
AST	Antimicrobial Susceptibility Testing
STR	Streptomycin
EMB	Ethambutol
INH	Isoniazide
RIF	Rifampicin
SIRE	Streptomycin, Isoniazide, Rifampicin, Ethambutol
OFL	Ofloxacin
MOX	Moxifloxacin
ETH	Ethionamide
KAN	Kanamycin
AMK	Amikacin
LZD	Linezolid
RT	Room Temperature
PZA	Pyrazinamide

4. RESPONSIBILITIES

The supervision and the correct application of the following instruction is a responsibility of the area coordinator. Preparation of drug stock solutions is responsibility of area technicians and coordinator.

5. EQUIPMENT AND MATERIALS

BactTec™MGIT™960 SIRE Kit (**Cat. N. 245123**) contains one each lyophilized vials of streptomycin, isoniazid, rifampin and ethambutol and 8 vials of SIRE supplement:

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

BD BactTec™MGIT™960 SIRE Supplement (**Cat. N. 245123**) contains 20 ml Middlebrook OADC enrichment. Approximate formula per liter of purified water:

- Bovine Albumin 50.0 g
- Dextrose 20.0 g

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- Catalase 0.03 g
- Oleic Acid 0.6 g

BactTec™MGIT™960 PZA Kit (**Cat. N. 245128**) contains two lyophilized vials of pyrazinamide and six vials of PZA supplement.

- Pyrazinamide 20000.0 µg

BactTec™MGIT™960 PZA Supplement (**Cat. N. 245128**) contains 15 ml of enrichment. Approximate formula per liter of purified water:

- Bovine Albumin 50.0 g
- Dextrose 20.0 g
- Catalase 0.03 g
- Oleic Acid 0.1 g
- Polyoxyethylene stearate (POES) 1.1 g

All these reagents must be used with BBL™MGIT™ 7 ml Mycobacteria Growth Indicator Tube.

Pure powders:

Drug	Description and Ingredients	Cat. N.	Storage	Manufacturer
Capreomycin	Capreomycin sulphate. 845 µg/mg	C4142	- 20 °C	Sigma-Aldrich
Ethionamide		E6005	2-8°C	Sigma-Aldrich
Ofloxacin		O8757	2-8°C	Sigma-Aldrich
Levofloxacin	> 98% HPLC	28266	2-8°C	Sigma-Aldrich
Amikacin	Amikacin disulfate salt 710 µg/mg	A1774	2-8°C	Sigma-Aldrich
Kanamycin	Kanamycin sulphate from <i>Streptomyces</i> <i>kanamyceticus</i> , animal component free, < 5% Kanamycin B. 770 µg/mg	K4000	RT	Sigma-Aldrich
Linezolid	Pure substance ca. 100%	165800-03-3	RT	Pfizer
Moxifloxacin	Pure substance ca. 100%	186826-86-8	RT	Bayer HealthCare

Other material:

- Sterile water
- 0.20 µm Polyethersulfone membranes
- 10 ml syringes
- 10-ml Falcon tubes
- Pipettor; 1ml/5ml/10ml pipettes
- 1 ml Eppendorf tubes
- sterile NaOH 0.1 N solution
- Ethylene glycol
- Analytic scales
- Blood or Chocolate Agar

6. PROCEDURES

6.1 Reconstitution of lyophilised first line drugs

Reconstitute each BactTec™MGIT™960 SIRE Kit drug vial with **4 ml** of sterile distilled/deionized water in order to make the desired stock solution (Table 1)

Reconstitute each BactTec™MGIT™960 PZA Kit drug vial with **2.5 ml** of sterile distilled/deionized water in order to make the desired stock solution (Table 1)

Table 1: Reconstitution volumes for first-line drugs

Drug lyophilised	Volume added	[C] of drug after reconstitution µg/ml	Volume added to MGIT tubes for DST (µl)	Final Concentration in MGIT Tubes
STR	4 ml	83	100	1.0 µg/ml
INH	4 ml	8.3	100	0.1 µg/ml
RIF	4 ml	83	100	1.0 µg/ml
EMB	4 ml	415	100	5.0 µg/ml
PZA	2.5 ml	8000	100	100 µg/ml

Once reconstituted each drug stock solutions should be split in several vials following these criteria:

- 420 µl-aliquot for 4 DSTs per time
- 1333 µl-aliquot for 13 DSTs per time

All aliquots should be frozen and stored at -80°C for up to six months, not to exceed the original expiration date. Once thawed the drug solutions should be used immediately.

6.2 Concentrations of stock solutions

Table 2: Concentrations of stock solutions

Drug	Concentration µg/ml	Solvent	Diluents	Concentration tested in MGIT tubes µg/ml
Amikacin	84.0	Sterile water	Sterile water	1.0
Kanamycin	420.0	Sterile water	Sterile water	5.0
Capreomycin	210.0	Sterile water	Sterile water	2.5
Ofloxacin	168.0	NaOH 0.1 N	Sterile water	2.0
Levofloxacin	84.0	NaOH 0.1 N	Sterile water	1.0
Moxifloxacin	21.0	NaOH 0.1 N	Sterile water	0.25
Linezolid	84.0	Sterile water	Sterile water	1.0
Ethionamide	420.0	Ethylene glycol	Sterile water	5.0

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6.3 Detailed Procedure

In order to achieve the desired concentrations of stock solutions, a Correction Factor (CF) must be applied to all weighs of drug powders that are not considered "Pure". This best approaches the theoretical weighs to the "real" quantity of drug powder to be weighed

- Correction Factor: 1 mg/drug potency in μmg^{-1}
- Theoretical grams of powder x Correction Factor = "Real" grams of powder to be weighed
- AMIKACIN ($710 \mu\text{mg}^{-1}$)
 - Dissolve 0.14 g of Amikacin powder in 10 ml sterile water = 10000 $\mu\text{g}/\text{ml}$ (**Solution A**)
 - 1 ml Solution A + 9 ml sterile H_2O = 1000 $\mu\text{g}/\text{ml}$ (**Solution B**)
 - 4 ml Solution B + 1 ml sterile H_2O = 800 $\mu\text{g}/\text{ml}$ (**Solution C**)
 - 1.05 ml Solution C + 8.95 ml sterile H_2O = **84 $\mu\text{g}/\text{ml}$ (STOCK SOLUTION)**

Notes:

- Before starting , prepare 5 Falcon tubes (15-ml tubes) under a safety cabinet with the exact volumes of sterile water (10 ml, 9 ml, 1 ml, 8.95ml in this order).
- Leave the last Falcon tube empty: it will contain the filtered solution at the end of the process.
- Pour the powder in the first tube (10 ml) and then proceed with subsequent dilutions.
- The powder should be completely dissolved before going on with the second dilution.
- Before splitting stock solution, filter it through a 0.20 μm Polyethersulfone membrane.
- Split the stock solution choosing one of the abovementioned options (420 μl -aliquot or 1333 μl -aliquot)
- Discard the first 10% of the filtered solution
- Seed two or three drops on a non-selective agar medium (Blood or Chocolate) and incubate for 48-72 hours at 37°C and 5% CO_2
- 0,14 g comes from the calculation based on the potency of the drug used ($710 \mu\text{mg}^{-1}$). The theoretical value is 0,10 g

- KANAMYCIN ($770 \mu\text{mg}^{-1}$)
 - Dissolve 0,13 g of Kanamycin powder in 10 ml sterile water = 10000 $\mu\text{g}/\text{ml}$ (**Solution A**)
 - 5 ml Solution A + 5 ml sterile H_2O = 5000 $\mu\text{g}/\text{ml}$ (**Solution B**)
 - 1 ml Solution B + 9 ml sterile H_2O = 500 $\mu\text{g}/\text{ml}$ (**Solution C**)
 - 4.16 ml Solution C + 0.84 sterile H_2O = **420 $\mu\text{g}/\text{ml}$ (STOCK SOLUTION)**

Notes: see *Amikacin*

- CAPREOMYCIN ($845 \mu\text{mg}^{-1}$)
 - Dissolve 0.12 g of Capreomycin powder in 10 ml sterile water = 10000 $\mu\text{g}/\text{ml}$ (**Solution A**)
 - 1 ml Solution A + 9 ml sterile H_2O = 1000 $\mu\text{g}/\text{ml}$ (**Solution B**)
 - 2.1 ml Solution B + 7.9 sterile H_2O = **210 $\mu\text{g}/\text{ml}$ (STOCK SOLUTION)**

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Notes: see *Amikacin*

- OFLOXACIN

- Dissolve 0.10 g of Ofloxacin powder in 2.5 ml sterile NaOH 0.1 N → Shake gently
- Add sterile water up to a final volume of 10 ml = 10000 µg/ml (**Solution A**)
- 1 ml Solution A + 9 ml sterile H₂O = 1000 µg/ml (**Solution B**)
- 1.7 ml Solution B + 8.3 ml sterile H₂O = **168 µg/ml (STOCK SOLUTION)**

Notes:

- Dissolving Ofloxacin in water could be quite hard. Sodium hydroxide at low concentrations (0.1 N) is advisable. Avoid using higher NaOH concentrations because of its bactericidal effect that could influence the viability of mycobacteria tested.
- Prepare 5 Falcon tubes with the following volumes: 1.5 ml NaOH 0.1 N, 9 ml sterile H₂O, 8.3 ml sterile H₂O.
- Pour the powder in the first tube containing 1.5 ml NaOH 0.1 N and start shaking. If the powder does not dissolve entirely, add small volumes of NaOH 0.1 N (one or two drops per time) and keep on shaking gently. The maximum volume achievable with NaOH 0.1 N should be 2.5 ml in order not to carry on big volumes of NaOH in the following solutions.
- These notes are to be taken into account for other fluoroquinolones too.
- See *Amikacin* for other general notes.

- LEVOFLOXACIN (> 98% HPLC)

- Dissolve 0.10 g of Levofloxacin powder in 2.5 ml sterile NaOH 0.1 N → Shake gently
- Add sterile water up to a final volume of 10 ml = 10000 µg/ml (**Solution A**)
- 1 ml Solution A + 9 ml sterile H₂O = 1000 µg/ml (**Solution B**)
- 4 ml Solution B + 1 ml sterile H₂O = 800 µg/ml (**Solution C**)
- 1.05 ml Solution C + 8.95 sterile H₂O = **84 µg/ml (STOCK SOLUTION)**

Notes: see *Ofloxacin*

- MOXIFLOXACIN (ca. 100%)

- Dissolve 0.10 g of Moxifloxacin powder in 2.5 ml sterile NaOH 0.1 N → Shake gently
- Add sterile water up to a final volume of 10 ml = 10000 µg/ml (**Solution A**)
- 1 ml Solution A + 9 ml sterile H₂O = 1000 µg/ml (**Solution B**)
- 1 ml Solution B + 9 ml sterile H₂O = 100 µg/ml (**Solution C**)
- 2.1 ml Solution C + 7.9 sterile H₂O = **21 µg/ml (STOCK SOLUTION)**

Notes: see *Ofloxacin*

- ETHIONAMIDE

- Dissolve 0.01 g Ethionamide crystals in 10 ml Ethylene glycol = 1000 µg/ml (**Solution A**)
- 5 ml Solution A + 5 ml sterile H₂O = 500 µg/ml (**Solution B**)

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- 8.3 ml Solution B + 1.7 ml sterile H₂O = **420 µg/ml (STOCK SOLUTION)**

- LINEZOLID (ca. 100%)
 - Dissolve 0.01 g of Linezolid powder in 10 ml sterile water = 1000 µg/ml (**Solution A**)
 - 4 ml Solution A + 1 ml sterile H₂O = 800 µg/ml (**Solution B**)
 - 1.05 Solution B + 8.95 ml sterile H₂O = **84 µg/ml (STOCK SOLUTION)**

Notes: see *Amikacin*

All drugs Stock solutions should be split into aliquots (110-µl aliquot and 420-µl aliquot) and stored at – 80°C for up to six months.

The aliquots should be stored in a dedicated cryobox indicating the name, the concentrations of the aliquots and the preparation date. Hazardous warnings for each drug solution should be hung on the freezer side.

7. RECORDING AND REPORTING

NA

8. RELATED DOCUMENTS

- BD BactTecTMMGITTM960 SIRE Kit Package Insert
- BD BactTecTMMGITTM960 PZA Kit Package Insert
- Guidance for Surveillance of Drug Resistance in Tuberculosis WHO/HTM/TB/2009.422
- Policy guidance on drug-susceptibility testing (DST) of second-line antituberculosis drugs WHO/HTM/TB/2008.392