UNIVERSIDAD PERUANA CAYETANO HEREDIA

Laboratorio de Investigación y Desarrollo (LID)

Laboratorio de Investigación en Enfermedades Infecciosas

Área de Mycobacterium

MODS

A user guide

Microscopic observation drug susceptibility assay

Lima-Perú

2008

Contents

1. Foreword 3
2. Substantive changes since last version 3
3. Introduction 4
4. Biosecurity – minimum requirements 4
5. Required equipment, supplies and reagents 5
   - 5.1. Equipment 5
   - 5.2. Supplies 5
   - 5.3. Reagents 5
6. Stock solutions 6
   - 6.1. Phosphate buffer 6
   - 6.2. NaOH–Na citrate stock solution for sputum decontamination 7
   - 6.3. Middlebrook 7H9 liquid medium with casitone and glycerol 7
   - 6.4. OADC 8
   - 6.5. PANTA 8
   - 6.6. Antibiotic stock solutions 8
   - 6.7. Positive TB control strains 10
7. MODS method – setting up the plates 11
   - 7.1. Final 7H9-OADC and 7H9-OADC-PANTA media preparation 12
   - 7.2. Antibiotic working solutions 13
   - 7.3. Sputum decontamination 15
   - 7.4. Preparation of final sample suspension and back up 16
   - 7.5. Final MODS plate preparation 16
   - 7.6. Plating out the positive internal quality control strains 17
8. Plate reading 18
   - 8.1. TB detection 18
   - 8.2. Drug resistance detection 19
   - 8.3. Internal controls 20
9. Plate disposal 22
10. Quality assurance 22
11. References 23

Appendix 1 – Possible suppliers of reagents and consumables 25
Appendix 2 – Calculating “g” from centrifuge rotor length & rpm 26
Appendix 3 – Alternative method for preparing antibiotic solutions 26
Appendix 4 – Preparing sample suspension and backups 28
Appendix 5 – Reading and interpreting results 29
Appendix 6 – Recovery & cryopreservation of positive MODS cultures 30

1. Foreword

This user guide describes how to perform the microscopic-observation drug-susceptibility (MODS) assay in detail, from the preparation of reagents and decontamination of the biological specimen, to the detection of mycobacterial growth and interpretation of the direct drug susceptibility results. The appendices contain ancillary information and procedures.

The current version (Version 12; April 2008) has undergone extensive updating and reformatting to make the presented information as clear, comprehensive and up to date as possible. We hope that the guide will be useful both to participants in the course "MODS: microscopic observation drug susceptibility assay for the diagnosis of TB and MDRTB directly from sputum" and to those implementing MODS independently.

We wish to thank the mycobacterium group and staff of the *Laboratorio de Investigación en Enfermedades Infecciosas* of Universidad Peruana Cayetano Heredia, led by Dr. Bob Gilman, who have brought their considerable experience to contributions and suggestions for the many iterations that have led to the current version of the guide.

2. Substantive changes since last version

This version replaces Version 11 (December 2007). Substantive changes in the layout and procedure descriptions include:

- Required equipment, materials and reagents updated
- Antibiotic stock and working solution contents and preparation procedures revised
- Positive TB control strain preparation revised
- Antibiotic dilution steps revised
- Final decontaminated sample preparation and plating procedures revised
- Plate reading and well result interpretation revised and expanded
- Suppliers and catalogue numbers for several consumables updated
- Procedures for recovery and cryopreservation of positive MODS cultures added

This version also updates Version 12 (April 2008), with some minor changes and clarifications identified as necessary during training

- In the light of discussions in operational settings, plate reading and interpretation tables have been simplified
- PANTA section (6.5) now more clearly details reconstitution procedure
- Tween preparation section (6.7) now explicitly describes concentration required
- NALC concentration in decontaminant solution corrected from 5% to 0.5% (7.3.1)
- Negative control media corrected from 7H9-OADC to 7H9-OADC-PANTA (7.5)
- Plating procedure modified (7.5) to reduce opportunity for cross-contamination

3. Introduction

MODS (Microscopic Observation Drug Susceptibility assay) is a liquid-culture based test that detects *Mycobacterium tuberculosis* and assesses isoniazid and rifampicin susceptibility directly from sputum samples. The method makes use of two important properties of *M. tuberculosis*: (1) markedly faster growth in liquid media than on solid media, and (2) an easily recognizable and characteristic microscopic ***cording*** appearance of that growth in liquid media. Using an inverted light microscope, 24-well plates inoculated with decontaminated sputum samples suspended in supplemented Middlebrook 7H9 medium are examined for microcolonies which can be detected in a median of 7 days, much earlier than macroscopic colony growth can be seen on solid medium. The incorporation of isoniazid and rifampicin in the testing process enables equally rapid MDR TB detection.

The simplicity of the technique, the greater sensitivity of liquid over solid media culture for TB detection, the specificity of the characteristic growth of *M. tuberculosis*, the evaluation of drug susceptibility in a short timescale, and the low cost of reagents are the major advantages of the method.

4. Biosecurity – minimum requirements

Safe MODS use requires Biosafety Level 2 (P2) laboratory facilities. Specific biosecurity measures necessary for working with MODS fall into two categories: appropriate infrastructure and safe laboratory practices. Essential elements are:

- Infrastructure
  - a well-maintained class II biosafety cabinet (BSC), in which all recirculated air passes through a HEPA filter
  - a laboratory space which is separated from the rest of the laboratory with well-sealed windows and lockable doors to prevent entry of personnel and air turbulence whilst samples are being handled
  - solid furniture which resists deterioration when disinfectants are applied
- Laboratory practice
  - staff trained in biosecurity procedures and the importance of biosafety in the laboratory
  - use of adequate protective clothing (gown, gloves)
  - use of appropriate personal respiratory protection at all times (N95 respirators)
  - proper MODS plate handling and disposal as described below

For additional detail, consult:

The MODS website at www.modsperu.org/Biosafety_FAQs.pdf

CDC's "Biosafety in Microbiological and Biomedical Laboratories (5th edition)
www.cdc.gov/OD/OHS/biosfty/bmbl5/bmbl5toc.htm

WHO's "Laboratory services in tuberculosis control. Part III: Culture"
http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_(part3).pdf

5. Required equipment, supplies and reagents

5.1. Equipment

Balance	to weigh isoniazid, rifampicin and NALC
Refrigerator/freezer	to store pre-prepared broth & antibiotic stocks
Autoclave	to sterilize medium, buffer and used plates
Vortex	to aid sample homogenization
Centrifuge (refrigeration not needed)	for sputum concentration (in 15ml conical base tubes)
Incubator (CO_2 enrichment not needed)	for culture incubation
Inverted light microscope (4x & 10x objective lens)	to read MODS plates
Automatic micropipettes (1000µl,200µl and 20µl)	to dispense OADC, PANTA and drugs
Multichannel pipette	to speed up plating out of antibiotic solution

5.2. Supplies*

Glass tubes (16x100 mm)	to store aliquots of prepared broth
0.2µm filters (for aqueous solvents)	to filter antibiotic stocks
0.2µm filters (for organic solvents)	to filter antibiotic stocks
Microcentrifuge tubes	to store aliquots of antibiotic stocks
15ml polypropylene centrifuge tubes (conical base)	for sputum decontamination and concentration
50ml tubes	to aliquot decontamination solution
10ml serological pipettes	to aliquot 7H9 and dispense OADC
Pasteur pipettes	to mix PANTA with medium
Micropipette tips (200µl, 1000µl;with/without filters)	to dilute antibiotics, dispense media
Tuberculin syringes (if micropipettes unavailable)	to dilute antibiotics, dispense media
24 well plates	for culture and reading
Sealable (ziplock-type) polythene bags	for biosecurity to contain 24 well plate

*all supplies must be sterile (except polythene bags)

5.3. Reagents

Middlebrook 7H9 broth	culture medium base
Casitone (pancreatic digest casein)	culture medium base
Glycerol	culture medium base
PANTA	antibiotic medium supplement
OADC	nutritional medium supplement
Antibiotic stocks (INH-RIF)	direct susceptibility testing
DMSO (Dimethyl sulphoxide)	to prepare rifampicin stock
Sodium hydroxide, pellets	sputum decontamination
Sodium citrate tribasic, dihydrate	sputum decontamination
N-acetyl-L-cysteine (NALC)	sputum decontamination – mucolytic agent
Sodium phosphate dibasic anhydrous (Na_2HPO_4),	sputum decontamination
Potassium phosphate monobasic, crystal (KH_2PO_4)	sputum decontamination
10% sodium hypochlorite	to discard contaminated waste
Disinfectant	to disinfect contaminated material or surfaces

6. Stock solutions

Stock solutions should be prepared in advance. The total volumes prepared at one time, and the volumes of aliquots that are stored can be adjusted to suit laboratory needs

6.1. Phosphate buffer – pH 6.8, 0.067M

- **Ingredients**

Sodium phosphate dibasic, anhydrous	Na_2HPO_4	9.47 g
Potassium phosphate monobasic, crystal	KH_2PO_4	9.07 g
Distilled water		2000ml

- **Preparation**

Solution A: Na_2HPO_4 **(Sodium phosphate dibasic)**
Dissolve 9.47g of sodium phosphate dibasic in 1000ml distilled water

Solution B: KH_2PO_4 **(Potassium phosphate monobasic)**
Dissolve 9.07g of potassium phosphate monobasic in 1000ml distilled water

6.1.1. Final phosphate buffer solution (pH 6.8)

(~1900ml – sufficient for ~190 samples)

- **Procedure**

1. Mix 950ml of Solution A with 950ml of Solution B and stir; keep back 50ml of each solution to adjust pH if necessary
2. Measure pH - should be pH 6.8 ± 0.2. To adjust:
   add solution A to raise pH
   add solution B to lower pH
3. Autoclave at 121-124°C for 15 minutes to sterilize.
4. Plate a 100µl aliquot of the phosphate buffer solution on nutrient agar medium in a Petri dish and incubate at 37°C for 48 hours to confirm sterility.
5. Store in refrigerator at 2-8°C for up to one month.

- **Notes**

Each sputum sample requires 10ml of phosphate buffer solution pH 6.8

The sterile buffer solution can be stored in sterile bottles containing 50-200ml for use in a single day, or in larger volumes. If large storage bottles are used, the amount needed for sample processing can be poured out using sterile technique on the day of processing.

6.2. NaOH–Na citrate stock solution (4% NaOH / 2.9% Na citrate for sputum decontamination)

(400ml – sufficient for 200 samples)

- **Ingredients**

Sodium hydroxide	8.0 g
Sodium citrate	5.8 g
Distilled water	400ml

- **Procedure**
  1. Dissolve 8.0g of sodium hydroxide in 200ml sterile distilled water
  2. Dissolve 5.8g of sodium citrate in another 200ml sterile distilled water
  3. Combine the sodium hydroxide and sodium citrate solutions (equal volumes)
  4. Mix and autoclave at 121-124°C for 15 minutes
  5. Store in refrigerator at 2-8°C for up to one month

- **Notes**

  Each sputum sample requires 2ml.

  Aliquots of smaller volumes can be stored in screw top tubes; the appropriate volume will depend upon the usual quantity of samples processed per day (see "NaOH-NALC decontamination solution, p14)

6.3. Middlebrook 7H9 liquid medium with casitone and glycerol (7H9)

(900ml - sufficient for 200 samples)

- **Ingredients**

Middlebrook 7H9 broth base	5.9 g
Glycerol	3.1ml
Casitone	1.25 g
Sterile distilled water	900ml

- **Procedure**
  1. Dissolve 5.9g of 7H9 medium powder in 900ml of sterile distilled water containing 3.1ml of glycerol and 1.25g of casitone.
  2. Mix with constant agitation until completely dissolved (use magnetic bead stir if available).
  3. Autoclave at 121-124°C for 15 minutes.
  4. Cool and divide the sterile medium into 4.5ml aliquots in sterile 16x 100 mm glass tubes for sample preparation, and 10.8ml aliquots for preparation of antibiotic solutions and internal controls.
  5. Incubate at 37°C for 48 hours to verify sterility (lack of turbidity).
  6. Store at 2-8°C with cap tightly closed for up to one month.

- **Notes**
  Each sputum sample and internal controls require one tube containing 4.5ml of 7H9 medium.

Each tube of 10.8ml 7H9 medium is sufficient for antibiotic solutions and internal controls for 15 sputum samples. The number prepared should be based on usual laboratory needs.

If large bottles are stored, smaller volumes can be poured out using sterile technique on the day of sample processing.

6.4. OADC

Enrichment supplement (oleic acid, albumin, dextrose and catalase): commercial preparation comes ready for use.

6.5. PANTA

Antibiotic supplement used to minimize contamination of MODS culture by oral flora micro-organisms not killed during the decontamination process. The BBL MGIT PANTA vial contains a lyophilized mixture of antimicrobial agents.

Reconstitute a lyophilized vial of BBL MGIT PANTA with 3 ml of sterile distilled water.

Antibiotic	Formulation per vial	Concentration per ml after reconstitution with 3ml sterile distilled water	Final concentration in the well with sample and 7H9-OADC media
Polimixin B	6000 units	2000/ml	40 Units/ml
Amphotericin B	600µg	200/ml	4µg/ml
Nalidixic acid	2,400µg	800/ml	16µg/ml
Trimethoprim	600µg	200/ml	4µg/ml
Azlocillin	600µg	200/ml	4µg/ml

6.6. Antibiotic stock solutions

6.6.1. Isoniazid stock (8 mg/ml)

- **Ingredients**
  Isoniazid 20 mg
  Sterile distilled water 2.5ml

- **Procedure**
  1. Dissolve 20 mg isoniazid completely in 2.5ml sterile distilled water.
  2. Filter with 0.2µm syringe filter **for aqueous solvent.**
  3. Store in 20µl aliquots in sterile micro centrifugation tubes at -20°C for up to 6 months.

- **Note**
  Each stored 20µl aliquot is sufficient for up to 100 samples (including wastage).

6.6.2. Rifampicin stock (8 mg/ml)

- **Ingredients**

Rifampicin	20 mg
Dimethyl sulphoxide (DMSO)	1.25ml
Sterile distilled water	1.25ml

- **Procedure**
  1. Dissolve 20mg rifampicin completely in 1.25ml DMSO.
  2. Add 1.25ml sterile distilled water and mix.
  3. Filter with 0.2µm syringe filter **for organic solvent.**
  4. Store in 20µl aliquots in sterile microcentrifuge tubes at -20°C for up to 6 months

- **Note**
  Each stored 20µl aliquot is sufficient for up to 100 samples (including wastage)

6.7. Positive TB control strains

Well-characterized positive TB control strains (one fully susceptible strain and one MDR strain) are plated out each day that clinical samples are processed for MODS. The positive controls test medium and antibiotic solution quality. To decrease risk of cross-contamination, the positive control strains are processed and placed in a separate plate after clinical samples have been plated and sealed.

- **Requirements**

  H37Rv. *M. tuberculosis* strain - ATCC 27294 (susceptible control)
  MDR control strain (local strain DM97 is used at UPCH)
  Middlebrook 7H11 Agar – 5% OADC in Petri dishes
  10% sterile Tween 80 40µl
  Sterile distilled water 10ml

- **Procedure**

  1. Reconstitute commercially prepared control strain with accompanying solution.
  2. Plate strain solution in Petri dish with Middlebrook 7H11 agar (see Appendix 6, "Recovery & cryopreservation of positive MODS cultures").
  3. Incubate at 37ºC for 15-20 days.
  4. On the day of strain solution preparation, mix 10ml sterile distilled water and 40µl of 10% sterile Tween 80 in a sterile tube (final Tween concentration = 0.04%).
  5. Using a sterile loop, harvest several colonies of mycobacteria and place in a sterile tube containing 100µl water-Tween solution and sterile glass beads.
  6. Cap tube tightly and vortex for 2-3 minutes; let stand 5 minutes.
  7. Open tube and add 3ml of water-Tween; cap tightly and vortex again for 20 seconds.
  8. Let stand for 30 min.
  9. Transfer the supernatant to another sterile tube using a transfer pipette.
  10. Adjust the turbidity to MacFarland scale 1 (approx. $3x10^8$ CFU/ml) with water-Tween.
  11. The MacFarland 1 suspension can be kept tightly sealed at 2-8°C for repeated use for 15 days.
  12. Remaining colonies in the Petri dish can be:
      - Replated in a new Petri dish with 7H11 agar to maintain a continuous supply.
      - Prepared for freezing for long term storage (see Appendix 6)

- **Notes**

  Preparation of MacFarland 1 suspensions involves manipulation of concentrated suspensions of mycobacteria and should only be carried out in a biological safety cabinet.

  If commercial control strains are not available, local strains with known drug resistance profiles (fully susceptible, and MDR or isoniazid and rifampicin monoresistant) can be utilized following the procedures described above and in Appendix 6. If preferred, the single positive control MDR strain can be replaced with two monoresistant strains: one isoniazid-resistant/rifampicin-susceptible strain and one isoniazid-susceptible/rifampicin-resistant strain.

7. MODS method – setting up the plates

Outline of steps on day of sample processing

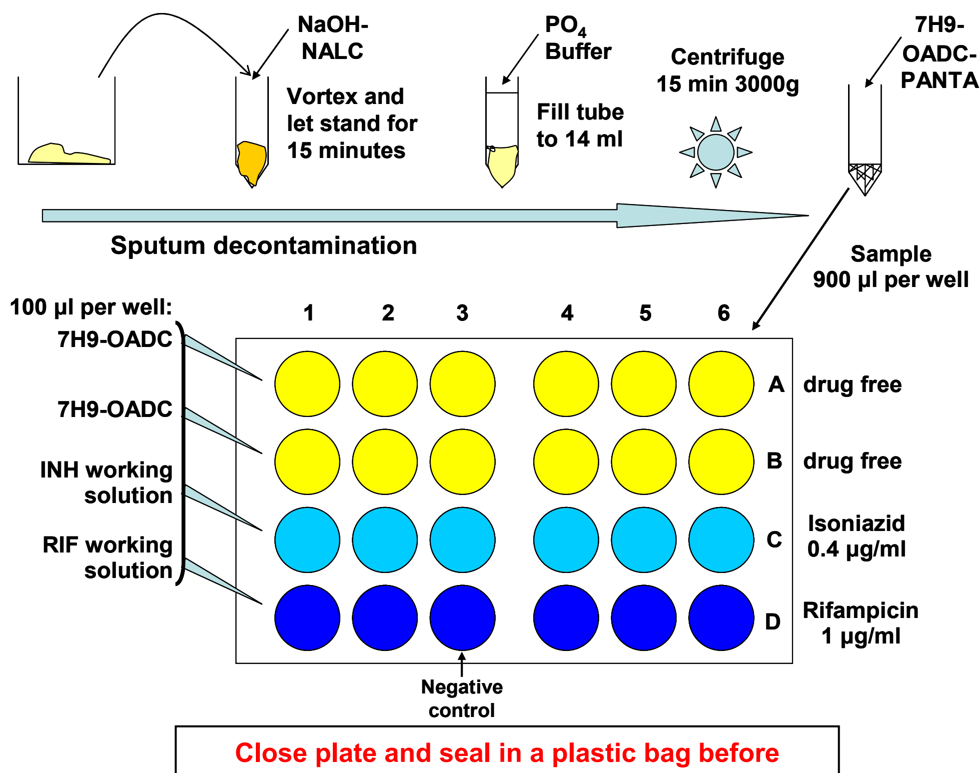
In a "clean" room (or the biosafety cabinet before bringing out samples), using sterile technique:

1. Add OADC to 7H9 medium (**7H9-OADC**).
2. Reconstitute PANTA and add to tubes with 7H9-OADC (**7H9-OADC-PANTA**).
3. Prepare antibiotic working solutions in a 24-well plate.
4. Pour out required volumes of stock NaOH-Na Citrate and phosphate buffer solutions (if stored in large bottles).
5. Weigh out required amount of NALC and add to NaOH-Na Citrate solution.

In the biosafety cabinet used for TB sample processing:

1. Prepare samples for decontamination and follow decontamination procedure.
2. Resuspend sample pellets with 7H9-OADC-PANTA.
3. Add antibiotic working solutions to plated samples.
4. Follow procedures for backup preparation and sample plating.
5. Close sample plates with lids, seal each in a ziplock bag, place in incubator.
6. Plate positive controls in the unused columns of the plate used for preparation of antibiotic working solutions; close and seal; place in incubator set at 37°C.
7. Start reading plates on day 5 of incubation.

Figure 1. MODS assay flowchart:



7.1. Final 7H9-OADC and 7H9-OADC-PANTA media preparation

Final media preparation should only be performed on the day of use.

- **Ingredients**
  7H9 medium (in tubes containing 4.5ml, 10.8ml)
  OADC
  PANTA
- **Procedure**
  1. Set out:
     a. 1 tube with 7H9 medium for every sputum sample to be processed, plus 1 additional tube for every plate (for the negative control column), plus
     b. 2 tubes of 7H9 medium for the positive controls (3 if monoresistant strains are used), plus
     c. 1-2 tubes with 10.8ml 7H9 for antibiotic solution preparation.
  2. Add 0.5ml OADC to the 4.5ml of 7H9 in each sample tube to yield 10% OADC in 7H9 medium (**7H9-OADC**: total volume=5ml).
  3. Add 1.2ml OADC to tubes with 10.8ml 7H9 (**7H9-OADC:** total volume=12ml).
  4. Set aside 2 tubes with 5ml 7H9-OADC for positive controls, and tube(s) with 12ml 7H9-OADC to be used for antibiotic solution preparation (these do not require PANTA).
  5. Reconstitute PANTA and add 0.1ml to each sample tube and to the negative control tubes (**7H9-OADC-PANTA**: total volume=5.1ml).

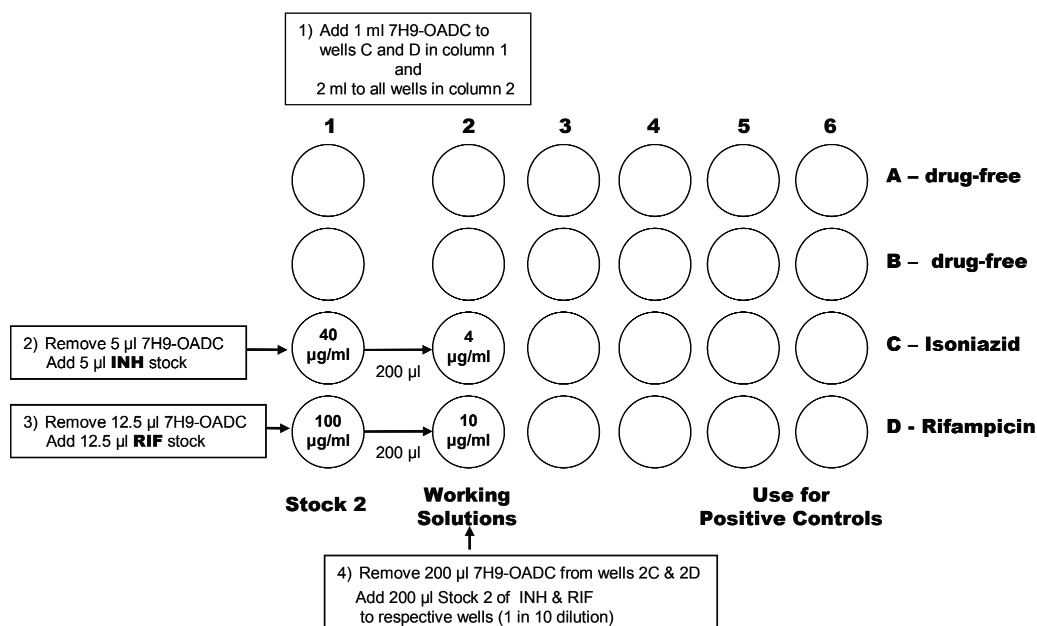
- **Note**

  Complete medium with PANTA (7H9-OADC-PANTA) is used for sputum samples and negative controls. Use 7H9-OADC without PANTA for positive controls and for antibiotic solution preparation.

7.2. Antibiotic working solutions

For accurate susceptibility results, the final antibiotic concentrations are critical. The following procedure is suitable for laboratories equipped with micropipettes. If micropipettes are not available, tuberculin syringes can be used with a different series of dilutions described in Appendix 3, “Alternative method for preparing antibiotic solutions”.

Working solutions of each drug are prepared on the day of use from stored aliquots of the 8 mg/ml stock solutions. Intermediate dilutions are performed using a 24-well plate (Figure 2). The remaining unused columns of that plate can be used later for positive controls.



7.2.1. Preparation of 24-well plate for antibiotic dilutions (Figure – step 1):

1. Add 1ml 7H9-OADC to wells 1C and 1D.
2. Add 2ml 7H9-OADC to all 4 wells in column 2 (wells 2A, 2B, 2C, 2D) (enough for 15 sputum samples plus 3 negative controls and 2 positive controls).

7.2.2. Preparation of Stock 2 solutions (Figure – steps 2 & 3):

1. Thaw aliquots of both antibiotic 8 mg/ml stock solutions.
2. Using a micropipette, remove 5µl of medium from the INH well (1C) and then add 5µl of INH stock; mix well (**0.04 mg/ml=40 µg/ml INH stock 2).**
3. Using a micropipette, remove 12.5µl of medium from the rifampicin well (1D) and then add 12.5µl of RIF stock; mix well (**0.1 mg/ml =100 µg/ml RIF stock 2).**

7.2.3. Preparation of working solutions (Figure – step 4):

1. Using a micropipette, remove 200µl from wells 2C and 2D -
2. Add 200µl of INH stock 2 solution to well 2C; mix well (**4 µg/ml INH working solution).**
3. Add 200µl of RIF stock 2 solution to well 2D; mix well (**10 µg/ml RIF working solution).**
4. The wells in column 2 now contain the solutions that will be added to the decontaminated sample suspensions - see “Final MODS plate preparation”, p.15.

- **Notes**

  Each frozen aliquot of 8 mg/ml antibiotic stock solution is sufficient for up to 100 isoniazid- or rifampicin- containing wells.

  The 2ml quantities of medium and antibiotic working solutions prepared in column 2 are sufficient for 3 full MODS plates (15 sputum samples plus 3 negative control columns plus 2 positive controls; these latter samples on a separate plate). If larger quantities are required column 3 (and column 4 if necessary) may be used in exactly the same way as column 2 (place 2ml 7H9-OADC in column 3 and follow the same procedure)

  When calculating the amounts of antibiotic working solutions needed, remember to include the negative control wells on each plate and the positive control strains and allow for wastage.

  Do not re-freeze or re-use antibiotic working or stock solutions as drug activity may be lost. Discard all unused antibiotic solutions at the end of the processing day.

Table 1. Dilution of antibiotic working solutions

Antibiotic stock solution	Dilution of stock in 7H9-OADC to generate stock 2	Dilution of stock 2 in 7H9-OADC to generate working solution	Final concentration in well when added to sample (μg/ml)	
Isoniazid **8 mg/ml**	5 μl stock/995 μl 7H9-OADC **(40 μg/ml)**	1/10 **4 μg/ml**	INH	0.4
Rifampicin **8 mg/ml**	12.5 μl stock/987.5 μl 7H9-OADC **(100 μg/ml)**	1/10 **10 μg/ml**	RIF	1.0

7.3. Sputum decontamination

Sputum decontamination is performed with **the sodium hydroxide–N-Acetyl-L-Cysteine (NaOH-NALC) method** described in "Public Health Mycobacteriology: A guide for the Level III Laboratory. 1985". NALC is a mucolytic agent; it must be added to the sterile NaOH-Na Citrate solution on the day of use, as activity wanes if stored. The sodium citrate in the NaOH solution prevents heavy metal ions that may be present from deactivating the NALC.

7.3.1. NaOH-NALC decontamination solution

Each sputum sample requires 2ml decontamination solution.

- **Ingredients**
  NaOH-Na citrate stock solution
  N-acetyl-L-cysteine (NALC)
  } See table 2 for amounts

- **Procedure**
  Dissolve 0.1g of NALC crystals in every 20ml of decontamination solution required. (0.5% NALC in NaOH-Na citrate = **NaOH-NALC** decontamination solution).

Table 2. Decontamination solution preparation

Number of Samples	Mix: NaOH-Na Citrate Stock (ml)	NALC (g)
10	20	0.1
20	40	0.2
50	100	0.5
100	200	1.0

- **Note**
  Discard any NaOH-NALC decontamination solution that remains unused after 24 hours as NALC loses its mucolytic activity over time.

7.3.2. Decontamination Procedure

1. Place 2ml of sputum sample into a 15ml centrifuge tube.
   (if less, make up to 2ml with phosphate buffer; if more, use only 2ml)
2. Add 2ml NaOH-NALC solution.
3. Cap tube tightly and vortex for 20 seconds; invert tube to ensure NaOH-NALC solution contacts the entire interior surface of the tube and lid
4. Let stand for a minimum of 15 minutes – can prolong by a few minutes if the sample is particularly viscous. To avoid over treatment, **should not exceed 20 minutes**.
5. Fill the tube to 14ml with phosphate buffer (pH 6.8) to neutralise alkali and terminate the decontamination process, and mix well by inverting the tube 4 times.
6. Centrifuge at 3000 g for 15 minutes.
7. Carefully pour off supernatant into a liquid waste container with 10% sodium hypochlorite or other suitable disinfectant and retain the pellet.

- **Notes**

NALC mucolytic activity may be reduced by excessive agitation. Avoid prolonged vortexing or forcefully shaking of the NaOH-NALC decontamination solution.

Centrifuge g-force varies with the length of the centrifuge rotor; thus different centrifuge models require different rpm speeds to obtain the recommended 3000g. See Appendix 2 for the calculation of rpm required to obtain 3000g based on centrifuge rotor length.

Overly harsh decontamination results in excessive killing of mycobacterial bacilli. If contamination rates of MODS cultures are less than 2%, or samples expected to be culture positive fail to grow, the possibility of excessive decontamination should be considered.

7.4. Preparation of final sample suspension and back up (also see Appendix 4)

- **Procedure**

1. Using 7H9-OADC-PANTA (from the tube containing 5.1ml), resuspend the sample pellet in a total volume of 2ml in the centrifuge tube with a Pasteur pipette; mix well.
2. Remove 1ml of sample suspension and store in a microcentrifuge tube at 2-8°C as a backup.
3. Add the second 1ml of sample suspension to the tube with the remaining 7H9-OADC-PANTA; mix well. This is the final sample solution ready for plating.

7.5. Final MODS plate preparation

- **Procedure**

1. Using a multi-channel pipette, carefully fill 4 tips with 100µl from the wells with 7H9-OADC and antibiotic working solutions (Column 2 in antibiotic dilution plate).
2. Add the 100µl aliquots to the column 1 wells in the 24-well plate.
3. Repeat until all columns have received the 100µl of medium (drug-free wells) or antibiotic solutions (including negative control column 3).
4. Place 900µl of the final sample suspension into each of the 4 wells of a single column
5. Repeat with additional samples until all columns of the plate, except Column 3, are filled (or until all samples are plated).
6. Place 900µl of 7H9-OADC-PANTA medium **without sample** in the 4 wells of Column 3 of each sample plate (negative internal controls).
7. Close the plate with its lid and place in a sealable polythene (Ziplock) bag and seal **(bag is not opened again from this point onwards).**
8. Incubate at 37°C (CO_2 enrichment is not necessary).

- **Notes**

See figure 1 (p11, MODS assay flowchart) for layout

7.6. Plating out the positive internal quality control strains

Two positive control strains must be run each processing day, one fully susceptible strain and one MDR strain - if laboratories prefer to avoid handling MDR strains then two separate strains can be used, one isoniazid monoresistant and the other rifampicin monoresistant. Positive controls test the medium and antibiotic solution quality used on the day of sample processing. If positive controls do not grow in the expected pattern, the results of samples plated on the same day **are not valid.** See section 8.3 - Internal Controls.

To minimize the risk of cross-contamination, the positive controls are set up in a separate plate after all plates with samples have been sealed and placed in an incubator. The unused columns of wells in the antibiotic dilution plate can be used for this purpose.

- **Procedure**
  1. Mix 5µl of each McFarland 1 control strain suspension with 5ml of 7H9-OADC medium.
  2. Place 900µl of each positive control suspension in the 4 wells of a column on the separate positive control plate.
  3. Using the multi-channel pipette, add the four 100µl aliquots of medium and antibiotic working solutions as was done for samples.
  4. Cap plate with lid and seal in a ziplock bag; incubate at 37°C with other plates processed the same day.

8. Plate reading [1]

8.1. TB detection

A positive result is defined as two or more colony forming units (≥2 cfu) in EACH OF THE TWO drug-free wells. See Table 3 and Appendix 5 for more detail on result interpretation

- **Procedure**
  1. Plates are removed from the incubator for examination under an inverted microscope
  2. Plates are examined within the transparent, sealed ziplock bags, which are **not opened**.
  3. Start examining drug-free wells on day 5.
     Early mycobacterial growth looks like small curved commas or spirals (days 5-9).
     Colony formation usually progresses to cords, and later more irregular tangled growth
     If two or more colonies (≥2 cfu) are detected in each of the two wells, the result is positive.
  4. If results are negative on day 5, continue reading drug-free wells daily (or on alternate days according to laboratory workload) until ≥2 cfu are observed in each of the two wells.
  5. When a positive result is observed, read the isoniazid- and rifampicin-containing wells *on the same day* - see section "Drug Resistance Detection" below.
  6. If no growth is observed by day 15, repeat reading on day 18 and day 21. If results are still negative on day 21 the final result is negative.
  7. If only 1 cfu appears in either drug-free well, or in both, the result is "indeterminate".
  8. Drug-containing wells (see 8.2 below) should not be read if the detection well result is negative or indeterminate.
  9. If bacterial or fungal contamination appears, backup samples should be re-decontaminated and re-processed, or a new sample requested. If contamination is present, results are uninterpretable.

Before final results can be considered valid, the internal negative and positive control wells must be examined and interpreted – see section 8.3 - Internal Controls.

- **Notes**

  For initial readings, examine wells with the 10X microscope objective to search for early colony forms (100X total magnification). For subsequent readings, use the 4X objective (40X total magnification) to examine the entire contents of each well.

  Bacterial or fungal contamination is uncommon but is usually apparent by day 5 (cloudy medium, florid growth). If contamination develops, backup samples, if available, should be re-decontaminated and re-cultured, or repeat patient samples requested (see Appendix 4).

  The culture medium **does not become cloudy** with growth of *M. tuberculosis*.

  Heavy debris can make detection of early mycobacterial forms difficult. With time, more mature mycobacterial colonies can be detected, particularly at the periphery of the wells.

[1] See photo library at www.modsperu.org

Growth in only one well, or less than 2 cfu in each of the two wells, should be considered an **indeterminate** result, and should prompt a request for a repeat sample and a search for evidence of cross-contamination.

Intervals between readings can be flexible to suit laboratory workload and schedule. More frequent readings yield faster results.

Table 3. Reading and interpreting drug-free wells

Reading

Observation in single drug-free well (A or B)	**Interpretation of well findings**
≥ 2 colony forming units (cfu)	Positive
no growth (0 cfu)	Negative
1 cfu	Indeterminate
bacterial / fungal overgrowth	Contaminated

Interpretation

Combined well findings (A & B)	**Overall culture interpretation**
Both wells positive	Positive
Both wells negative	Negative
Either well indeterminate	Indeterminate
One well positive, other well negative	Indeterminate
One well positive, other well indeterminate	Indeterminate
Either well contaminated	Contaminated

8.2. Drug resistance detection

Antibiotic-containing wells should only be examined if and when drug-free wells are positive (≥2 cfu).

Resistance is defined as mycobacterial growth of ≥2 cfu in drug-containing wells on the same day that both drug-free wells are positive. See Table 4 and Appendix 5 for more detail on result interpretation.

- **Procedure**
    1. *On the same day* that both drug-free wells have definite mycobacterial growth of ≥2 cfu, examine the isoniazid- and rifampicin-containing wells.
    2. If there is any growth of ≥2 cfu in a drug-containing well, the sample is resistant to that drug (at the concentration present); no growth means the sample is sensitive to the drug.
    3. If there is positive growth in both isoniazid- and rifampin-containing wells, the sample is multi-drug resistant (MDR).
    4. Drug-containing wells should NOT be re-examined after the reading performed on the day that drug-free containing wells are identified as positive. Scant breakthrough growth in drug-containing wells after prolonged incubation is not indicative of resistance.

- **Notes**

  Growth of drug resistant *M. tuberculosis* in drug-containing wells is usually readily identifiable when drug-free wells are positive.

  Growth may be less florid in the drug-containing well but the presence of ≥ *2 cfu* indicates resistance (this is not a proportions-type test).

  Only very rarely is a single cfu detected in drug-containing wells (read at the correct timepoint), however if this is encountered the interpretation is indeterminate.

  Growth in drug-containing wells should only be considered as indicating resistance if drug-free wells for the same sample have also shown growth

Table 4. Reading and interpreting wells containing isoniazid and rifampicin

Reading

Observation in antibiotic containing well (C or D)	**Interpretation of well findings**
No growth (0 cfu)	Susceptible
Growth of ≥ 2 cfu	Resistant
Growth of only 1 cfu	Indeterminate
Bacterial / fungal overgrowth	Contaminated

Interpretation

Combined well findings (C & D)	**Overall drug susceptibility interpretation**
No growth in either drug-containing well	Susceptible (non-MDR)
Growth in isoniazid well only	Isoniazid resistant (non-MDR)
Growth in rifampicin well only	Rifampicin resistant (non-MDR)
Growth in both drug-containing wells	Multidrug-resistant (MDR)
Either drug-containing well with only 1 cfu	Indeterminate for that drug
Either drug-containing well contaminated	Indeterminate for that drug

8.3. Internal controls

Use of the internal negative controls (every sample plate) and positive controls (every plating session) are essential for ensuring valid MODS results. The internal controls MUST be examined and correctly interpreted before sample results can be considered valid. See Table 5.

Internal control wells are read and interpreted in the same manner as sample wells.

8.3.1. Negative controls

These are the wells with culture medium but no sample which are run on every plate).

All 4 wells in column 3 (3A, 3B, 3C & 3D) should have no growth.

If any mycobacterial colonies are observed in any well, there has been cross-contamination. The entire plate should be discarded and backup samples reprocessed if available, or new samples requested. A search for potential sources of cross-contamination should occur and if the source is identified appropriate remedial action should be taken.

8.3.2. Positive *M. tuberculosis* controls

These are the wells containing drug-susceptible and drug-resistant control strains that are run on a separate plate every processing day.

8.3.2.1. Drug-free wells

All 4 drug-free wells (2 with sensitive strain, 2 with MDR strain) should have positive mycobacterial growth (≥2cfu).

Absence of growth in all drug-free wells suggests that the medium does not support growth and sample results are not valid. All samples plated out on the same day should be re-processed with a new batch of medium.

If only one of the two positive control strains grows in drug-free wells, the strain that did not grow may not be viable. A fresh strain suspension should be used with re-testing.

8.3.2.2. Antibiotic-containing wells

As with sputum samples the drug-containing wells should only be read if drug-free control wells are positive.

The drug-susceptible control strain (H37Rv or other) should not grow in either of the drug-containing wells. Growth indicates incorrect (low) antibiotic concentrations or inadequate isoniazid and/or rifampicin activity.

The drug-resistant control strain (1 MDR strain, or 2 mono-resistant strains) should grow in the drug-containing wells. Absence of growth indicates that the final isoniazid and/or rifampicin concentrations are too high.

If positive controls do not perform as expected in antibiotic-containing wells, the susceptibility results for samples plated at the same time are **invalid** (indeterminate) – discard all plates and re-process backup or repeat samples using freshly prepared antibiotic stock and working solutions.

- **Notes**

  Absence of control strain growth in all drug-free wells may also indicate strain non-viability. Consider use of fresh control strain preparations for re-testing.

  Reduced drug activity may be due to incorrect concentrations, or reduced potency related to improper handling or storage of original (undissolved) drug or drug stock solution.

Table 5. Expected Internal Control Results

Control Type	Strain	Medium	Expected Result
Negative	none	4-well column (2 drug-free; 1-INH, 1-RIF)	No growth
Positive	fully sensitive	drug-free wells INH- and RIF-containing wells	Growth No growth
	MDR*	drug-free wells INH- and RIF-containing wells	Growth Growth

*or INH- & RIF monoresistant strains

9. Plate disposal

- **Procedure**

1. Keep all plates sealed inside their original ziplock plastic bags and seal in an autoclave bag.
2. Autoclave at 121-124ºC for 45-60 minutes.
3. Discard the sealed sterilized bags in the site designated for this purpose.

- **Notes**

Once MODS plates are loaded with samples, they must remain sealed within their ziplock bags whether they contain positive cultures or not.

10. Quality assurance

The MODS method described in this guide includes sterility checks for stock solutions and medium. The positive internal controls test medium and antibiotic solution function; the negative internal controls test for cross-contamination.

A systematic QA plan including quality control (QC) and external quality assessment (EQA) procedures is under evaluation and will be added when validated. A draft version can be found at www.modsperu.org. Suggestions and contributions are welcomed.

11. References

11.1. MODS

www.modsperu.org

Ejigu GS, Woldeamanuel Y, Shah NS et al.
Microscopic-observation drug susceptibility assay provides rapid and reliable identification of MDR-TB.
Int J Tuber Lung Dis 2008; 12(3):332-337.

Tovar M, Siedner MJ, Gilman RH et al.
Improved diagnosis of pleural tuberculosis using the microscopic observation drug susceptibility technique
Clin Infect Dis 2008; 46: 909-12

Caws M, Ha MDT, Torok E et al.
Evaluation of the MODS culture technique for the diagnosis of tuberculous meningitis.
PLoS ONE 2007; 11: e1173

Moore DAJ, Roper MH
Diagnosis of smear-negative tuberculosis in people with HIV/AIDS.
Lancet 2007; 370: 1033

Moore DAJ.
Future prospects for the MODS assay in multidrug resistant tuberculosis diagnosis.
Future Microbiol 2007; 2: 97-101

Caviedes L, Moore DAJ.
Introducing MODS – a low-cost, low-tech tool for high-performance detection of tuberculosis and multidrug resistant tuberculosis.
Ind J Med Micro 2007; 25 (2): 87-8

Mello FCQ, Arias MS, Rosales S et al.
Clinical evaluation of the microscopic observation drug susceptibility (MODS) assay for detection of Mycobacterium tuberculosis resistant to isoniazid or rifampicin.
J Clin Microbiol 2007; 45 (10): 3387-9

Shiferaw G, Woldeamanuel Y, Gebeyehu M et al.
Evaluation of microscopic observation drug susceptibility assay for detection of multidrug-resistant Mycobacterium tuberculosis.
J Clin Microbiol 2007; 45 (4): 1093-1097

Arias M, Mello FCQ, Pavón A et. al
Clinical evaluation of the microscopic observation drug susceptibility assay for detection of tuberculosis.
Clin Infect Dis 2007; 44: 674-80

Moore DAJ, Gilman RH, Friedland JS.
MODS assay for the diagnosis of TB.
N Engl J Med 2007; 356 (2): 189

Moore DAJ, Evans CA, Gilman RH et. al.
Microscopic-Observation Drug-Susceptibility Assay for the Diagnosis of TB.
N Engl J Med 2006; 355: 1539-50

Moore DAJ, Caviedes L, Coronel J et. al.
Low rates of Mycobacterium tuberculosis liquid culture cross-contamination in the microscopic observation drug susceptibility assay (MODS).
Diagn Micro Infect Dis 2006; 56: 35-43

Oberhelman RA, Soto-Castellares G, Caviedes et al.
Improved recovery of Mycobacterium tuberculosis from children using the microscopic observation drug susceptibility method.
Pediatrics 2006; 118 (1): 100-106

Moore DAJ, Mendoza D, Gilman RH et al,
Microscopic observation drug susceptibility assay – a rapid, reliable diagnostic test for multidrug-resistant tuberculosis suitable for use in resource-poor settings.
J Clin Microbiol 2004; 42 (10): 4432-7

Caviedes L, Lee TS, Gilman RH et al.
Rapid, efficient detection and drug susceptibility testing of Mycobacterium tuberculosis in sputum by microscopic observation of broth cultures. The Tuberculosis Working Group in Peru.
J Clin Microbiol 2000; 38(3): 1203-8.

11.2. Biosafety and laboratory methods

CDC

Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition.
http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

Kent, B.D. and Kubica, G.P. Public health mycobacteriology.
A guide for the level III laboratory 1985 p.36-39, 47-69, and 185-187. US. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

WHO

Laboratory Services in Tuberculosis Control Series (Part III: Culture). Geneva, 1998.
http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_(part3).pdf

Appendix 1 – Possible suppliers of reagents and consumables

The following table indicates some suppliers and product codes for the reagents and consumables required for MODS. The list is neither exhaustive nor necessarily intended as a recommendation; other suppliers may be able to provide the same or similar products at a better price in some regions. However, we are often asked where the materials can be purchased so this list is intended to address that need and includes most of the suppliers that we generally use or have used.

Item	Supplier	Product code	Unit
Middlebrook 7H9 broth (Difco)	Fisher	DF0713-17-9	500gr/bottle
Casitone (pancreatic digest casein)	Fisher	DF0259-17-9	500gr/bottle
Glycerol (glycerin)	Sigma	G-33-500	500ml/bottle
PANTA (Antibiotic mixture lyophilized BD)	Fisher	B4345114	6 bottles/pack
OADC (Middlebrook OADC enrichment BD)	Fisher	B11886	10 x 20ml/pack
Dimethyl sulphoxide (Hibri-Max)	Sigma	D-2650	100ml/bottle
Antibiotic stocks isoniazid rifampicin	Sigma	I-3377 R-3501	50 gr/bottle 1gr/bottle
Sodium hydroxide (pellets)	Sigma	221465	500gr/bottle
Sodium citrate (trisodium salt dihydrate)	Sigma	S-4641	500gr/bottle
N-acetyl-L-cysteine	Sigma	A-7250	50gr/bottle
Potassium Phosphate Monobasic crystal. KH_2PO_4	Sigma	P0662	500gr/bottle
Sodium Phosphate Dibasic, anhydrous. Na_2HPO_4	Sigma	S0876	500gr/bottle
Sodium hypochlorite	household bleach		
15ml centrifuge tubes (polypropylene 15ml Falcon 35-2096)	Fisher	14-959-49B	500ea/case
24 well plates (Plates Tissue 24 wells BD Falcon 35-3047)	Fisher	08-772-1	50 plates/case
Sealable polythene bags 6 X 6 " (ziplock)			
Glass tubes with lid (16 x 100mm)	Fisher	14932-1B	144 tubes/package
Screw cap microcentrifuge tubes (1.5ml)	Fisher	05-669-22	1000ea/case
0.22µm filters (aqueous solvents) Syringe filter Millex blue	Fisher	SLGL 025 OS	50 units/case
0.22µm filters (organic solvents) Syringe filter Millex yellow	Fisher	SLGV 033 RS	50 units/case
Disposable Pasteur pipettes borosilicate glass 9″	Fisher	13-678-20C	720ea/case
Aerosol barrier tips 1000-1300µl	Fisher	02-707-51	1000ea/pk
USA Scientific Tips One 1-200µl yellow tips	Fisher	1111-0006	1000 tips/bag

Appendix 2 – Calculating "g" from centrifuge rotor length & rpm

g **= (1.118x10^{-5}) x (rotor radius in cm) x (rpm^2)**

To calculate the rpm needed to achieve the recommended 3000g,

rpm = √ (3000 / ((1.118x10^{-5}) x (rotor radius)))

Remember: 3000g is NOT the same as 3000rpm (revolutions per minute)

Appendix 3 – Alternative method for preparing antibiotic solutions

This alternative method is designed to prepare accurate concentrations of antibiotic working solutions using tuberculin syringes if micropipettes are unavailable.

1. Antibiotic stock solutions

1.1. Isoniazid stock (0.4 mg/ml)

- **Ingredients**

Isoniazid	4 mg
Sterile distilled water	10ml

- **Procedure**
  1. dissolve 4mg isoniazid completely in 10ml sterile distilled water
  2. filter with 0.2µm syringe filter **for aqueous solvent**
  3. store in 120µl aliquots in sterile micro centrifugation tubes at -20°C for up to 6 months

- **Note**
  Each 120µl aliquot is sufficient for 100 samples

1.2. Rifampicin stock (1 mg/ml)

- **Ingredients**

Rifampicin	10 mg
Dimethyl sulphoxide	5ml
Sterile distilled water	5ml

- **Procedure**
  1. dissolve 10mg rifampicin completely in 5ml Dimethyl sulphoxide (DMSO), then add 5ml sterile distilled water and mix
  2. filter with 0.2µm syringe filter **for organic solvent**
  3. store in 120µl aliquots in sterile micro centrifugation tubes at -20°C for up to 6 months

- **Note**
  Each 120µl aliquot is sufficient for 100 samples

Do not re-freeze or re-use antibiotic solutions as drug activity may be lost. Discard all unused antibiotic solutions at the end of the processing day.

2. Dilutions for antibiotic working solutions

Working solutions of each drug are prepared *on the day of use* from the storage stock. Dilutions are made using 7H9 with 10% OADC (7H9-OADC)

2.1. Isoniazid 4µg /ml working solution

- **Ingredients**
  Isoniazid 0.4 mg/ml stock aliquot 120µl
  7H9-OADC medium

- **Procedure**
  1. Using a tuberculin syringe, add 100µl of thawed stock solution (400µg/ml) to 900µl 7H9-OADC (yields **INH Stock 2, 40 µg/ml**).
  2. Add 100µl of INH Stock 2 to every 900µl 7H9-OADC required (yields **4 µg/ml INH working solution)**.

- **Notes**

  The total amount prepared will depend on the number of samples to be processed.

  Each sample requires 100µl of INH working solution. An additional 200µl are necessary for the two positive control samples.

2.2. Rifampicin 10µg /ml working solution

- **Ingredients**
  Rifampicin 1.0 mg/ml stock aliquot 120µl
  7H9-OADC medium

- **Procedure**
  1. Using a tuberculin syringe, add 100µl of the thawed stock solution (1 mg/ml) to 900µl 7H9-OADC (yields **RIF Stock 2, 100µg/ml**).
  2. Add 100µl RIF Stock 2 to every 900µl 7H9-OADC required (yields **10µg/ml RIF working solution)**.

- **Notes**

  The total amount prepared will depend on the number of samples to be processed.

  Each sample requires 100µl of RIF working solution. An additional 200µl are necessary for the two positive control samples.

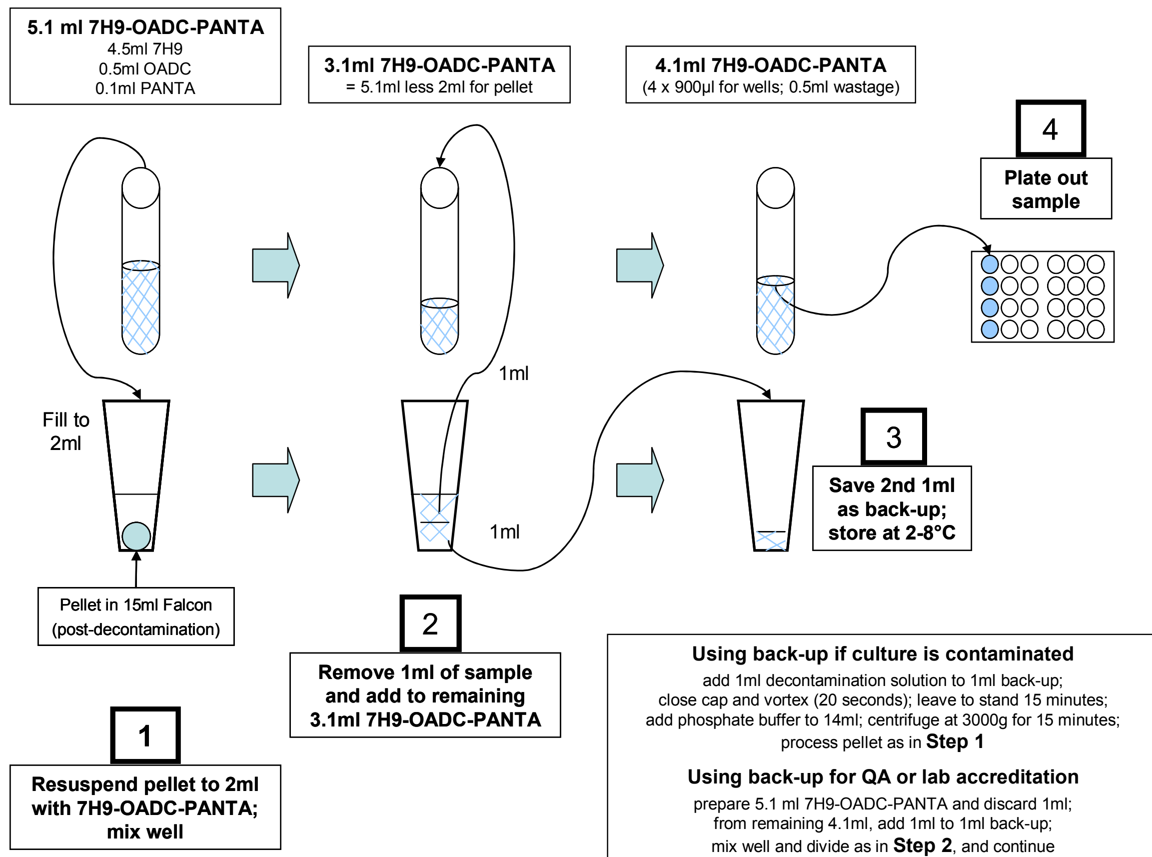
Dilution of antibiotic working solutions – alternative method

Antibiotic Stock	Dilution of stock in 7H9-OADC to generate Stock 2	Dilution of stock 2 in 7H9-OADC to generate working solution	Final concentration in well when added to sample (µg/ml)	
Isoniazid **400 µg/ml**	100µl stock / 900µl 7H9-OADC (40µg/ml)	1/10 **4µg/ml**	INH	0.4
Rifampicin **1000 µg/ml**	100µl stock / 900µl 7H9-OADC (100µg/ml)	1/10 **10µg/ml**	RIF	1.0

Appendix 4 – Preparing sample suspension and backups

Backup sample solutions should be prepared each time a sample is processed. The backups can be used for reprocessing if bacterial or fungal contamination occurs, for blind rechecking as part of an external quality control program, or for additional testing.

The following illustrates recommended backup preparation and re-processing procedures:



Appendix 5 – Reading and interpreting results

Results for the majority of samples processed in MODS are clearly positive (many colonies) or clearly negative (no growth at all). Difficulty arises rarely and only when growth is minimal, or if contamination is present.

TB detection		
Well A	**Well B**	**Interpretation**
+	+	POSITIVE
-	-	NEGATIVE
+	C	CONTAMINATED
C	+	CONTAMINATED
-	C	CONTAMINATED
C	-	CONTAMINATED
C	C	CONTAMINATED
+/-	C	CONTAMINATED
C	+/-	CONTAMINATED
+	-	INDETERMINATE
-	+	INDETERMINATE
+	+/-	INDETERMINATE
+/-	+	INDETERMINATE
-	+/-	INDETERMINATE
+/-	-	INDETERMINATE
+/-	+/-	INDETERMINATE

\+ means ≥ 2cfu in the well
+/- means 1cfu in the well
\- means no growth in the well
C means contamination in the well and no visible TB growth

Isoniazid and rifampicin susceptibility		
Well C INH 0.4µg/ml	**Well D RIF 1.0 µg/ml**	**Interpretation**
+	+	MDR
-	-	Susceptible to rifampicin and isoniazid
+	-	Resistant to isoniazid, susceptible to rifampicin
+	C	Resistant to isoniazid, rifampicin indeterminate
+	+/-	Resistant to isoniazid, rifampicin indeterminate
-	+	Resistant to rifampicin, susceptible to isoniazid
C	+	Resistant to rifampicin, isoniazid indeterminate
+/-	+	Resistant to rifampicin, isoniazid indeterminate

\+ means ≥ 2cfu in the well
+/- means 1cfu in the well
\- means no growth in the well
C means contamination in the well and no visible mycobacterial growth

Appendix 6 – Recovery & cryopreservation of positive MODS cultures

When additional tests on positive MODS cultures are needed, or long term storage of samples is desired, the strains must be isolated first.

Here we explain how to recover the strains from positive cultures in MODS plates and how to cryopreserve them. The same culture expansion and cryopreservation techniques can be used for preparation of positive control sample stores.

1. Recovery of MODS positive culture strains

1.1. Preparation of Middlebrook 7H11 Agar - 5% OADC

- **Ingredients**

Middlebrook 7H11 agar base	21 g
Glycerol	5ml
Distilled water	945ml
OADC	50ml

- **Procedure**
  1. Dissolve 21g of 7H11 agar medium base in 945ml of distilled water.
  2. Add 5ml of glycerol.
  3. Heat and stir with constant agitation until fully dissolved (magnetic stirrer should be used). The medium should appear translucent. Do not boil or overheat the medium.
  4. Autoclave at 121-124° C for 15 minutes.
  5. Cool down to 50-55°C and add 50ml of OADC using sterile technique. Mix well for 5 minutes and pour into Petri dishes (25-30ml per dish).
  6. Leave to solidify (protect against direct exposure to light).
  7. Verify medium sterility by incubating at 37° C for 24-48 hours.
  8. Store at 2-8ºC.

1.2. Recovery of strain from a positive MODS culture

- **Procedure**
  1. Place the MODS plate containing the positive culture to be recovered inside the Biosafety Cabinet. Open the plastic bag and carefully withdraw the plate. Remove the plate cover aseptically.
  2. Withdraw the contents of the 2 positive control wells with a sterile Pasteur pipette (only use positive cultures from wells which do not contain isoniazid or rifampicin).
  3. Inoculate 50-100µl of the recovered sample onto 7H11 agar prepared as above. Using a sterile loop, streak the sample throughout the surface of the agar; then cover the Petri dish.
  4. Place the remaining sample in a sterile glass tube to keep at room temperature for additional use or in case of contamination.
  5. Incubate the inoculated Petri dish at 37°C for 15-20 days to obtain enough growth for cryopreservation.

2. Cryopreservation of strains from MODS cultures in Middlebrook 7H9 broth

2.1. Preparation of Middlebrook 7H9 broth with 10% Glycerol for strain cryopreservation

- **Ingredients**

Middlebrook 7H9 base	0.94 g
Glycerol	20ml
Sterile distilled water	160ml
ADC supplement	20ml

- **Procedure**

1. Dissolve 0.94g of 7H9 medium base in 160ml of sterile distilled water and add 20ml of glycerol.
2. Stir with constant agitation (magnetic stirrer) until completely dissolved.
3. Autoclave at 121-124° C for 15 minutes.
4. Let cool and add the enrichment supplement ADC.
5. Mix well and verify medium sterility by incubating at 37° C for 24-48 hours.
6. Place 0.9ml aliquots of the medium in cryovials using sterile technique; seal tightly and store at 2-8°C.

- **Note**

ADC and **not OADC** is used for this procedure

2.2. Cryopreservation of strains

1. Place the 7H11 agar Petri dishes containing the cultures of strains obtained from MODS inside the Biosafety Cabinet.
2. Open the Petri dishes carefully and harvest the colonies with a sterile loop. Add one full loop to each cryovial containing 7H9 broth with 10% glycerol.
3. Seal the vial tightly and vortex for 10-15 seconds to homogenize the strains.
4. Store at -70°C (-20°C can be used if -70°C is not available).
5. Remaining colonies can be used for additional tests (molecular tests, indirect drug susceptibility tests, etc.)
6. Place all leftover infectious materials in an autoclave bag and seal.
7. Autoclave at 121-124°C for 45-60 minutes.

Jorge Coronel
Martha Roper
Luz Caviedes
David Moore

Lima – Peru
August 2008