STANDARD OPERATING PROCEDURE FOR MYCOBACTERIOLOGY LABORATORY

DEPARTMENT OF BACTERIOLOGY

TUBERCULOSIS RESEARCH CENTRE, ICMR,

MAYOR V.R. RAMANATHAN ROAD, CHETPET,

CHENNAI- 600 031, INDIA

2010

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2010

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FOREWORD

I am delighted to write this foreword for the Standard Operating Procedures (SOP) manual compiled by the Department of Bacteriology, Tuberculosis Research Centre (TRC), (Indian Council of Medical Research / Department of Health Research, Ministry of Health, Government of India) Chennai. The Department deserves to be congratulated for its efforts in promoting quality assured bacteriology procedures that have put TRC on the Global map of TB research. I am glad that the department has produced this manual utilizing the vast reservoir of knowledge and research experience accumulated over several decades. The manual documents the precision and quality that is required for the diagnosis of tuberculosis and also catalogues the improvisations made over the years. I earnestly hope that this manual will go a long way in further raising the standards and help in maintaining the recognition which the Centre commands.

I wish the department and all the dedicated and committed staff all success in their endeavors for the cause of TB control. Finally, I congratulate the head of the department and the entire staff for their sincere effort, unrelenting support and cooperation in the compilation of the SOPs.

Dr. V. Kumaraswami,
Director-in-Charge
Tuberculosis Research Centre (ICMR)

PREFACE

This Standard Operating Protocol (SOP) manual is a guideline intended to assist as a reference material for routine laboratory activities in mycobacteriology lab. This guideline clearly spells out what is expected and required of personnel while handling mycobacteria during their day-to-day activities in the laboratory. SOPs provide a mechanism to, identify necessary changes, implement Institute's policies, enhance quality of training, and enhance desired operational performance that set the standards under which the team will perform. As a result there is an improved operational efficiency, greater accountability and increased safety. They provide the desired outcome and allow decision makers a great deal of flexibility in their decision making processes and empower the laboratory staff. The committed and dedicated members of Bacteriology department (list attached) of Tuberculosis Research Centre (Indian Council Medical Research/ Department of Health Research, Ministry of Health, Government of India) have executed a mammoth task of compiling the SOPs for all the laboratory procedures, right from the sputum reception to result tabulation including critical biosafety and maintenance protocols, in a manner which will guide the laboratory technologists working in the mycobacteriology laboratory. The onus lies on the department as it has the recognition of being the WHO Supranational Reference Laboratory for Tuberculosis for South East Asia region. It is the flagship to set the trend for the TB research and control programs nationally and globally. This version of the SOP will be useful to the laboratory personnel involved in the fight against control of TB. It is dedicated to the cause of the disease and ensures the safety of the people involved in combating it by adhering to these guidelines.

DR. N. SELVAKUMAR, Ph. D.

Scientist - F & HOD

ABBREVIATION

AFB	:	Acid Fast Bacilli
AC	:	Actual Concentration
ADC	:	Albumin Dextrose Catalase
AMC	:	Annual Maintenance Certificate
AMI	:	Amikacin
AST	:	Antimicrobial Susceptibility Testing
BAL	:	Broncho Alveolar Lavage
BAP	:	Blood Agar Plate
BSA	:	Bovine Serum Albumin
BSC	:	Biological Safety Cabinet
CA	:	Chocolate Agar
CAP	:	Capreomycin
CFU	:	Colony Forming Unit
CLED	:	Cysteine-Lactose-Electrolyte-Deficient
CLSI	:	Clinical and Laboratory Standards Institute
CPC	:	Cetyl Pyridinium Chloride
CSF	:	Cerebro Spinal Fluid
DC	:	Desired Concentration
DST	:	Drug Sensitivity Testing
EMB	:	Ethambutol
EP	:	Extra Pulmonary
EQA	:	External Quality Assurance
FM	:	Fluorescence Microscopy
GHTM	:	Government Hospital Thoracic Medicine
GI	:	Growth Index
$H_{37}RV$	:	Human Rough Virulent
HCL	:	Hydro chloric acid

HEPA	:	High efficient Particulate Air
HPF	:	High Power Field
HPLC	:	High Performance Liquid Chromatography
I	:	Isoniazid
ID	:	Identification
IQC	:	Internal Quality Control
ITM	:	Institute of Thoracic Medicine
K	:	Kanamycin
LED	:	Light Emitting Diode
LJ	:	Lowenstein Jensen
LT	:	Laboratory Technician
MA	:	MacConkey Agar
MDR	:	Multi Drug Resistant
MGIT	:	Mycobacterium Growth Indicator Tube
MHA	:	Muller Hinton Agar
MIC	:	Minimum Inhibitory Concentration
NALC	:	N-Acetyl L- Cysteine
NaOH	:	Sodium Hydroxide
NAP	:	ρ-nitro-α-acetyl amino-β-hydroxypropiophenone
NRL	:	National Reference Laboratory
NTM	:	Non tuberculosis mycobacteria
OF	:	Ofloxacin
OI	:	Opportunistic Infection
PBS	:	Phosphate Buffer Saline
PNB	:	Para Nitro Benzoic Acid

PANTA	:	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PZA	:	Pyrazinamide
R	:	Rifampicin
RCF	:	Relative Centrifugal Force
RF	:	Reference
RNTCP	:	Revised National Tuberculosis Program
RPM	:	Rotation Per Minute
RRT	:	Relative Retention Time
S	:	Streptomycin
SK	:	Kirchner's
SP	:	Sodium Pyruvate
SSMG	:	Salt Solution Malachite Green
STLS	:	Senior Tuberculosis Laboratory Supervisor
SWG	:	Standard Wire Gauze
T. No	:	Treatment Number
Th	:	Ethionamide
UMB	:	Unidentified Mycobacterium
UV	:	Ultraviolet
ZN	:	Ziehl Neelsen

LIST OF STAFF IN DEPARTMENT OF BACTERIOLOGY

S. No.	Name	Designation
1	Dr. N. Selvakumar	Scientist – F
2	Dr. Vanaja Kumar	Scientist – F
3	Dr. Ranjani Ramachandran	Scientist – D
4	Dr. K. Jayasankar	Technical Officer – B
5	Mr. A. Syam Sundar	Technical Officer – A
6	Mr. S. Jagadeesan	Technical Officer – A
7	Mr. J. Samuel Vasanthan	Technical Officer – A
8	Mr. S. Manoharan	Technical Officer – A
9	Mrs. Lakshmi Sambandam	Technical Officer – A
10	Dr. N. S. Gomathi	Technical Officer – A
11	Dr. Gomathi Sekar	Technical Officer – A
12	Mrs. K. Silambuchelvi	Technical Officer – A
13	Dr. L. Prabakaran	Technical Assistant (Research)
14	Ms. Mariam George	Technical Assistant (Research)
15	Mrs. D. Saraswathi	Technical Assistant (Research)
16	Mr. A. Radhakrishnan	Technical Assistant (Research)
17	Mrs. S. Sivagamasundari	Technical Assistant
18	Mr. M. Anandan	Technical Assistant
19	Mrs. V. Girijalakshmi	Technical Assistant
20	Mr. C. Thirukumar	Technical Assistant
21	Mr. M. Asokan	Technical Assistant
21	Mr. D. Thangaraj	Technical Assistant
22	Mrs. B. Mahizaveni	Technical Assistant
23	Mrs. G. Vadivu	Technical Assistant

S. No.	Name	Designation
24	Mr. K. Rajasekaran	Technician -C
25	Mrs. K. Devika	Technician -C
26	Mr. V. N. Azgar Dusthakeer	Technician -C
27	Mr. B. Daniel	Technician -C
28	Mr. M. Baskaran	Technician -C
29	Mr. V. Thiyagarajan	Technician -C
30	Mr. D. Ravikumar	Technician -C
31	Mr. S. Govindarajan	Technician -C
32	Mr. K. Ramakrishnan	Technician -C
33	Mr. Michel Premkumar	Technician -C
34	Mr. K. Rajaraman	Technician -C
35	Mr. M. Mohan	Technician -C
36	Mr. D. Venugopal	Technician –B
37	Mrs. K. Shanthi	Technician –B
38	Mr. M. Thanigachalam	Technician –A
39	Mr. V. Sundararajan	Attendant
40	Mr. K. Kuttappan	Attendant
41	Mr. A. Rajavarman	Attendant
42	Mr. V. Mohan	Attendant
43	Mr. S. Venkatesan	Attendant
44	Mr. P. Senthilvelan	Attendant
45	Mrs. H. Ponrose	Attendant
46	Mr. J. Stanly Gnanadhas	Stenographer

LIST OF PROJECT STAFF IN DEPARTMENT OF BACTERIOLOGY

S. No.	Name	Designation
1	Dr. S. Prabu Seenivasan	Consultant Microbiologist
2	Dr. R. Radhakrishnan	Consultant Microbiologist
3	Mr. S. Balaji	Senior Research Fellow
4	Mr. M. Radhakrishnan	Senior Research Fellow
5	Mrs. R. Lakshmi	Senior Research Fellow
6	Mrs. I. Jerrine Joseph	Senior Research Fellow
7	Ms. R. Vasanthi	Research Assistant
8	Mr. P. Nagarajan	Sr. Lab. Technician
9	Mr. S. Anbarasu	Sr. Lab. Technician
10.	Mrs. M. Devisangamithrai	Sr. Lab. Technician
11	Mrs. G. Radhika	Lab. Technician
12	Ms. K. Jeyasree	Lab. Technician
13	Mr. Sanjay	Lab Attendant
14	Mr. Navalan	Lab Attendant

CONTENTS

1. SPECIMEN RECEPTION & NUMBERING

- A good sputum sample should consist of recently discharged material from the bronchial tree.
- Quality of the specimen implies the presence of mucoid or mucopurulent material.
- Ideally a sputum specimen should have a volume of approximately 5ml

Materials required

- 5% phenol solution – melt 500 gms of phenol crystals in a boiling water-bath and add to 10 litres of water slowly in a plastic tub
- Racks (12 bottle rack)
- Tongs
- Cotton
- Tissue paper

Type of Specimen received

- Sputum specimen.
- Extra pulmonary specimens such as Urine, Bronchial wash, ascitic fluid CSF, pus, biopsy material, etc.
- Urine specimen for analysis in biochemistry department.

Centers and Sub Centers

- Tuberculosis Research Centre
- Sub centers include; Tambaram Sanatorium, GH and Vellore.
- Receive these specimens directly from patients or through health visitors or attendants.
- From Madurai and Thiruvallur sputum specimens are received in bulks through Lab messengers.
- Madurai specimens
- Thiruvallur – Base line survey, Tobacco survey study and Chennai disease survey

Referred Specimens

Patients may be directly referred to TRC from outside such as Institute of Thoracic Medicine (ITM) and Government Hospital of Thoracic Medicine(GHTM) for Sputum AFB culture and sensitivity testing.

- Extra pulmonary specimens may be referred from other hospitals and clinics (Govt. & Private) for AFB culture and sensitivity.
- Sputum or Urine specimen received for opportunistic infection
- Direct cultures may be received for DST/ ID

Procedure

- TRC registered patients bring sputum specimen and sputum card.
- Check if the bottle is tightly closed; if not, instruct the patient to close it tightly.
- Check if the bottle number on the bottle cap and the sputum card
- If the sputum volume is less than 5ml instruct the patient to collect more sample
- Place the sputum sample in the 12 rack and keep in the 5% phenol tank in such a way that the bottles and caps are completely immersed.
- Leave the bottles for at least 30 minutes in phenol tank.
- Remove the bottles from the tank using tongs and rinse in running water.
- After receiving 12 samples allot consecutive lab numbers and enter on the corresponding cards
- Enter the details in the TRC SPUTUM REGISTER FORM - the patient name, Treatment number (T. No), Bottle number and Lab number given on the sputum cards
- Mention the time of receipt of the 12^{th} specimen on the corresponding sputum card.
- Note the volume and consistency of each sample in the duplicate copy of the sputum register form.
- Send the completed set (12 samples) to the lab through bacteriology lab attendant along with the sputum cards and sputum register forms.

- Do the same for sputum samples received for opportunistic infections and send to the Opportunistic Infection Laboratory
- Send the urine specimens received for biochemical investigations to the biochemistry department along with the urine cards through their staff.

Specimen received from Tiruvallur and Madurai:

- Decontaminate Specimen with 5% of Lysol tank for 30 minutes in wash up room
- Check the bottle number with request form
- Align lab number and document in separate note.

Specimen received from sub centers of TRC

- Specimens are received through Health visitors along with sputum register form mentioning the patients name, T. NO and bottle number
- Check the number on the form and the bottle cap.
- Decontaminate as described early.
- Assign lab numbers for specimens and enter in sputum forms.
- Sent the samples to bacteriology Lab through attendant.
- When extra pulmonary specimens are received from TRC or sub centers: Assign "SP" prefix lab number (SP1023).

Lab numbers that are allotted to Sub centers of TRC specimens

Study center	**T. No.**	**Prefix lab no**
San (Tambaram)	HT	B (eg. , B13424)
GH	HG	S (eg. , S14001)
VELLORE	VO	E (eg. , E5476)
MDR STUDY		DP
MADURAI	M	D (D2424786)
TOBACCO SURVEY STUDY		TS
CHENNAI DISEASE SURVEY		CS
REFERRAL and GHTM OP CASES		RF
STUDY PATIENTS -EXTRA PULMONARY		SP
REFERRAL CASES (EXTRA PULMONARY)		RFEP

Procedure for Referred Samples:

- Check the requisition or Referral slip or letter from the patients.
- Read the request form carefully and get it attested by HOD, Bacteriology or Clinic.
- If sputum for AFB and Culture & Sensitivity is requested, issue a numbered sterile McCartney bottle wrapped with tissue paper to the patient.
- Instruct the patients to collect 5. 0 ml of the sputum into the bottle. (Approximately mark the level using marker)
- Document the patient's name, age, sex, sputum collection date, referral doctor or institute and Patient's / Clinician's address in the sputum Referral form.
- After receiving the sputum specimen, note the consistency and volume of the specimen and immerse it in 5% phenol.
- Assign the lab number in the form and issue the perforated acknowledgement slip to the patient which has patient's name, age, sex, date of specimen and lab number Eg. RF16414.
- For Sputum specimen: Instruct the Patient to bring this slip after three months for the report.
- For Extra Pulmonary specimen: Instruct the Patient to bring this slip after three months for the report.
- If the specimen is the extra pulmonary enter the type of specimen, date of collection.
- Allot RFEP lab number and send to the lab immediately
- If a patient or an attendant comes with a request for collection of extra pulmonary specimen (biopsy, tissue) issue a Kirchner's liquid medium bottle / a numbered sterile bottle (pus, CSF, gastric lavage etc. ,)
- Instruct the patient attendant to bring the sample on the same day of collection.
- Issue 3 sterile wide mouthed bottles for collection of urine samples. Instruct the patient to collect early morning and mid stream urine and to bring it as early as possible

Specimen Received in a Packed Container (by courier)

- Receive all the packed containers from the couriers or individuals and send to the laboratory.

- Open the parcel in the bio safety cabinet and note the nature of the specimen. If the specimen is sputum, send the same to the lab reception along with the request form / letter, for decontamination or decontaminate in the lab in bread boxes.

- Allot the corresponding RF number on the RF form and send to the lab along with specimens.

- If the specimen is direct culture, Send the request form to the Lab reception for allotting the referral lab number

- Whenever there is a special request for BACTEC (RF,SP specimen study 25) write "Bactec" on the bottle cap or on the side of the bottle and the request form

- Document in separate note

Work Procedure at the end of the day

- In the lab reception diary enter the first and the last number against each of the following studies:

- TRC

- RF

- GH (S)

- Vellore (E)

- San (B)

- MDR (DP)

- Extra pulmonary (RF EP)

- Opportunistic infection (OI)

- CPC samples: samples collected in CPC should be stored at room temperature

- Samples received on holidays should be stored accordingly:

(Sputum and other biological specimens should be stored in the cold room)

2. WASHING PROCEDURE

Objective and scope

To describe the washing procedure for glassware

Material required

- Source of hot and cold water
- Containers for distilled water and commercial (5%) hydrochloric acid.
- Continuous supply of distilled water.
- Washing brushes of various sizes for test tubes, conical flasks, McCartney bottles and measuring Cylinders.
- Discard bucket with lid.
- Large size wire baskets (1 x 1) for bottles and small size baskets (½ x ½) for test tubes.
- Acid-resistant gloves
- Washing sink and draining board.
- Adequate bench space.
- Thread
- Gauze

Reagents Required

- 5% Lysol - (9500ml water in 500 ml of commercial Lysol) (sriram chemicals) for main lab use
- 10% Dichromate solution
- 1%Soap solution (Commercial) (Qualigens)
- 5% HCl - (Commercial)
- Non absorbent cotton
- Absorbent cotton
- Aluminum foil
- Acetone

Washing of Glassware from media room

Different quantities of flasks (5, 3, 2 and 1 Lt; 500, 250, 100 and 50 ml)

- Clean the flask with running water
- Rinse with acetone (~ 250 ml to a flask and transfer to next flask) and clean in running water
- Rinse them in 5% HCl (~250 ml to a flask and transfer to next flask) solution
- Clean in running tap water
- Clean with soap solution using a brush
- Clean in running tap water for 4-5 times
- Rinse/soak in distilled water
- Dry it for 5-10 minutes
- Cotton plug is prepared (~12 gm,15 gm,18 gm and 22 gm of non absorbent cotton covered with gauze and tied with thread) for the flask and cover the plug with aluminum foil
- Dry it in oven at 160°C for 1 hr

Washing of McCartney bottles (universal bottles) containing Media

- Autoclave at 121°C for 30 minutes
- Remove the caps separately in plastic box
- Wash the numbered caps only with hot water
- Remove the media by using bottle washing machine (1400 rpm, anticlockwise) by using the brush
- Immense the bottle in a plastic tub containing hot water
- Add 100 ml (5 %) of HCl to the tub containing luke warm water, keep it for overnight.
- Use soap solution and clean the bottles and wash them in running tap water
- Immerse in distilled water for 5 to 10minutes.
- Dry the bottles in inverted position in the wire baskets

Washing Metal caps of McCartney bottles (universal containers)

- Autoclave the McCartney bottles
- Remove the rubber liners from the caps
- Erase the numbers on the caps with acid-acetone (add 50ml acetone into 5 -10 ml of HCl)
- wash in soap water and then in running tap water before replacing them in the caps Check the liners, for their good condition
- Soak the Bijou bottles (7ml capacity) in 10% dichromate solution for 24 hrs
- Wash in running tap water and keep them immersed in distilled water for 5 to 10 minutes
- Air dry in Hot air oven for 3-4 hrs
- Add to the Bijou bottle 3mm dm ~10-12 beads for DST.
- Add one 5mm bead in 0. 5 ml of distilled water for sub culture and ID
- Plain Bijou bottles without beads
- Close the cap before autoclaving

Others

- Wash Culture bin, Lysol bin (rectangular, Lysol bucket), Cotton bin (clinic use), safety disposal bucket (clinic use) in tap water only.

Washing procedures for new slides

- Soak New slides overnight (~1500) in freshly prepared (3 litre 10% dichromate solution) for overnight and wash them in running tap water
- Soak Bijou bottles, niacin tubes, Horlicks bottles, Honey jar bottles in dichromate solution
- Keep them immersed in distilled water for 5 to 10 minutes; drain the water; and keep them at 190 °C for 1 hr in Hot air oven.
- After cooling; arrange in the slide box for main lab use

Autoclave

Five Autoclaves are available in bacteriology Department

The types are

a) 4 horizontal
b) 1 vertical

Autoclaving materials

- Pack clean Catalase tubes, test tubes for swab, grinding tube, Teflon rod (big or small), scissors, forceps, vials, pourer, rod in distilled water and plug cotton packed in brown paper for autoclaving
- Wrap the Funnel inside (½ x ½ size) gauze with brown paper and pack with aluminum foil for autoclave
- Keep clean numbered bottles separately for TRC, Tiruvallur, Madurai, Vellore, CA other studies and autoclave.
- Autoclave Mc Cartney bottles, NaOH, SSMG, Bijou bottles, swab, all tubes, and clinical cotton bin for 121°C for 30 minutes
- For discarded materials / culture, autoclave for 50 minutes to 1hour.

Note:

Autoclave media bottles (culture) at 121°C for 45 minutes; if the media does not turn yellow re - autoclave

Log book: Maintain time and temperature for autoclave and hot air oven by entering in the log book.

Date	Starting Time	Loading time	Setting Temp	Closing time	Opening time	Remarks	signature

Sterilization Indicators for Checking (weekly) Autoclave and Hot air oven

- Place the Temp tubes inside the autoclave while loading
- Note the colour change in tube (red) after autoclave
- Note the colour change in tube (blue) after hot air oven

Indicator Tape

- Paste the indicator tape to all the materials before autoclave
- Note the colour (black lines) change in the tape

Safety Conditions

Follow strictly bio-safety, chemical safety and disposal guidelines.

Note

- Consider all articles discarded in the laboratory excepting those from media preparation room as infectious and sterilized before for washing.
- The caps of culture slopes and sputum cups should be loosened slightly before putting them into the autoclave for sterilization.
- All containers into which infectious material are discarded should be sterilized before washing.
- Sterilize sputum cups and culture slopes are sterilized separately and do not mix with the media containers

3. MEDIA ROOM

PREPARATION OF STAINING SOLUTION

Auramine phenol

Auramine phenol stain (Modification of Blair's method)

Chemicals required:

Auramine (BDH)	:	25 g
Ethanol	:	3000ml
Phenol	:	250 g
Distilled water	:	5300 ml

- Take 25 gm (1 bottle) of Auramine powder & empty the contents into a conical flask (while emptying the bottle, rinse with little ethanol and pour it into the flask)
- Add 500 ml of ethanol (absolute alcohol- TNG *) slowly and stir to dissolve the dye.
- Put the magnet in the flask & place the flask on the magnetic stirrer
- Keep it on the stirrer till the Auramine dye dissolves completely
- Place the phenol bottle (500 gm-Qualigen) in the water bath (80 °C) for 1 hr to melt the phenol crystals.
- Remove from the water bath, measure 250 ml of melted phenol using a measuring cylinder (wear gloves while handling phenol)
- Measure 5300 ml of distilled water & pour it into a plastic bucket (exclusively used for Auramine dye preparation)
- Now add 250 ml of phenol slowly into the bucket taking care to avoid spillage of phenol & mix well using a plastic mug.
- Pour the dissolved Auramine into the phenol solution
- Measure 2500 ml ethanol and pour into the Auramine in successive small volumes so as to dissolve the Auramine dye completely

- Mix thoroughly the Auramine-phenol solutions & distribute in amber colored bottles. Label bottles as 0. 3 % Auramine phenol solution and date of preparation.
- Store in a cool dark place

Acid- alcohol

chemicals required

Sodium Chloride (AR –Qualigen)	:	50g
Hydrochloric acid	:	50 ml
Distilled water	:	1250 ml
Absolute Alcohol	:	3750 ml

- Weigh 50 gm of sodium chloride powder (AR-Qualigen)
- Dissolve this completely in 1250 ml of distilled water in sterile 5 litre conical flask
- Measure 50 ml of Conc. HCL in a sterile measuring jar
- Add the acid slowly to the conical flask taking care not to spill the acid
- Measure 3750 ml of ethanol in a measuring jar & add it the salt acid mixture
- Mix well by rotating the flask
- Store the acid-alcohol mixture in a cool place and label as 1% acid-alcohol
- Write the date of preparation

Potassium Permanganate

Chemicals required

Potassium permanganate (AR Qualigen)	:	5g
Distilled water	:	5000 ml

- Weigh 5 gm of potassium permanganate crystals accurately into a 5 litre conical flask.

- Dissolve this by thorough mixing
- Transfer to a 5litre glass jar & label it as 0. 1 % potassium permanganate ($KMnO_4$)
- Write the date of preparation.

Carbol fuchsin

Chemicals required

Basic fuchsin (Hi media)	:	10g
Absolute alcohol	:	100 ml
Phenol	:	50 g
Distilled water	:	900 ml

- Weigh 10 gm of basic fuchsin dye in a balance & transfer it to 250 ml Erlenmeyer flask
- Add 100 ml of absolute alcohol & dissolve the dye by placing it in a water bath at 60° C. Avoid direct heating
- Place the phenol bottle in the water bath at 80 `C for melting
- Measure 50 ml of phenol and add to the basic fuchsin solution and mix gently
- Transfer the contents into a 1000 ml measuring cylinder
- Add distilled water to make up the final volume to 1000 ml
- Pour the solution through filter paper (whatmann No 1) and store filtered solution in a glass bottle. Label the bottle as 1% Carbol Fuchsin
- Write the date of preparation

25% sulphuric acid (H_2SO_4)

Chemicals required

Conc. H_2SO_4 : 250 ml

Distilled water : 750 ml

- Take 750 ml distilled water in a flask
- Carefully add concentrated sulphuric acid to the water (do not add water to the acid)
- Mix gently and store it in amber coloured bottle and label it as 25 % Sulphuric acid
- Write the date of preparation.

0. 1 % Methylene blue

Chemicals required

Methylene blue (BDH) : 0. 5 g

Distilled water : 500 ml

- Weigh 0. 5 gm of Methylene blue and transfer to a 1L flask
- Add 500 ml of distilled water
- Shake well & dissolve
- Store in a glass bottle with the label as 0. 1 % Methylene blue
- Write the date of preparation.

PREPARATION OF REAGENT & SOLUTION

4 % Sodium hydroxide (NaOH)

Sodium Hydroxide (Qualigen)	:	40 g
Distilled water	:	1000 ml

- Weigh 40 gm of Sodium hydroxide and transfer to a 1 litre flask
- Add 1000 ml of distilled water to it and mix thoroughly until it dissolves
- Distribute in 100 ml aliquot into 250 ml conical flasks and cover the mouth of the flask with cotton bunk
- Autoclave at121^{0}C
- Store at 37^{0}C until use.

Dichromate solution (10 %)

Potassium dichromate powder	:	400 gm
Con. Sulphuric acid	:	1000 ml
Distilled water	:	3000 ml

Preparation

- Weigh 400 gm of potassium dichromate powder
- Dissolve this in 3000 ml Distilled water in 5L Conical flask keep it in the running tap water basin
- Add 1000ml of con H_2SO_4 little by little (so that to prevent heat from the flask) shake well and use it after cooling.

2% Malachite green

Malachite green dye : 25 g

Distilled water : 1250 ml

- Empty the dye in to the mortar and grind the powder using a pestle and by adding little water
- Transfer the dye solution into a conical flask and repeat the grinding procedure till all the malachite green power has been completely removed from the mortar.
- Make up the solution to 1250ml by adding the remaining distilled water.
- Label it as 2% Malachite green solution with date of preparation and store it for 1 week.
- Autoclave it at 121° C for 15 minutes and store it in refrigerator until use

Mineral Salt Malachite Green Solution (SSMG)

Potassium dihydrogen orthophosphate AR, KH_2PO_4	:	14. 4g (0. 4%)
Magnesium sulphate AR	:	1. 44 g (0. 4%)
Magnesium citrate	:	3. 6 g (0. 1%)
L-Asparagine AR	:	21. 6 g (0. 6%)
Glycerol	:	72 ml (2%)
Malachite green 2%solution	:	120 ml
Distilled water	:	3600 ml

- Dissolve the salts in about 300 ml of distilled water
- Add 72 ml of glycerol and 120 ml of malachite green solution
- Make up the volume to 3600 ml with distilled water
- Distribute the solution in 600 ml amounts in 1 litre conical flasks autoclave 121°C for 30 minutes and after cooling store in the refrigerator
- Label it as SSMG with date of preparation.

MEDIA PREPARATION

Precautions

- Media preparation must be done in the media preparation room.
- The room must be kept clean and dust free.
- Working cabinet should be sterilized by UV lamp daily for 20 minutes.
- The benches in the media room should be cleaned with 5% Lysol / phenol solution every day.
- To avoid contamination of media the door must be closed.
- All glasswares have to be sterilized daily.
- Aseptic techniques must be observed at all time by flaming the mouth of the flask before and after removal of the bunk.
- All used glasswares have to be send for cleaning.

Purpose

- The definite diagnosis of tuberculosis demands that *M. tuberculosis* is recovered on culture media and identified using differential in vitro tests.
- Many different media have been devised for cultivating tubercle bacilli and three main groups can be identified viz. Egg based media, Agar based media and Liquid media.
- The ideal, less expensive and most widely used medium for *M. tuberculosis* culture is Lowenstein Jensen medium.

LOWENSTEIN-JENSEN (LJ) MEDIUM (DRUG FREE)

Principle

- Lowenstein-Jensen (LJ) medium is most widely used for tuberculosis culture.
- LJ medium containing glycerol favors the growth of *M. tuberculosis* while LJ medium without glycerol but containing Pyruvate encourages the growth of *M. bovis* as well as drug resistant strains of M. tuberculosis.
- The malachite green suppresses the growth of non acid fast organisms. (L-Asparagine for nitrogen source).

Homogenisation of egg

- Select eggs not older than 7 days for the preparation of egg fluid
- (Note: Hens should be fed on food without antibiotics)
- Check Fresh eggs for minimum air space are checked for viability and is done by candling method.
- Clean eggs with soap water; Place in a basin and wash in running water until the water is clear, then rinse in distilled water and then again immerse finally in 70% alcohol for 5 minutes; Place the eggs on a clean towel to dry.
- Break the eggs individually and transfer into a stainless steel beaker and transfer the egg fluid into a 2 litres round flat bottomed flask.
- Homogenise the egg fluid using a mechanical egg churner
- Filter the egg fluid using a sterile gauze and funnel
- Measure one litre of egg fluid using a sterile measuring cylinder and transfer into a 3 or 5 litres conical flask.
- Transfer 600 ml of the sterilized mineral salt malachite green solution to the egg fluid
- Gently shake to mix thoroughly.
- Fix the pourer to the mouth of the conical flask and distribute approximately 6 ml of medium in Universal container (McCartney bottle).

Coagulation of media

- Pour distilled water into the Inspissator tank through the side opening up to the mark

- Place the bottles in the Inspissator to coagulate the media for 60 minutes at 85°C -90 °C
- Remove after 60 minutes from the Inspissator and leave at room temperature.
- Record the Inspissator temperature periodically in a note book (every 15 minutes)
- Re Inspissator the bottles at 85^0-90^0 C for 30 minutes on the consecutive day after overnight storage at room temperature.
- Label the media tray with batch number and date of preparation. The same should be recorded in the Media Preparation Register.

Sterility check

After inspissation randomly the whole media batch should be incubated at 37 ^{0} C for 24 hours select 2 bottles of plain LJ for sterility check and record in the Media Sterility register.

LOWENSTEIN- JENSEN MEDIUM WITH ANTI TB DRUGS

- Media preparation must be done in the media preparation room.
- The room should kept clean and dust free.
- Sterlise working cabinet by UV lamp daily for 20 minutes.
- The benches in the media room should be cleaned with 5% Lysol / phenol solution or 70% alcohol every day.
- To avoid contamination of media the door must be closed.
- All glasswares must to be sterilized daily.
- Aseptic techniques must be observed at all time by flaming the mouth of the flask before and after removal of the bunk.
- All used glasswares have to be sending for cleaning.

Lowenstein Jensen medium with drug

- To one litre of egg fluid add 600 ml of SSMG; mix well, till uniform pale green color is obtained.
- Preparation of drug containing media is done according to the requirement.
- Place the bottles in the Inspissator and coagulate the medium for 60 minutes at 85-90^0 C.
- After 60 minutes bottles are removed from the Inspissator and arranged in a tray.
- Label the media tray with its drug name, batch number and date of preparation.
- The same should also be recorded in the Media Preparation Register.

First line drugs	**Second line drugs**
Streptomycin (S)	Kanamycin (K)
Isoniazid (I)	Ethionamide (TH)
Rifampicin (R)	Ofloxacin (OF)
Ethambutol (E)	

Preparation of stock solution and various concentration of the drugs.

STREPTOMYCIN SULPHATE (S)

Stock solution

- Weigh accurately 250 mg of streptomycin sulphate (S) using butter paper and electronic balance.
- Transfer the weighed drug into a sterile McCartney bottle
- Pipette out 20 ml of sterile distilled water to the McCartney bottle
- The final concentration of the stock solution is 10, 000 µg/ml.

Working dilutions:

- 4 ml of 10,000 µg/ml+16 ml distilled water =2000 µg/ml
- 1 ml of 2000 µg/ml +19 ml distilled water =100 µg/ml

Media solution:

- For 100 slopes each of 8, 16, 32 &64 µg/ml and 16 slopes each of 2 & 4 µg/ml.

ML of stock (µg/ml)	L-J fluid	Final Conc. (µg/ml)
2. 0 ml of 100 µg/ml	100	2
4. 0 ml of 100 µg/ml	100	4
2. 4 ml of 2000 µg/ml	600	8
4. 8 ml of 2000 µg/ml	600	16
9. 6 ml of 2000 µg/ml	600	32
3. 84 ml of 10,000 µg/ml	600	64

SD_4 for Proportion sensitivity testing (PST)

Stock solution

- Weigh accurately 125 mg of dihydrostreptomycin sulphate powder (SD4) using butter paper and electronic balance.
- Transfer the weighed drug into a sterile McCartney bottle
- Pipette out 10 ml of sterile distilled water to the McCartney bottle
- The final concentration of the stock solution is 10, 000 µg/ml.

Working solution

- 2 ml of 10,000 µg/ml+8 ml distilled water = 2000 µg/ml.

Media Solution

ML of stock solution (µg/ml)	**L-J fluid**
1. 2 ml of 2000 µg/ml	600

ISONIAZID (I)

Stock solution

- Weigh accurately 200 mg of isoniazid (I) powder using butter paper and electronic balance.
- Transfer the weighed drug into a sterile McCartney bottle.
- Pipette out 20 ml of sterile distilled water to the McCartney bottle.
- The final concentration of the stock solution is 10, 000 µg/ml.
- Shake well to dissolve completely.
- Filter the solution using a membrane filter.
- Keep the filtered solution frozen and use it up to 1 month.

Working solution

ML of stock solution (µg/ml)	Distilled Water(ml)	Final Conc(µg/ml)
1 ml of 10,000	9	1000
2 ml of 1000	18	100
1 ml of 100	19	5
2 ml of 5	8	1

Media preparation

For 100 slopes each of 0. 2, 1 & 5 µg/ml and 16 slopes each of 0. 025, 0. 05 & 0. 1 µg/ml.

ML of Stock solution (µg/ml)	L-J Fluid (ml)	Fluid Conc. (µg/ml)
2. 5 ml. (1 µg/ml)	100	0. 025
5 ml. (1 µg/ml)	100	0. 05
2 ml. (5 µg/ml)	100	0. 1
1. 2 ml. (100 µg/ml)	600	0. 2
6 ml. (100 µg/ml)	600	1. 0
3 ml. (1000 µg/ml)	600	5. 0

RIFAMPICIN

Stock solution

- Weigh accurately 200 mg of rifampicin using butter paper and electronic balance.
- Transfer the weight drug into a sterile McCartney bottle.
- Pipette out 20 ml of dimethyl formamide to the drug and shake well.
- The final concentration of the stock solution is 10, 000 µg/ml.

Working solution:

Stock solution (µg/ml)	Distilled Water	µg/ml
4 ml of 10,000	16 ml	2000
2 ml of 2000	18 ml	200

Media preparation:

For 100 slopes each of 32, 64 & 128 µg/ml & 16 slopes of each 4, 8 &16 µg/ml.

ML of stock solution (µg/ml)	LJ fluid (ml).	Final Conc. (µg/ml)
2. 0 ml of 200 µg/ml	100	4
4 ml of 200 µg/ml	100	8
8 ml of 200 µg/ml	100	16
9. 6 ml of 2000 µg/ml	600	32
3. 84 ml of 10,000 µg/ml	600	64
7. 68 ml of 10,000 µg/ml	600	128

Proportion sensitivity testing (PST) (R 40)

ML of stock solution (µg/ml)	LJ fluid (ml).	Final Conc. (µg/ml)
2. 4 ml of 10,000 µg/ml	600	40

ETHAMBUTOL (EMB)

Stock solution:

- Weigh accurately 270 mg of Ethambutol hydrochloride using butter paper and electronic balance.
- Transfer the weighed drug into a sterile McCartney bottle
- Pipette out 20 ml of sterile distilled water to the drug and shake well.
- Sterilize by filtering through a membrane filter.
- The final concentration of the stock solution is 10,000 µg/ml.

Working solution:

- 2 ml of 10,000 µg/ml +18 ml water = 1000 µg/ml
- 10 ml of 1000 µg/ml +10 ml water = 500 µg/ml
- 1 ml of 500 µg/ml + 9 ml water = 50 µg/ml

Media preparation:

For 100 slopes each of 2,4 & 8 µg/ml and 16 slopes of each 0. 5 & 1. 0 µg/ml.

ML of stock solution (µg/ml)	L-J fluid (ml)	Fluid Conc (µg/ml)
1. 0 ml. (50 µg/ml)	100	0. 5
2. 0 ml. (50 µg/ml)	100	1. 0
2. 4 ml. (500 µg/ml)	600	2. 0
4. 8 ml. (500 µg/ml)	600	4. 0
4. 8 ml. (1000µg/ml)	600	8. 0

KANAMYCIN (K)

Stock solution:

- Weigh accurately 128 mg of Kanamycin using butter paper and electronic balance.
- Transfer the weighed drug into a sterile McCartney bottle
- Pipette out 10 ml of sterile distilled water to the drug and shake well.
- The final concentration of the stock solution is 10,000 µg/ml.

Working dilutions

4 ml of 10,000 µg/ml + 6 ml water = 4000 µg/ml

2 ml of 10,000 µg/ml +18 ml water = 1000 µg/ml

1 ml of 1000 µg/ml + 19 ml water = 50 µg/ml

Media preparation

For approximately 50 slopes of 8, 16, 32 & 64 µg/ml and 8 slopes of each 2 & 4 µg/ml

ML of Stock solution (µg/ml)	**LJ fluid (ml)**	**Final Conc (µg/ml)**
2. 0 ml of 50 µg/ml	50	2
4. 0 ml of 50 µg/ml	50	4
2. 4 ml of 1000 µg/ml	300	8
4. 8 ml of 1000 µg/ml	300	16
2. 4 ml of 4000 µg/ml	300	32
4. 8 ml of 4000 µg/ml	300	64

Proportion sensitivity testing (PST) (K 30)

ML of stock solution (µg/ml)	**LJ fluid (ml)**	**Final Conc. (µg/ml)**
4. 5 ml of 4000 µg/ml	600	30

ETHIONAMIDE (ETH)

Stock solution:

- weigh accurately 200 mg of Ethionamide using butter paper and electronic balance
- Transfer the weighed drug into a sterile McCartney bottle
- Pipette out 20 ml of Triethylene glycol (Trigol), to the drug.
- Mix well and keep at 37°C overnight.
- The final concentration of the stock solution is 10,000 µg/ml.

ML stock solution	water (ml)	µg/ml
6. 0 (10000 µg/ml)	14	3000
8. 55 (10000 µg/ml)	11. 45	4275

Media preparation:

For approximately 50 slopes of each concentration.

Solution (ml)	LJ fluid (ml)	Fluid Conc (µg/ml)
2. 0ml. (3000µg/ml)	300	20
2. 0 ml. (4275µg/ml)	300	28. 5
4. 0 ml. (3000µg/ml)	300	40
4. 0 ml. (4275µg/ml)	300	57
8. 0 ml. (3000µg/ml)	300	80
8. 0 ml. (4275µg/ml)	300	114

OFLOXACIN (OF)

Stock solution

- Weigh accurately 100 mg of Ofloxacin using butter paper and electronic balance.
- Transfer the weighed drug into a sterile McCartney bottle.
- Pipette out 10 ml of 0. 1N sodium hydroxide solution (1ml of 4% NaOH + 9 ml of distilled water) to the drug and shake well.
- The final concentration of the stock solution is 10,000 µg/ml.

Working solution

Stock (µg/ml)	Water (ml)	Final Conc (µg/ml)
2 ml of 10,000	18	1000
1ml of 1000	19	50

Media preparation

For 100 slopes each of 2, 4 & 8 µg/ml and 16 slopes of each 0. 5 & 1. 0 µg/ml

ML of stock (µg/ml)	LJ (ml)	Final Conc (µg/ml)
1. 0 ml of 50 µg/ml	100	0. 5
2. 0 ml of 50 µg/ml	100	1. 0
1. 2 ml of 1000 µg/ml	600	2. 0
2. 4 ml of 1000 ug /ml	600	4. 0
4. 8 ml of 1000 µg/ml	600	8. 0

AMIKACIN

Weigh 0. 139 g of Amikacin in 10 ml sterile distilled water to give 10000 µg/ml or (0. 209 mg of Amikacin in 15 ml of Distilled water)(potency of Amikacin =716 mg /gm)

Working solution

4ml of 10000 µg/ml +16 ml of Distilled water = 2000 µg/ml

1ml of 2000 µg/ml +19 ml Distilled water = 100 µg/ml

Median Preparation

For approximately 100 slopes of 8, 16,32,40,64 and 16 slopes of each 2& 4

Stock solution	LJ fluid	Final Conc. (µg/ml)
2ml of 100 µg/ml	100	2
4 ml of 100 µg/ml	100	4
2. 4 ml of 2000 µg/ml	600	8
4. 8 ml of 2000 µg/ml	600	16
9. 6 ml of 2000 µg/ml	600	32
2. 4 ml of 10000 µg/ml	600	40
3. 84 ml of 10000 µg/ml	600	64

CAPRIOMYCIN

Weighed 0.117 mg Capreomycin powder dissolved in 10 ml of sterile distilled water to give 10,000 µg/ml (potency differs recalculate accordingly)Potency of Capreomycin =8. 55 mg /gm

Working Solution

4 ml of 10000 µg / ml + 16 ml water = 2000 µg/ml

1 ml of 2000 µg / ml +19 ml water = 100 µg / ml

Media Preparation

For approximately 100 slopes 8,16,32,40 & 64 and 16 slopes of 2, 4

Stock solution (µg/ml)	LJ fluid (ml)	Final Conc (µg/ml)
2ml of 100	100	2
4 ml of 100	100	4
2. 4 ml of 2000	600	8
4. 8 ml of 2000	600	16
9. 6 ml of 2000	600	32
2. 4 ml of 10000	600	40
3. 84 ml of 10000	600	64

Lowenstein- Jenson medium with Sodium Pyruvate (SP)

- Add 8 gm of Sodium Pyruvate to every 600 ml of autoclaved SSMG (without glycerol)
- Add 1000ml of egg fluid.
- Distribute and inspissate.

LOWENSTEIN -JENSEN MEDIUM WITH P-NITROBENZOIC ACID (PNB)

Stock solution

Weigh out 800mg of PNB and add 25 ml of dimethyl formaldehyde. Mix to dissolve, add 1600ml of L-J fluid giving a final concentration of 500 µg/ml. Distribute and inspissate. This will give approx. 250 slopes.

Sterility Check

- After inspissations the whole media batch should be incubated at 37^0 C C for 24 hours.
- The media bottles are randomly selected (1set) and incubated at 37^0 C for 14 days.
- Sterility should be recorded in the Media Sterility Register.
- If bacterial and fungal contamination is noted the entire batch is to be rejected.

Selective Kirchner's Medium

(For culture of extra pulmonary specimens)

Composition

Disodium hydrogen phosphate,Na_2HPO_4. 12 H_2O	:	19. 0 g (7. 5g of anhydrous salt)
Potassium dihydrogen phosphate, KH_2PO_4	:	2. 0 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	:	0. 6 g.
Trisodium Citrate	:	2. 5 g.
L-asparagine	:	5. 0 g.
Casein hydrolysate (Bacto casitone)	:	0. 5 g.
Glycerol	:	20. 0 ml
Phenol red, 0. 4% solution*	:	3. 0 ml
Distilled water to	:	1 litre

- Check pH to 6. 9 – 7. 2. Autoclave at 15 lbs/15 minutes. The autoclaved salt solution can be stored in the cold until used. Prepare by dissolving 0. 4g phenol red dye in 100 ml distilled water. Can be stored indefinitely.

- On the day of preparation of the final medium, the antibiotics mixture as well as the calf serum is added as described below.

- Into a dry sterile universal container add the following:

- Polymyxin B : 200,000 units

- Polymyxin is usually available in vials of 5,000,000 units (Sigma). To this vial, 10ml of distilled water is added. The resulting solution contains 500,000 units/ml. From this, 0. 4 ml is used per litre of medium (200,000 units). The remaining solution can be stored frozen for later use.

Amphotericin B, solubilized	:	0. 01g
Carbenicillin	:	0. 100g
Trimethoprim	:	0. 01g
Vancomycin	:	0. 01g

- Dissolve the above in 5 ml sterile distilled water and add to 1 litre of the sterile salt solution.

- Finally, add sterile calf serum 100 ml (filter through 0. 45µ membrane filter).

- Mix well; distribute in 5-6 ml amounts in sterile universal containers. Check sterility by overnight incubation at 37^0C and store in the cold.

MIDDLEBROOK'S 7H 9 LIQUID MEDIUM

Salt solution:

Disodium anhydrous hydrogen phosphate (Na_2HPO_4)	:	2. 5g
Potassium dihydrogen orthophosphate (KH_2PO_4)	:	1. 0 g
Ammonium sulphate (NH_4SO_4)	:	0. 5g
L-sodium glutamate	:	0. 5g
Trisodium citrate ($2H_2O$)	:	0. 1g
Pyridoxine hydrochloride	:	1. 0 ml of 0. 1% aq. soln.
Biotin	:	1. 0 ml of 0. 05% aq. soln.
Ferric ammonium citrate (green)	:	0. 5 ml of 8% aq. soln.
Magnesium sulphate ($MgSO_4.7H_2O$)	:	1. 0 ml of 5% aq. soln.
Calcium chloride ($CaCl_2.2H_2O$)	:	1. 0 ml of 0. 05% aq. soln.
Zinc sulphate ($ZnSO_4.7H_2O$)	:	1. 0 ml of 0. 1% aq. soln.
Cupric sulphate ($CuSO_4.5H_2O$)	:	1. 0 ml of 0. 1% aq. soln.
Tween-80, 10% (For obtaining dispersed cultures)	:	5. 0 ml
(Or)		
Glycerol	:	5. 0 ml
Distilled water to	:	900 ml.

Mix well, distribute in 95 ml amounts and sterilize at 15 lbs/15 mins.

The salt solution can also be prepared by using Difco dehydrated powder.

Weigh 4. 7 g of dehydrated base into a 2 litre flask, add 900 ml distilled water and 0. 5 ml of Tween-80 or Glycerol. Mix well, distribute in 95 ml amounts and autoclave at 15 lbs/15 minutes.

Before use, to each 95 ml salt solution add aseptically 5 ml sterile ADC (bovine albumin-dextrose-catalase) solution and mix well.

Distribute in 5-10 ml amounts in sterile universal containers, check sterility by overnight incubation at 37^{o}C and store in the cold.

ADC supplement

Bovine albumin, Fraction V : 10g.

Glucose, A. R. (dextrose) : 4g.

Catalase : 3 mg*

* Dissolve 30 mg catalase in 10 ml water by vigorous shaking and add 1 ml of this solution.

Distilled water : 100ml.

Mix well and sterilize by Seitz filtration or membrane filtration.

Documentation in Media room

Inspissation time register

Date	Time		Media	I/II	Inspissator temperature (^{0} C)				
03-05-2010	11. 00	12. 00	Plain	I	85. 5	86. 2	85. 4	87. 1	88. 2
04-05-2010	11. 30	12. 00	Plain	II	86. 1	85. 2	85. 5	86. 8	87. 1
	12. 15	1. 15	Plain/drug	I	86. 5	86. 2	86. 8	86	87. 5
05-05-2010	11. 00	11. 30	Plain	II	85. 5	86. 2	85. 4		
	12. 00	1. 00	Plain/drug	I	87. 2	87. 3	85. 8	86. 8	85. 8
06-05-2010	11. 00	11. 30	Plain	II	87. 1	86. 2			
	12. 15	1. 15	Plain	I	87. 2	85. 2	86. 8	87. 1	87. 3

Media request register

Date of request	Request of drug media	Number of slopes(Conc)	Study	Name of the request person	Prepared by	Date of preparation
3-5-2010	Strep	16(2)16 (4) 100(8) 150 (16)100 (32)100 (64)	Routine	Devika	Anandan	4-5-2010
4-5-2010	EMB	16(0. 5) 16(1. 0) 100 (2. 0)150(4. 0) 100(8. 0)	Routine/ RF	Rajaraman	Anandan	5-5-2010
7-5-2010	PNB	250	ID	Daniel	Anandan	11-5-2010

Batch number register

Date	Plain/drug	Batch number	Remarks
4-5-2010	Plain	18464	
5-5-2010	EMB	18465	
	Plain	18466	
6-5-2010	INAH	18467	
	Plain	18468	
7-5-2010	KANA	18469	
	Plain	18470	

Media sterility register

Date	Plain/drug	Batch number	14th day	Remarks
7-4-2010	RMP	18432	22-4-2010	Nil
	R40	18433	22-4-2010	Nil
8-4-2010	Plain	18434	23-4-2010	Nil
9-4-2010	Plain	18435	26-4-2010	Nil
12-4-2010	Plain	18436	27-4-2010	Nil

ZIEHL NEELSEN (ZN) STAINING

Introduction

Dr. **Franz Ziehl** (1857-1926) was a German bacteriologist in Lubek. He introduced the Carbol fuchsin stain for the tubercle bacillus in 1882. With **Friedrich Neelsen** (1854-1894), pathologist Ziehl developed the Ziehl-Neelsen stain, which is used to identify acid-fast mycobacteria.

Ziehl described a new method in a paper published on 12th August 1882 which showed that the solutions could be acidic rather than alkaline. The new stain was less damaging to tissue preparations of tubercles while still permitting the visualization of the causative organisms. With minor modifications this is the stain used in routine today.

Principle

The property of acid-fastness of Mycobacteria is based on the presence of Mycolic acid in their cell wall. Primary stain (fuchsin) binds to cell wall Mycolic acids. Intense decolourization (strong acid) does not release primary stain from the cell wall and AFB retain the red colour of fuchsin. Counter stain (Methylene blue) provides contrasting background.

Materials required

- Sputum container to collect sputum.
- Sterile 1 oz. universal containers with identification number engraved cap.
- Wire loop with an inner diameter of 5 mm to spread sputum on the slide
- Clean new, washed microscopy slide (no grease and no scratches on the slide)
- Diamond marker to enter identification number on the microscopy slide
- Forceps to hold slide with sputum smear
- Bunsen burner to fix smear
- Metal waste bin with disinfectant (5% phenol solution) to discard infected material

- Staining rack to hold the slides
- Slide rack to place stained smear slides to dry in the air
- 1% Carbol-fuchsin
- 25% H_2SO_4
- 0. 1% Methylene blue
- Tap Water

Collection of Sputum collection, selection of the purulent portion for smear preparation and making smear is critical for good quality of smears.

Size: Take purulent portion of sputum and prepare 2 - 3 cm length X 1 - 2 cm wide **or** 3 X 2 cm (100-150 fields to be counted in one length) smear in the center of the slide.

Evenness: Firmly make smear perpendicular to the slide (move in small concentric circles or coil like patterns).

Thickness: Place the slides on the piece of printed-paper. If letters cannot read it is too thick.

Allow the smear to air dry completely at room temperature. After air drying, fix the slide by passing it on the flame 3-4 times

Staining Procedure

- Place the slides on a staining rack in batches (maximum 12) with the smeared side facing up. Ensure that the slides do not touch each other
- Flood entire slide with filtered 1 % Carbol-fuchsin.
- Heat each slide slowly until it is steaming. Do not boil. Maintain steaming for five minutes by using intermittent heat.

- Rinse each slide individually in a gentle stream of running water until all free stain is washed away
- Flood the slide with the 25 % H_2SO4 solution for 2-3 minutes.

- Rinse the slide thoroughly with water. Drain off excess water from the slide.
- Flood the slide with 0. 1% Methylene blue for 30 seconds
- Rinse the slide thoroughly with water. Drain excess water from the slide. Allow smear to air dry. Do not heat or use blotting paper.

Examination and Reporting (ZN Microscopy)

- Use the objective 100x
- Apply one drop of Liquid paraffin oil (heavy) immersion oil to the left edge of the stained smear
- Scan the stained smear systematically from left to right side
- Count AFB in low positive smears for quantification. (Scanty &1+)
- Always search for useful areas, i. e. those containing mucoid threads and pus cells; do this by moving up or down when arriving at an almost empty area, till another useful zone has been found, then continue moving to the left.
- Grade the smear according to WHO guidelines (Table-1)
- Place the slide smear-down on a piece of absorbent paper (absorbent tissue paper,) after examination; let the oil soak in and do not rub
- At the end of the day, store the slides in a slide box
- Do not write the result on the slide
- Clean the objective lens at the end of each day using lens or soft tissue

Reporting

The number of bacilli seen in a smear reflects severity of illness and patient's infectivity.

Table: Grading Chart for ZN Microscopy

(100x oil immersion objective and 10x eye piece)

ZN staining grading (RNTCP)		**Reporting /Grading**
>10 AFB/field after examination of 20 fields	:	Positive, 3+
1-10 AFB/field after examination of 50 fields	:	Positive, 2+
10-99 AFB/100 field	:	Positive, 1+
1-9 AFB/100 field	:	Positive, Scanty
No AFB per 100 fields	:	Negative

Reason for false Positive

- If old slides are re-used for smear microscopy
- If Un filtered Carbol fuchsin is used
- If Smears are insufficiently decolourised
- If Oil applicator touches the sputum smear
- If Oil immersion touches the sputum smear

Consequences of False positive results

- Wrong diagnosis
- Unnecessary extension of Intensive Phase
- Wastage of Drugs

Reason for false Negative

- If saliva is selected for making smears
- If Smear is not fixed properly
- If Carbol fuchsin is not heated
- If Carbol fuchsin is boiled
- If the sputum smears are excessively decolourised

- If the sputum smears are not examined sufficiently (5 mints)

Consequences of False Negative results

- Patients miss the Diagnosis of Tuberculosis
- Intensive phase will not be extended
- Patient loose the faith in the programme

Reason for both false positive and false negative

- If the Laboratory number is not written properly
- If the sputum results are not recorded clearly
- If the sputum results are not reported correctly

Internal QC

Internal QC for freshly prepared staining solutions

- Prepare batches of manufactured slides as per SOP.
- Prepare at least 20 control smears from positive (1+) and Negative specimens
- Check every newly prepared staining reagents with unstained control smears, using at least one positive, with approximately known number (1+) of AFB, and one negative slide
- Examine the controls as above, and note the results in the QC logbook, under the batch number (and /or preparation date) of the new reagents

Unacceptable control results include the following

- If AFB in the positive control (s) are not stained strongly red, or are clearly too few in number than the excepted average AFB/100 fields
- If Positive control background remains red or contains precipitates
- If Negative control shows AFB (possibly from contaminated water)
- If unacceptable control results observed discard the entire batch of reagents

AURAMINE PHENOL STAINING

Principle

Mycobacteria retain the primary stain even after decolorizing with acid-alcohol; Hence the term "acid-fast". A counter-stain is employed to highlight the stained organisms for easier recognition. With Auramine staining, the bacilli appear as slender golden yellow fluorescent rods, standing out clearly against a dark background

Materials required for staining

- 0. 3% Auramine Phenol solution
- 1% Acid alcohol
- 0. 1% Potassium permanganate solution

Staining Procedure

- Place the slides on a staining rack, with the smeared side facing up, the slides should not touch each other
- Flood the slides with freshly filtered 0. 3% Auramine-phenol.
- Let it stand for 7-10 minutes
- Wash well with running tap water, taking care to control the flow of water so as to prevent washing away of the smear
- Drain the water from the slides
- Decolorize by covering completely with 1% acid-alcohol for 1-2 minutes
- Wash well with running tap water
- Drain the water from the slides
- Counter stain with 0. 1% potassium permanganate for 30 -45 seconds
- Wash well with tap water and allow the slides in slanting position to dry in hot plate maintained at 75- 80 °C

EXAMINATION & REPORTING

(FLUORESCENCE MICROSCOPY)

Smear examination procedure and reporting results

- Switch on the mercury vapor lamp. The bulb takes approximately 10 minutes to reach full intensity.
- Using the low power objective (magnification 100-150x) first examine a known positive slide to ensure that the microscope is correctly set up.
- Report positive if a smear contains a minimum of 4 AFB of typical bacilli in at least 50 fields.
- If less than 4 bacilli are present, report as negative. However, the number of bacilli, is noted in the smear record book.
- Confirm bacillary morphology with a high power objective (magnification 400x) Do it for all doubtful smears as well as smears that are scanty positives.
- For positive smears at least 50 fields have to be screened.
- Report the smear as negative if no AFB is seen in at least 100 fields.

Grade positive smears into three degrees of positivity using the high power field (HPF) as below

No. of bacilli		**Grade**
At least 4 in 100 HPF or <6 per HPF in at least 50 HPF	:	1+
6-100 in at least 50 HPF	:	2+
> 100 in at least 50 HPF	:	3+
No bacilli (0-3) 100 HPF	:	Negative

ISOLATION OF *M. TUBERCULOSIS*

4 % SODIUM HYDROXIDE (NaOH) METHOD /MODIFIED PETROFF'S PROCEDURE

Objective & Scope

- To isolate *M. tuberculosis* from sputum specimens for diagnosis, speciation and drug susceptibility testing.

- *M. tuberculosis* grows slowly, taking three to six weeks or longer to give visible colonies and requires specific media for isolation. Due of the long incubation time required, cultures are usually made in McCartney bottles (or standard 25ml metal stopped bottles) and to prevent drying of the cultures.

- Clinical specimens submitted to the tuberculosis culture are contaminated to varying degrees by more rapidly growing unwanted normal flora.

- Most specimens must therefore, be subjected to a harsh digestion and decontamination procedure that liquefies the organic debris and eliminates contaminants.

- However care should be taken not to kill tubercle bacilli to obtain a good recovery.

The clinical specimens submitted for culture in mycobacteriology are of 2 types.

a. Specimens like sputum, urine and pus from sinuses are contaminated with other organisms

b. Specimens like CSF, lymph node aspirates, biopsy materials etc. are aseptically collected under sterile conditions.

- Satisfactory quality is indicated by mucoid (or) mucopurulent material. Ideally, a sputum specimen should have a volume of 4-5ml, although smaller quantities are acceptable

- Specimens should be transported to the laboratory as soon as possible after collection. If delay is unavoidable, the specimens should be refrigerated at 4°C to inhibit the growth of unwanted microorganisms.

- If refrigeration is not possible and a delay of more than 3 days is excepted a suitable preservative 1% Cetyl Pyridinium chloride (CPC) along 2 % sodium chloride solution is recommended.

- Always digest/decontaminate the whole specimen, i. e. , do not attempt to select portions of the specimen.

Material required

- Incubator set at- 37°C.
- Aerosol containment Centrifuge (Megafuge-1, Megafuge-11), capable of speed up to at least 3000 g, fitted with rotor to take at least 12 Mc Cartney bottles.
- Pan balance.
- Bio-safety cabinet.
- One culture bin
- Two 5 mm wire loops (Nichrome wire of 27 SWG).
- One Lysol bin with lid for disposable waste.
- Wire baskets or racks to hold 12 Mc Cartney tubes.
- Wire racks for holding 150 universal containers.
- Stock of clean, sterile Mc Cartney bottles.
- Stock of LJ slopes.
- Sterile 4% NaOH solution (as per SOP)
- Sterile distilled water in 500ml conical flask.
- Diamond marker pencils.
- Timer.

Specimens are to be checked against the accompanying lab cards

- Label the caps with the lab serial number of the specimen

Sputum processing by 4% NaOH method

- Add double the volume of sterile 4% NaOH
- Tighten the caps of the McCartney bottles and mix it well by hand for 1 minute.
- Invert each bottle to ensure that NaOH solution contacts all the sides and inner portion of caps
- In case of Leakage, change the caps of McCartney bottle.

- Place the bottles in shaker for 10-15minutes
- At the end of 15 minutes, Insert the bottles in centrifuge bucket
- Balance the buckets properly in pan balance before keeping for centrifuge at 3000 g for 15 minutes
- At the end of 15 minutes remove the McCartney bottles from the centrifuge without shaking.
- Discard the supernatant fluid into a Lysol bin
- Add sterile distilled water up to the neck of the McCartney bottles
- Mix it well and centrifuge at 3000 g for 15 minutes
- At the end of 15 minutes remove McCartney bottles from the centrifuge without shaking.
- Discard the supernatant fluid slowly into a Lysol bin
- From the sediment, inoculate two slopes of LJ medium
- Use one loopful of sediment for each slope (using a sterile twisted wire loop made up of Nichrome wire (27 SWG).
- Incubate the bottles in rack.
- Label the rack with study name and rack number.

Sterility checks

- Inoculate one loop of randomly selected distilled water flask or 2 LJ slopes and write the date on the bottles.
- Incubate all the LJ media slopes at 37°C
- Keep them in a rack in which that particular batch number was used.
- Check the growth weekly for eight weeks

Documentation:

Record the study name and lab numbers in the respective diary.

Note:

Avoid splashing, minimize aerosols. Whole process should be carried out in a bio-safety cabinet

ISOLATION OF MYCOBACTERIUM TUBERCULOSIS BY CETYL PYRIDINIUM CHLORIDE (CPC) METHOD

Principle

CPC is used to decontaminate and liquefy the specimen. The use of this method not only reduces the number of specimens lost by contamination as a result of prolonged transit time, but also decreases the laboratory time required for processing the specimens.

Materials required

- Vortex Mixer, Same as modified Petroff's method except 4%NaOH

Reagents required

CPC solution

- 1% Cetyl Pyridinium chloride (CPC) (weigh 10 gms of CPC and 20 gm of sodium chloride and dissolve in 1 L of distilled water)
- Distribute 5 ml quantity in bottles and autoclave in 121 °C for 15 minutes.

Procedure

- CPC containing specimens should be processed as described below:
- To the specimen with CPC, add 10-15 ml sterile distilled water (to reduce the viscosity)
- Tighten cap of container and mix well by inversion
- Centrifuge at 3000 g for 15 minutes
- Carefully pour off the supernatant in discarding bin containing Lysol and vortex for 2-3 minutes
- Add approximately 20 ml sterile distilled water and resuspend the sediment
- Centrifuge again at 3000 g for 15 minutes
- Decant supernatant, prepare the deposit smear and inoculate the deposit on to two slopes of LJ medium.
- Incubate all the LJ media slopes at 37°C

Documentation

- Record the study name and lab numbers in the respective diary.

Note

- CPC specimen should be processed after 5 days but within 10 days.
- Use CPC reagent before 1 month.

PROCESSING EXTRA-PULMONARY SPECIMENS

Mycobacteria may not be suspected as the causative agent of an extra-pulmonary disease because the chest x-ray is normal or the tuberculin test is negative, or both. However, based on clinical symptoms and because mycobacteria can infect almost any organ in the body, the laboratory should expect to receive a variety of extra-pulmonary specimens such as body fluids, surgically excised tissues, aspirated or draining pus and urine. Extra Pulmonary specimens divided into two groups: -

Specimens that are free from contaminating normal flora: spinal, pleural, pericardial, synovial, ascitic, blood, pus, bone marrow, tissues (lymph node or tissue biopsies).

Specimens known to contain contaminating normal flora: gastric lavage, bronchial washings and urine.

Since extra-pulmonary specimens, in general, are paucibacillary in nature, their processing methods require milder decontamination. Further, these specimens are inoculated onto multiple media, viz. LJ, LJ, SK medium enriched with sodium pyruvate (SP), and a liquid medium Kirchner's, made selective by incorporating polymyxin B, amphotericin B, carbenicillin, vancomycin and trimethoprim to inhibit growth of other microorganisms (PACT).

Cerebrospinal fluid (CSF) / Broncho Alveolar Lavage (BAL) & Pericardial Fluid

Smear

- Place one loopful of CSF in the middle of a slide, without spreading and allow to air dry.
- Place one more drop of the CSF on the same spot and allow to dry. Place a third drop of the deposit after centrifugation on the same spot, air dry, fix, stain and examine.

Culture

- Culture of CSF is done in two steps 1. Direct inoculation and 2. After decontamination.

Direct

- Inoculate one loopful of CSF onto one slope each of LJ and SP.
- Add 0. 2 ml of CSF into one bottle of SK medium and label these as set **'A'**
- Incubate at $37^{o}C$

Decontamination

- Add 1 ml of 5% H_2SO_4 to CSF
- Mix well and allow it to stand for 15 minutes.
- Fill up bottle with sterile distilled water and centrifuge at 3500 rpm for 15 minutes.
- Discard supernatant carefully
- Inoculate one loopful onto one slope each of LJ and SP.
- Transfer remaining deposit into one bottle of SK.
- Label this set as 'B' and incubate 37 °C.

BAL

Process BAL as CSF except direct inoculation.

Gastric aspirate

- Process immediately after receipt in the laboratory to minimize the lethal action of the high gastric acid content on the mycobacteria.
- Make direct smear and process by modified Petroff's method for culture.
- Add one drop on direct smear, dry and stain
- Inoculate two slopes each of LJ, SP and SK media.

Tissues/biopsy material

Processing of Tissue and Biopsy Specimen for smear and culture

- Cut into small pieces using sterile scissors
- Transfer into a sterile tissue grinder tube and add 5 ml of sterile distilled water and homogenize with sterile Teflon grinding rod.
- Make a direct smear from the homogenate
- Centrifuge the homogenate at 3500 rpm for 15 minutes
- Decant the supernatant carefully
- To deposit add 1 ml sterile distilled water
- Add one drop to the direct smear, dry and stain.
- Add 1 ml 5% H_2SO_4
- Mix well and let stand for 15 minutes
- Fill up bottle with sterile distilled water
- Centrifuge at 3500 rpm for 15 minutes
- Discard supernatant carefully
- To deposit add 0. 2 ml sterile distilled water
- Inoculate onto two slopes each of LJ and SP.
- Transfer remaining deposit into two bottle of SK. Incubate at 37^oC

Needle biopsy specimens

In needle biopsy, a very tiny bit of the tissue is obtained and is transferred directly into SK medium

- Incubate SK at 37^o C.
- If the biopsy is received without SK medium
- Pool the contents of two bottles of SK medium in a single bottle. Add the needle biopsy material. Shake and allow to stand for 10 minutes
- Divide into two aliquots and incubate

Pus

Smear

- Make a direct smear, air dry, fix, stain, and examine.

Culture

If the pus is thick or purulent

- Process by Petroff's method (4% NaoH) as sputum
- Inoculate on two slopes each of LJ slopes, SP and SK

If the pus is thin /diluted

Process as per procedure described for CSF

Urine and Ascetic fluid

- Distribute entire specimen in 20 ml volumes into sterile universal containers and centrifuge at 3500 rpm for 15 minutes ("Centrifuge"). Process the deposits and supernatant as follows:
- **Deposits:** Pool all deposits in a single bottle and Process by 5% H_2SO_4 method
- Inoculate 2 bottles each of LJ, SP and SK media
- **Supernatant:** Collect 1ml of top layer of supernatant and Transfer 3-4 bottles
- To each bottle add 1 ml of 5% H_2SO_4 and 1 ml Sterile distilled water
- Mix and allow to stand for 15 minutes
- Fill up bottle with distilled water and centrifuge
- Inoculate the deposit of Supernatant into two SK medium bottles
- Label as 'DSD' and incubate
- From the supernatant Transfer 1 ml from each bottle into SK medium(Maximum 2 bottles)
- Label as 'DSS' and incubate at 37ºC

Swabs

- If two swabs are provided, Use one swab for making smear and other one for culture.

- If only one swab is provided, do culture alone.

Procedure for Culture of swab

- Immerse the swab in a tube containing 5 ml of sterile 4% sulphuric acid for10 minutes.
- Transfer the swab to another tube containing 5 ml of 1% NaOH and Immerse in it for 1 minute.
- Remove the swab from NaOH.
- Inoculate on LJ, SP.
- Finally transfer the swab into SK medium and Incubate at 37ºC

Decontamination of SK cultures

Examine SK bottles consequently for 6 weeks.

Decontaminate by two ways

- Remove SK bottles within 6 weeks at any time of culture reading if gross turbidity is seen or if granular growth is seen.
- Decontaminate SK bottles using NaOH method:
- Inoculate deposits on 2 LJ slopes.
- Remove the SK bottles at the end of 6 weeks of incubation.
- Decontaminate by NaOH method
- Inoculate deposit on 2 LJ slopes.

CULTURE READING

- Mondays are designated as culture reading days for the purpose of examining cultures.
- Cultures are examined starting from 8th week cultures to 1st week. (1st wk is decided after completing 10 days {up to previous Friday} of inoculation).
- Note: Culture reading can be done a day later if Monday happens to be a holiday.
- Typical colonies of *M. tuberculosis* are rough, buff, tough, non-pigmented (cream coloured) and slow- growers, i. e. Colonies appearing after one to two weeks after inoculation.

Grading of Positive cultures:

<19 colonies	:	Write actual number of colonies
20-100 discrete colonies	:	1 +
> 100 colonies	:	2+
Confluent growth	:	3 +
Contaminated	:	* / * ID

- While taking reading, keep typical cultures, doubtful cultures (atypical morphology), and contaminated cultures are kept separately in a rack for further confirmation (Selection of cultures).
- Entries of cultures during reading: Enter the results in the culture volume and in non negative sheet separately for each study.
- Enter the Lab number and No of slopes of contaminated cultures
- For TRC/Madurai/S/E/B/ TB Spine:
- In the Non negative sheet record 8th week starting number, last number and 1st week last number.

- If both slopes are positive in the same week record the highest degree of growth in the final column.

- If one slope is positive and the other slope is negative, report the culture as positive in the final result column

- Continue reading the second slope until 8th week, if it becomes positive keep this as duplicate.

- If both slopes shows > 20 Cols record 1+ in the final result Column.

- Record the total number of colonies if the sum is less than 20 Cols on both slopes.

- If one slope shows UMB and the other is Neg, enter the final result as Neg/UMB with grading at 8th week.

- If both slopes are UMB on different week enter the growth of first slope as the final result.

- If one slope is contaminated the final result depends on the second slope.

Note Book Entries

- Enter starting and last number of cultures completing 8th week

- These slopes are to be rechecked by a senior person before discarding.

- Contaminated lab numbers should be entered in 1st week note book.

- In 48 Hrs note book cultures contaminated within 48hrs should be entered.

- In 48 hrs if both slopes contaminated verify the sputum card and inform the clinician, if necessary.

Selection of cultures by HOD / Deputy

- Head of the department will check all contaminated cultures, UMB and Positive slopes.

- Doubtful cultures should be confirmed by Ziehl-Neelsen (ZN) staining for AFB. (SOP for ID)

- When culture reading is completed, select the patient's Laboratory culture cards for all positive cultures and enter the laboratory number under the appropriate month of treatment and at the same time indicate the sensitivity tests to be set up in the appropriate column in the culture register.

- Receive Culture cards of new patients from the Statistical Department.

- For pre treatment cultures, select two (different lab numbers) positive cultures for sensitivity tests
- Store a third positive culture in the deep-freeze (-80 ^{0}C) as per SOP.
- In the case of change of treatment, select two cultures during the month of change.
- Set up DST to the old drugs as well as the new drugs
- Place a tick on the lab card against the culture selected for DST and on requests in the appropriate column of the culture register for the selected cultures. Cross out the request for DST on the remaining cultures for that month in the culture register.
- Give Positive cultures not selected for DST and duplicate slope of the positive for sub culture (Refer SOP for sub culture).
- In case of two cultures positive on the same week one is given for the storage.
- Give Duplicate slopes (if it becomes positive in subsequent week) for subculture.
- Enter the selected positive cultures separately according to study wise in the selected note book.

For ID test: Refer SOP for ID

Culture reading

First slope	Second slope	Final reading
*	Neg	Neg
*	*	Cont
*	*ID UMB	UMB
Neg	*ID UMB	UMB
+	Neg	+
+	++ (different week)	+
+	++ (same week)	++
*ID UMB	+	+
*	+	+

Reading of extra pulmonary specimen cultures

- The reading of the LJ slopes is similar to that for sputum cultures.
- Observe the SK medium bottles up to six weeks only.
- Remove the SK bottles or when a gross turbidity is seen
- Decontaminate with NaOH and inoculate onto two slopes of L-J medium.
- Read weekly for a further period of eight week. Decontaminate At the end of six weeks all the remaining SK bottles with NaOH (as for sputum without shaking) and inoculate onto two slopes each of LJ medium
- Incubated for a further period of 8 weeks.

IDENTIFICATION OF AFB AMONG SUSPECTED CULTURES

Following the culture reading on Tuesdays HOD will select and separate positives, query positive and contaminated cultures. Take the query positives for Identification

- Arrange the culture slopes to study wise in ascending order.
- Write the serial number on the lid of the culture slopes
- Write the serial number, lab number and culture morphology in the ID note book shown below

S. No	Lab No.	T. No	Pt. Name	Colony Morphology	ZN Smear Morphology
1	099999	G90752	Ranjitha	1 smooth buff col.	Short beaded
2	034410	G91196	Rajesh	1+ Mucoid yellow B/G	Long beaded
3	D245861	M161218	Premananda	1 Mucoid yellow	Short thick
4	B13663	HT0901	Ambiga	2 big smooth yellow cols	Slender
5	D246014	M16887	Amaravathy	1 tiny buff col	Coccoid
6	D246000	M17098	Lailamajnu	1+ mucoid orange	Negative

Following characters are recorded for describing **colony morphology**:

Colour : White, yellow, buff, orange etc

Appearance : Smooth/ Rough

Grading of culture : <19 colonies write the actual number.

1 +, 2 +, 3 + According to standard method

- Write the serial number on the slide, 3 per slide
- Clean the slide with dry cotton
- Place one drop of sterile Distilled water on the slide using wire loop
- Pick the colony with a sterile 3 mm nichrome wire (inner diameter 3 mm 24 SWG) and make a thin smear of approximately 1cm in diameter

- Incubate If one or few colonies present in the slope, spread the colony and further
- Allow the slide to air dry for 5-10 minutes
- Transfer the slide to hot plate (75-80° C) for fixing the smear
- Wait for 5-7 minutes
- Take the slides from hot plate then allow to cool at room temperature.
- Proceed for ZN staining (as per SOP for ZN method)

Result

Look for

- AFB
- Non-AFB
- If AFB, describe the bacterial morphology : Short beaded; slender beaded ; long beaded ; short thick ; coccoid;
- If non AFB seen ---Report as negative and discard the culture slope and document it as negative in the culture register
- If positive for AFB, Incubate the culture slopes for ID setup.

Note: The smear results will be sent to stats. Department on the same day

For ID setup following tests should be performed:

1. Niacin
2. PNB
3. Catalase

ID setup

- Select Sufficient growth containing culture slopes
- Arrange the slopes study wise in ascending order
- Enter the lab number and serial number (1, 2, 3, 4, 5, etc) in the daily diary and ID register
- Write the lab number and serial number using different colour marker in Bijou bottle cap (last 3 digit only)

- Take 1/3 loop of colonies (3 mm internal diameter 24 SWG) and transfer into the sterile Bijou bottle containing 0. 5 ml of distilled water with one glass bead (4 mm diameter)
- Seal the Bijou bottle with Para film
- Vortex it for 30 seconds
- Allow it for 5-7 minutes for settling aerosols
- Write lab number on two LJ slopes and one PNB slope
- Take one loop of culture suspension from Bijou bottle and inoculate into two LJ and one PNB slope using thin wire loop of external diameter 3 mm (27 SWG)
- Incubate at 37^{o}C for 28 days
- Enter serial number, Lab number, T. No. , Patient name, Colony Morphology and smear morphology in the ID smear note book
- Enter the details subsequently in ID culture note book leaving columns for 28th day reading on 2 LJ and 1 PNB slopes
- Allot Separate columns for entering niacin, catalase and HPLC results.

Lab No.	T. No	Pt Name	Colony Morphology	Smear Morphology	LJ	PNB	Niacin	Catalase	HPLC

- Enter the date of set up against the lab numbers in ID smear note book.
- After 28 days, read the slopes and grade the growth along with pigmentation if present in ID register
- Select one of the slopes from each culture and ensure that it contains more than 2+ growth with water of condensation for performing Niacin test.
- Give Serial number for the slopes.
- Enter in the niacin notebook along with the Lab no.
- Give the culture slopes for autoclaving.

- If the water condensation is very less add 0. 5 ml of sterile distilled water into the slopes

Finalisation & Documentation

- Report the culture as UMB in the ID culture note if there is growth on PNB and the test is negative for Niacin
- Report it as typical, if no growth is seen in PNB and the Niacin test is positive.
- Amend the report accordingly in the ID culture note book, culture register (volume) and the amendment note book.
- Pick up the culture card, if available, and assign the DST accordingly in consultation with T. O in charge in culture reading room.
- If the card is not available, give the slopes for storage
- Give the slopes for HPLC if no growth is seen in PNB and Niacin is negative. Repeat ID.
- Give the slope for HPLC if Growth is seen in PNB and Niacin Positive, Repeat ID
- If LJ or PNB are contaminated repeat the test
- Report as Test not possible – TNP(Y^2) if contaminated on repeated testing
- If no growth seen in either slope repeat ID
- For all the cultures reported as UMB, the slope should be sent to HPLC for further ID

Table: Identification for *M. tuberculosis*

LJ	PNB	Niacin	Report
+	+	Neg	UMB
+	Neg	+	Typical
+	Neg	Neg	Give it for HPLC after repeat test
+	+	+	Give it for HPLC after repeat test
Contaminated	Contaminated	-	Repeat ID from original
Neg	Neg	-	Repeat ID from orginal

NIACIN TEST

Principle

All mycobacteria produce niacin (nicotinic acid). *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis. Niacin negative *M . tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests. Cultures grown on egg medium (LJ medium) containing Asparagine yield consistent results.

Equipment and materials

- Test culture on LJ medium (A culture must be at least three to four weeks old and must have sufficient growth of at least 2+ growths).
- Positive Control: *M. tuberculosis* H37Rv
- Negative Control: Condensed water from uninoculated LJ medium
- O-toluidine - 1. 5% (Weigh 1. 5 g of O-toluidine and add 100 ml of ethanol; mix in an amber coloured bottle; prepare fresh weekly).
- Cyanogen bromide 10%

Cyanogen bromide being highly toxic is hazardous to weigh alternatively, a saturated aqueous solution of Cyanogen bromide which is approximately 10%, is used for the test. Add excess distilled water to the bottle and leave at least overnight at 4^{o}C in the refrigerator.

Procedure

- Select 4 weeks old LJ culture (from all studies after DST reading) with minimum 2+ growth and include positive and negative controls
- Enter the Lab number in the niacin register.
- Mark the corresponding serial number on the slopes
- Check for water of condensation in the culture tube
- If needed add 0. 5 or 1 ml of sterile water to the tube accordingly
- Place the bottles in the autoclave at 121ºC for 30 minutes
- Cool to room temperature
- Switch on the fume hood cabinet

- Remove 0. 25ml of the water of condensation to a clean tube (75x12 mm)
- Add 0. 25ml of 1. 5% O-toluidine using micropipette
- Add 0. 25ml of 10% Cyanogen bromide and mix well
- Observe for color development within 5 minutes
- Pink color (Niacin positive)
- White precipitate (Niacin negative test)
- Discard the tubes into the bin containing 4% sodium hydroxide solution

Precautions:

- Cyanogen bromide is a severe lachrymator and toxic, if inhaled. Prepare the solution in a well ventilated fume hood Do the test in a biological safety cabinet.
- In acid solutions, Cyanogen bromide hydrolyses to hydrocyanic acid, which is extremely toxic. Discard all reaction tubes into a disinfectant solution made alkaline by addition of sodium hydroxide.

CATALASE TEST

Principle

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually all mycobacteria possess catalase enzymes, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis.* Mycobacteria posses several kinds of catalase that vary in heat stability. Drug susceptible strains of *M. tuberculosis* lose catalase activity when heated to 68^0C for 20 minutes. For this test cultures on LJ should be used.

Equipment and materials

- Test culture on LJ medium
- Positive Control: *M. tuberculosis* H37Rv
- Positive control of NTM (Non tuberculosis Mycobacterium)
- Negative Control: uninoculated tube of medium
- Test tubes (16 x 125 mm)
- Water bath
- Timer
- Measuring cylinder
- 2 Honey jar bottle
- Pipette tips
- Loop -24 SWG (3mm)
- Glass beads single beads (3mm)
- Catalase rack (10 into 2)
- Catalase note book
- Vortex

Reagents

- 0. 067 M Disodium hydrogen ortho phosphate (Na2HPO4) anhydrous
- 0. 067M Potasium dihydrogen phosphate (KH_2PO_4) buffer solution, pH 7. 0

Solution 1

- Label the conical flask as solution-1
- Dissolve 9. 47 g disodium hydrogen ortho phosphate in a 1 litre of distilled water to provide 0. 067 M solution

Solution 2

- Label the conical flask as solution-2
- Dissolve 9. 07 g of dibasic potassium phosphate in 1 litre of distilled water dissolve to give 0. 067 M solution)
- Autoclave solution 1 and 2 at 121°C / 15 lbs for 10 minutes
- Allow to cool. Store in the refrigerator.

Preparation of Hydrogen Peroxide

Store 30% of Hydrogen peroxide, (commercial) solution in the refrigerator.

Preparation of 10% Tween-80

- Mix 10 ml of tween-80 with 90 ml of Distilled water
- Store in a refrigerator in a brown bottle.

Procedure

- Switch on the water bath
- Arrange the screw capped tubes with a single beads (5mm) in a catalase rack in ascending order
- Arrange the test cultures arranged in a culture rack
- Label the Lab number on the Lid of the culture slope.
- Enter the lab number in the catalase note.

Preparation of Phosphate Buffer Solution (freshly prepared)

- Measure 61. 1 ml of prepared solution -1 in a measuring cylinder and pour into the honey Jar bottle

- Add 38. 9 ml of (solution -2) into honey jar bottle which contains 61. 1 (solution -1) to make up of 100 ml and mix well (for 200 tests).
- Remove the caps of screw caps
- With a sterile pipette, aseptically add 0. 5ml (500µl) of Phosphate buffer to (16 x 125 mm) screw capped test tubes
- Close the cap
- Suspend a loopful of test culture in the buffer solution, using sterile loop (24SWG)
- Vortex slowly for 30 seconds (without avoiding splash)
- Place the tubes containing the emulsified culture in a heated water bath at 68 ºC for 20 minutes.
- Set the Timer for 20 minutes
- Mix equal volume of 10% Tween-80 and 30% hydrogen peroxide (Tween – peroxide mixture)
- Switch off the water bath
- Remove the catalase tube rack from water bath and cool to room temperature
- Remove the cap of the tubes
- Add 0. 5ml of freshly prepared 10% Tween -peroxide mixture to each tube
- Close the cap of the tubes
- Observe the control tubes (positive and negative)
- Observe the formation of bubbles appearing on the surface of the liquid.
- Do not shake the tubes because Tween-80 may also form bubbles when shaken, resulting in false positives.
- Hold negative tubes for 20 minutes before discarding

Results and interpretation

- Report as positive, if bubbles are seen and report negative, if no bubbles are seen

Positive catalase test

- Atypical (NTM)
- **MTB and resistant to Isoniazid**

Negative catalase test

No formation of bubbles if strain is *M. tuberculosis* and sensitive to Isoniazid

Documentation

Date	Sr. No	Lab. No	Result
12/3/2010	1	BS99560	Neg
	2	BS99764	Neg
	3	H37RV	Neg
	4	+VE (control)	+

Inform the catalase test results to the concerned personnel.

SUB CULTURE

Principle

All our presumptive positive culture slopes are subjected to subculture to confirm whether the growth is typical or atypical.

Procedure

Subculture sample from various studies

Receive / Collect the positive slopes from different studies for subculture

Materials Required

- Autoclave Bijou bottles with 0. 5 ml of distilled water containing (3mm) of

  Glass beads 3 mm 24 SWG and 27 SWG nichrome wire loops

- Bijou bottle racks
- LJ-Slopes
- PNB medium
- Marker pen
- Diary
- Subculture Register

Procedure

- When culture reading is completed sub culture slopes will be selected by the head of the department
- If sufficient growth is not seen in culture slopes, spread the colonies in a loop and kept it for further incubation (1-2 weeks)
- Arrange the selected culture slopes in rack according to studies in ascending order
- Mark the Lab number and Serial number on the Bijou bottle
- Arrange one LJ Slope and one PNB in a rack
- Mark the Lab number and Serial number on the LJ Slopes according to the study

Preparation of Bacterial Suspension

- Prepare a suspension by adding approximately 2/3 loopful of culture using 24 SWG of (internal diameter of 3 mm) in O. 5 ml of distilled water
- Keep it for 15 -20 minutes for the homogenization.
- Wrap the Bijou bottle and seal with Para film to avoid aerosol
- Vortex the Bijou bottle for 1 minute for uniform suspension
- Keep it for 5- 10 minutes for uniform mixing (clumps to settle down)

Inoculation

- Inoculate one loop(27 SWG of external diameter of 3 mm) of suspension in one LJ and one PNB Slopes
- Discard Bijou bottle after inoculation into bin

Incubation

- Incubate the slopes at 37^0 C for 4 weeks
- Store orginal slopes in Anti-cold room up to 1 month
- If needed collect the orginal slope for repeat testing.

Recording:

- Record the Date, Serial Number and Lab number in a daily diary and in sub culture register
- Store the original slopes after finalizing the Culture results in cold room for 6 months.

DRUG SUSCEPTIBILITY TESTING (DST)

Drug susceptibility tests should be performed in the following instances

- For relapse or re-treatment cases
- To change the drug regimen when drug resistance is suspected
- Undertaking drug resistance surveillance studies in a region/country

Definitions and abbreviations

- LJ medium: egg based solid medium (Lowenstein-Jensen) used for culture of TB bacilli
- DST: Drug sensitivity Testing.
- MIC: Minimum inhibitory concentration
- CFU: colony forming units

There are three methods used for determining drug susceptibility of Mycobacterium tuberculosis

- Absolute concentration method /Minimal Inhibitory concentration method (MIC method)
- Resistance ratio method
- Proportion method

Safety Conditions

- All the work should be carried out in the bio-safety cabinets.
- Care should be taken that aerosols are not generated.
- Wipe the BSC with 5% phenol prior to work and leave it for 5-10 minute.
- Arrange all the equipment and media necessary for the DST prior to performing procedure to reduce the chances of contamination and errors.
- In case of difficulties, notify the laboratory in-charge.

Precautions

- Check the Bijou bottles for any cracks and dust particles before use.
- Cool down the loop sufficiently before picking the colonies.

- Avoid touching the media while picking the colonies.
- Take representative colony of the culture slope (Try to take 2/3 loop-full of colonies from different growth area, by touching all colonies on the LJ slope).
- Avoid touching the water of condensation while scrapping the colonies
- Tip of the pipette should not touch the bottle's brim while delivering distilled water
- Wait for sufficient time after vortexing to avoid aerosol.
- Inoculate uniform drops of suspension in to all slopes.

Materials required

- Drug-free LJ media bottles
- Drug containing media- as per the table
- Calibrated loops: 3mm external diameter loop made with 27 SWG nichrome wire (thin) and 3mm internal diameter loop 24 SWG nichrome wire (thick).
- Double sterile distilled water
- Test strains
- Control culture- M. tuberculosis H37RV
- Known positive strain (IQC).
- Pipette aid
- Racks to hold McCartney bottles and Bijou bottles
- Sterile Bijou bottle containing 8-10 glass beads of 3 mm diameter
- Vortex mixer
- Pipettes
- Cotton
- 5% Phenol
- Known positive culture

1. The Absolute Concentration Method

Objective and Scope

This method uses a standardized inoculum grown on drug-free media and media containing graded concentrations of the drugs to be tested. Resistance is expressed in terms of the lowest concentration of the drug that inhibits growth (≥20 colonies) i. e. , minimal inhibitory concentration (MIC). This method is greatly affected by inoculum size and the viability of the organisms.

Procedure

Following are the drug containing media used for the absolute concentration method:

Drug	Concentration of Drug (µg/ml)		
	H37RV (control strain)	**Test strain**	**Test strain of Referred (RF)cultures**
First line			
Isoniazid	0. 025,0. 05,0. 1,0. 2,1, 5	0. 2,1, 5	0. 2
Rifampicin	4,8,16,32,64,128	32,64,128	64
Ethambutol	0. 5,1,2,4,8	2,4,8	4
Second line			
Ofloxacin	0. 5,1,2,4,8	2,4,8	2, 4
Kanamycin	2,4,8,16,32,64	8,16,32,64	32
Ethionamide	20,28. 5,40,57,80, 114	20,28. 5,40,57,80,114	80

PNB -500 µg/ml

Inoculum preparation

- With 3mm internal diameter loop (24 SWG nichrome wire) take 2/3 loop full of a representative sample (approximately 4 mg of moist weight) from the primary culture and place on the side wall of a Bijou bottle containing 0. 3ml double sterile distilled water.

- Vortex the bottle for 20–30 seconds
- Add 0. 7 ml of double sterile distilled water
- Wait for 10 minutes
- With 3mm external diameter loop (27 SWG nichrome wire) inoculate the all media containing the graded concentration of first and second line drug from lower to higher concentration

First line DST: 12 slopes to be inoculated (Isoniazid-3, Rifmapicin-3, Ethambutol-3, plain LJ-2 and PNB-1).

Second line DST: 16 slopes to be inoculated (Ofloxacillin-3, Kanamycin-4, Ethionamide-6, plain LJ-2 and PNB-1).

2. Resistance Ratio (RR) Method

Objective and Scope

It compares the resistance of unknown strains of tubercle bacilli with that of a standard laboratory strain. Parallel sets of media, containing two fold dilutions of the drug, are inoculated with a standard inoculum prepared from both the unknown and standard strains of tubercle bacilli. Resistance is expressed as the ratio of the minimal inhibitory concentration (MIC) of the test strain divided by the MIC for the standard strain in the same set.

Drug	H37RV (control strain) (µg/ml)	Test strain (µg/ml)	Test strain of Referred (RF)cultures (µg/ml)
Streptomycin	2,4,8,16,32,64	8,16,32,64	16

Inoculum preparation

- Same as Absolute concentration method (MIC)
- Inoculate slopes for control and test strains as shown in table.
- Inoculate only LJ 16 µg/ml for RF samples

3. Proportion method

Bacterial Suspension

- Prepare a suspension by scrapping approximately 4 mg moist weight of a representative sample (visualized as 2/3 loopful of 3mm internal diameter 24 SWG (Thick) wire loop) into 0. 3 ml of sterile distilled water in a Bijou bottle
- Vortex for 30 seconds to produce a uniform suspension.
- Add 3. 7 ml of sterile distilled water to the suspension to make it approximately 1mg/ml (S).
- Keep this suspension is kept on the bench for 15-20 minutes to allow the coarser particles to settle down.
- From this suspension make a 10-fold dilution is made by carefully adding 0. 2 ml to 1. 8 ml sterile distilled water (S1, 10^{-1}).
- Prepare Two further serial dilutions 10^{-2}(S2) and 10^{-3} (S3) are prepared in a similar manner.
- Inoculate One loopful (3 mm external diameter, 27 SWG Thin) on drug free and drug containing LJ slopes for S1 & S2 as shown in table.

Dilution	**Plain**		**Drug**				
			S	**H**	**R**	**E**	**PNB**
S	X						X
S_1	X	X	X	X	X	X	
S_2	X	X	X	X	X	X	
S_3	X	X					
S_4	X	X					

Concentrations used

First line Drug

Dihydrostreptomycin : 4 µg/ml

Isoniazid : 0. 2 µg/ml

Rifampicin : 40 µg/ml

Ethambutol : 2 µg/ml

Second line Drug

Kanamycin : 30 µg/ml

Ethionamide : 40, &57 µg/ml

Ofloxacin : 2 µg/ml

Amikacin : 40 µg/ml

Capriomycin : 40 µg/ml

Note: storage of Drug containing slopes up to 1 month in 4^0 C.

List of registers

- DST set up diary
- Selected culture note book
- Repeat DST note book
- Drug susceptibility testing register
- Culture register

List of entries

- After setting up of DST, enter the list of lab numbers setup on that particular date in the corresponding diary along with the technician's name. Mention the corresponding batch numbers of the drugs used.
- Later score the lab numbers for which the DST is setup in the selected culture note book along with the date.
- Make entries in the DST register - the details of the lab numbers along with the patient's name (from the culture register), drugs for which the DST is setup and their batch numbers.
- Enter the date of DST setup for the respective lab numbers in the culture register along with the page numbers in the DST register in which the results are to be entered.
- Similarly if the lab numbers are from repeat DST, enter the details of the date of setup and score against the respective numbers in the repeat DST note book.

DRUG SUSCEPTIBILITY READING

Incubation and reading of the tests

Examine the inoculated slopes for growth after 28 days of incubation. In the MIC method 'growth' is defined as the presence of 20 colonies or more. As each slope is examined, record the results as follows:

3+ : Confluent growth

2+ : Innumerable colonies (>100 colonies)

1+ : 20-100 colonies

1-9 : Actual number of colonies

If the drug-free control slope yields 100 colonies or less, repeat the test from the control slope. However, if the control slope shows no growth or is contaminated, repeat the test from the original positive culture or from an alternate culture from the same patient, if available. Record the lowest concentration of the drug inhibiting growth (MIC).

Definitions of resistance

Drugs	**Resistant**	**Doubtful**
Isoniazid	MIC $\geq$ 5	MIC = 1*
Rifampicin	MIC $\geq$ 128	
Ethambutol	MIC $\geq$ 8	MIC = 4*
Ofloxacin	MIC $\geq$ 8	
Kanamycin	MIC $\geq$ 64	
Ethionamide	MIC $\geq$ 114	

*Repeat the test from control slope

Interpretation (Streptomycin)

Record the lowest concentration of drug inhibiting growth (MIC). Divide the MIC of the test strain by the MIC of the standard strain, $H_{37}Rv$ to obtain the Resistance Ratio (RR).

Definition of resistance:

Sensitive	:	RR of 2 or less
Doubtful*	:	RR of 4
Resistant	:	RR of 8 or more

- Repeat the test in case of a doubtful result

PST METHOD

Interpretation of Tests

Interpretation of all tests is based on the 42-day readings. However, if a strain shows clear-cut resistance based on the 28-day reading, no further reading is needed and the report may be sent as such. Strains that are susceptible at 28 days must be read again at 42 days and the report is based on the later reading only.

For each strain, express the number of organisms resistant to each drug concentration as a percentage of the number of organisms growing on the drug -free slope. Make the selection of slopes for estimating the growth on the drug-free and drug containing media in the following order of preference:

Drug-free & Drug containing slope

1. 20-70 colonies
2. 5–19 colonies
3. More than 70 colonies

Definitions of resistance- proportion method

Drug	Concentration (mg/l)	Proportion
Dihydro Streptomycin	4	1% or more
Isoniazid	0. 2	
Rifampicin	40	
Ethambutol	2	

Calculation of proportions – An illustration

Suspension	Drug-free medium		Drug concentration (mg/l)	
			INH $_{0.2}$	Rif $_{40}$
S_1	3+	(50,000)	2+	2+
S_2	3+	(5000)	2+	**26**
S_3	2+, 2+	(500)	**32**	3
S_4	**46, 54**	**50**	4	1
Proportion resistant			6. 4% R	O. 52% S

More examples for illustration

Dilution	Drug-free	H $_{0.2}$
S_1	3+ (40,000)	2+
S_2	3+ (4000)	2+
S_3	2+, 2+ (400)	85
S_4	38, 42 (40)	7
% Res: 85/400 = 21. 25% R		

Dilution	Drug-free	H $_{0.2}$
S_1	3+ (60,000)	4
S_2	3+ (6000)	-
S_3	2+, 2+ (600)	-
S_4	64, 56 (60)	-
% Res: 4/60,000 = 0. 007% = 0. 01$^-$% S		

Dilution	Drug-free	$H_{0.2}$
S_1	3+ (1700)	2+
S_2	3+ (170)	2+
S_3	16, 18 (17)	18
S_4	1, 2	7
% Res: 18/17 = 105. 9% = 100% R		

Dilution	Drug-free	SM_4
S_1	3+ (2400)	-*
S_2	* (240)	-
S_3	21, 27 (24)	-
S_4	1, 4	-
% Res: 1/2400 = 0. 04- % S		

* Calculate assuming a growth of 1 colony

Dilution	Drug-free	Emb_2
S_1	3+ (7200)	2+
S_2	3+ (720)	65
S_3	76, 68 (72)	3
S_4	3, 6	1
% Res: 65/720 = 9. 0% R		

Dilution	Drug-free	Emb_2
S_1	3+ (8500)	22
S_2	3+ (850)	2
S_3	82, 88 (85)	-
S_4	3, 2	-
% Res: 22/8500 = 0. 26% S		

Enter the Niacin result and growth in PNB in the culture volume

If Niacin Negative PNB negative ---Repeat both

If Niacin and PNB both positive---Repeat Both

If Niacin Negative and PNB positive after repeat

Write DNR (Do not Record) in the DST volume book

PNB	Niacin	Report
+	Neg	UMB
Neg	+	Typical
Neg	Neg	Give it for HPLC
+	+	Give it for HPLC

Culture reading

First slope	Second slope	Final reading
*	Neg	Neg
*	*	Cont
*	*ID UMB	UMB
Neg	*ID UMB	UMB
+	Neg	+
+	++ (different week)	+
+	++ (same week)	++
*ID UMB	+	+
*	+	+

Results of DST should be entered in the appropriate study note book and compare with previous culture (it may be the same month /different month). If any discrepancies in the result repeat the test and it should be entered in the repeat note book.

PRIMARY ISOLATION OF MYCOBACTERIA BY BACTEC 460

Introduction

BACTEC has been widely used for the rapid recovery of mycobacteria from sputum and other clinical specimens. Both pulmonary and extra pulmonary specimens can be processed for primary isolation by BACTEC.

The sample is inoculated into the BACTEC 12B vial with a syringe through the rubber septum and incubated at 37^{o}C. The culture vial is periodically placed in the system to assay the radioactive CO_2 content. A positive reading indicates the presence of viable microorganisms in the vial.

Principle

BACTEC 12B medium is an enriched Middlebrook 7H9 broth based liquid medium. Microorganisms, if present in the test sample inoculated into the BACTEC vial, begin utilizing a ^{14}C labeled substrate (fatty acid) present in the medium and release ^{14}CO2. The gas is aspirated from the vial and the ^{14}CO2 radioactivity is determined quantitatively as growth index (GI) from 0-999. Increase in the GI is directly proportional to the rate and amount of growth in the medium.

For blood specimens BACTEC 13A medium is used.

Specimens and processing methods

Pulmonary and extra-pulmonary specimens

- Both NaOH and NALC-NaOH decontamination procedures can be employed for specimen processing wherever applicable
- Paucibacillary specimens are processed as per the standard protocol

Preliminary requirements

- The BACTEC 12B vial should be examined for breakage, deterioration and visible contamination
- The vial should be test-run in the instrument prior to inoculation to ensure 5-10% CO_2 in the medium and to screen for presence of contamination.

- BACTEC 12B vial can be used as stand alone medium; but for maximum recovery and reduced contamination, antibiotic supplement such as PANTA should be added.

Preparation of PANTA supplement

- PANTA plus kit includes lyophilized antibiotics such as Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin.
- Reconstituting fluid consists of POES (poly oxy ethylene stearate) which is a growth promoting substance.
- Lyophilized PANTA vial should be reconstituted with 5ml of reconstituting fluid.
- PANTA for Blood culture should be reconstituted with 2. 5ml of reconstituting fluid.

Preparation of decontaminated deposit

- Suspend the decontaminated deposit in 1 ml of sterile 0. 067M phosphate buffered saline (PBS) and vortex slightly for even mixing and dilution of deposit

Inoculation into 12 B vial

- Mark the vial with appropriate specimen number and the date of inoculation
- Clean the rubber septum of the 12B vial with 70% alcohol for surface sterilization
- Add 100 µl of reconstituted PANTA supplement using tuberculin / insulin syringe (4 units = 100µl)
- Inoculate 500 µl of the reconstituted sputum deposit into the 12B vial using a similar syringe (20 units= 500 µl)
- In case of extra pulmonary specimens, inoculate the whole of the reconstituted deposit
- Clean the rubber septum immediately with 70% alcohol to disinfect the surface in case of any droplet spillage
- Incubate the vial at 37^{o}C without shaking in an incubator

Inoculation of Blood specimens in to 13A medium

- The medium vial is sent to the clinic. Instructions are given to the Health visitor to aseptically collect and inoculate 5ml of blood in to the medium.

- Upon receipt of the inoculated medium in the lab, the vial is inspected for any spillage of blood, and patient' details. In case of spillage, wipe the outer surface with 70% alcohol.

- Allot a lab number with BL as prefix

- Add 0. 5ml of enrichment provided exclusively for the medium

- Add 0. 1 ml of reconstituted PANTA (meant for Blood culture)

NOTE: Separate racks are provided for 12B (red) and 13A vials (white)

Reading algorithm

- Read the vials every two to three days for the first two weeks, twice weekly for the 3rd and 4th week and weekly thereafter for a maximum of 6 weeks

Results and interpretation

- The instrument is programmed to read the radio activity of the radio-labeled carbon which is interpreted as growth index. Hence, no specific calculation is required.

- GI is a measure of the $^{14}CO_2$ radioactivity aspirated from each vial and is directly proportional to the amount of active growth in the vial.

- A GI of 100 is considered as the cut off for positivity (this cut off is recommended since only cultures crossing this cut off offer clear picture with ZN stain, though manufacturer's recommend 30 as the cut off GI)

- Vials showing GI of 100 are subjected for confirmation of growth

Confirmation of growth

Cultures selected as positive need to be confirmed since species identification of mycobacteria using morphology is not possible in broth based systems. Hence, the culture is subjected to ZN staining as follows:

- Mix the culture well

- Place a drop of poly–L-lysine or BSA (fixatives) on a clean slide

- Using a tuberculin syringe transfer a few drops of the culture on to the slide.

- Air dry, heat fix and stain by ZN staining procedure

- Report AFB positive cord forming units / any other growth with different morphology (The manufacturer has provided an identification kit, called NAP test kit for differentiation of *M. tuberculosis* from NTM. Kit identification is done wherever necessary)

Time to detection

The average time to detection is 8-12 days for *M. tuberculosis* and 5-6 days for NTM.

Trouble shooting for Contamination

- The incidence of contamination varies with laboratories and depends upon the method of decontamination procedure followed. The contamination rates in 12B vials is usually less than the conventional media and rates up to 5% is acceptable.

- In case of any contamination encountered during primary isolation, the entire medium should be aspirated, reprocessed by usual decontamination procedure followed in the laboratory and inoculated into a fresh 12B vial.

Storage instructions

- The media, supplements, other reagents & chemicals required for the test should be sterile and stored at appropriate temperature indicated in the test kit.

- The kit should be used within the expiry date. If any color change or breakage is noted in any of the vial in the test kit, it should be documented and safely disposed.

Disposal of used medium

- The used 12B vials should be stored for a minimum of up to 6 months and then discarded.

- The vials should be autoclaved and safely disposed following the procedures indicated for safe radioactive disposal. Reuse of any material in the test kit should be avoided.

Disposal of needles

- The tuberculin / insulin syringe used for inoculation should be disposed into a separate screw capped bottle half filled with Lysol / 5% phenol.

- The needle cap should not be replaced back on to the used needle. The bottle should be autoclaved and safely disposed.

General Precautions

- Always keep the BSL I cabinet clean and keep the materials handy within the cabinet.
- Clean the surface of the cabinet with Lysol / 5% phenol before and after use.
- Use of needles requires special attention and care must be taken during inoculation and disposal.
- In case of any spillage or breakage, the concerned authority Senior Technical Officer (STO) must be informed and necessary action should be taken immediately as liquid cultures are highly infective and generate more aerosols.
- Surface sterilization of the vials is mandatory as the inoculated 12B vials are incubated in an open environment.
- Use of bacterial culture from 12B vials for other testing procedures should be performed carefully
- The septum of the 12B vial should be monitored during culture reading for any tears that may cause leakage of culture material.
- PANTA should be discarded if any turbidity is noted.
- The print out of the culture reading must be preserved till results are dispatched. Recording of results should be performed on the day of culture reading to avoid confusion between two readings.
- Care should be taken while transferring the culture numbers on the print out to avoid any visual error; preferably the readings can be cross checked by two individuals.
- Do not break the crimp of the 12B vials as they are required to hold the rubber septum during reading process and to serve as seal to the vial.

Procedure for sub culture from 12B to 12B vial (whenever required):

- Check the GI of the 12B vial and confirm the growth as pure culture using confirmation tests such as smear and contamination check on nutrient agar plates.
- Mark a fresh 12B vial with culture number, date and mention it as subculture.
- If required add 100µl of PANTA into the vial.
- Aseptically aspirate 50-100µl of the primary culture from the 12B vial after mixing and add into the subculture vial.

- The sub culture vial should be incubated at 37^{o}C and from the 2nd day onwards, take daily readings till the GI reaches 450.

- The culture may be confirmed for *M. tuberculosis* and can be used for setting up drug susceptibility testing.

- Disposal of the vials is similar to that of primary isolation.

IDENTIFICATION OF CULTURES BY BACTEC 460

Rationale

Though primary isolation by liquid culture is rapid, it does not give a clear indication whether the growth is of *M. tuberculosis* complex or non tuberculosis mycobacteria (NTM).

BACTEC 12B medium does not allow observation of colony morphology, though GI output can be characteristic for certain species of mycobacteria. *M. tuberculosis* complex exhibits slow growth with a 2-3 fold increase in daily output while NTM may show daily increases as high as 10 fold. Confirmation for the presence of acid fast bacilli is usually performed by Ziehl- Neelsen staining (ZN). Quality of the primary isolate is determined using sterility check on blood agar/ nutrient agar plate. Hence, a test to differentiate *M. tuberculosis* complex from NTM is essential.

p-nitro-α-acetylamino-β-hydroxy-propiophenone (NAP) TB kit helps to differentiate *M. tuberculosis* complex from other mycobacteria. This test should be used in conjunction with colony morphology on smear and growth characteristics in BACTEC 12B medium.

Principle:

A sample of actively growing culture is inoculated into the NAP vial which contains a disc containing 5µg of NAP. A control vial is included without the NAP disc. Both vials are incubated and growth in the presence and absence of NAP is monitored. Analysis of daily readings within the next 3-5 days helps in the differentiation of the TB complex from other mycobacteria.

NAP, an intermediate compound in the synthesis of Chloramphenical, inhibits *M. tuberculosis* complex, while NTM show either slight or no inhibition. This is indicated by either absence of increase in GI or a decrease. This effect on growth, depicted by GI is used as tool for identification.

KIT components

10 vials each containing a paper disc impregnated with NAP at 5 µg concentration.

Storage

Store the vials at 2-6^{o}C out of direct sunlight. Do not expose to heat or high temperature as NAP is heat labile.

Methodology

- When BACTEC 12B vial shows GI of 10 or more, read the vial daily till the GI reaches 50-100
- Confirm the quality of culture by performing a contamination check on BA/NA plate
- Homogenize the culture with a tuberculin syringe by aspirating in & out 3-4 times inside the BACTEC 12B vial
- From the culture, transfer 1 ml to NAP vial.
- If the GI exceeds a GI of 100, then it needs to be diluted accordingly;

GI 50-100	No dilution
GI 101-200	0. 8ml into a fresh BACTEC 12B vial
GI 201-400	0. 6ml into a fresh BACTEC 12B vial
GI 401-600	0. 4ml into a fresh BACTEC 12B vial
GI 601-800	0. 3ml into a fresh BACTEC 12B vial
GI 801-999	0. 2ml into a fresh BACTEC 12B vial
GI 999 or more than 1 day	0. 1ml into a fresh BACTEC 12B vial
GI >999	Sub culture and test daily until desired GI is obtained

- Transfer 1ml of the diluted culture to the NAP vial.
- Include the original culture / the diluted culture as the control vial
- Swab the septum of the two vials with 70% alcohol and read them in the instrument (for initial CO_2 filling in the vials – Disregard the GI value)
- Read the vials daily for 3-5 days; Record the readings of NAP vial and control vial daily

Testing growth from solid medium

- Prepare suspension by scraping a few representative colonies and emulsify using sterile distilled water or BACTEC diluting fluid (volume of the liquid can be customized)
- Adjust the turbidity to McFarland 1. 0 standard
- Add 0. 1ml of the suspension into a fresh BACTEC 12B vial and incubate at 37^{o}C and read daily in the instrument
- Once the GI reaches 50-100, perform NAP differentiation test

Methodology

- Aseptically transfer 1ml of the homogenized culture into the NAP vial
- Swab the septum of the vial before and after inoculation
- Use the original vial as the control
- Swab the septum of the two vials with 70% alcohol and read them in the instrument (for initial CO_2 filling in the vials – Disregard the GI value)
- Read the vials daily for 3-5 days; Record the readings of NAP vial and control vial daily

Interpretation of results

- Daily GI of the control vial will continue to increase
- In the NAP vial, increase or decrease of the GI depends upon the species of mycobacteria present
- A decrease or unchanging GI indicates *M. tuberculosis* complex and increase in GI indicates NTM

Algorithm for result interpretation

***M. tuberculosis* complex**

- 2 consecutive significant decrease in GI after inoculation
- Slight, but not significant increase in first 2 days and then decrease or no increase in GI

NTM

- Daily GI reading increases to over 400 within 4 days

- Slight decrease or no increase during 0-3 days after inoculation and then 2 consecutive daily significant increases

- A "significant" increase or decrease means a 20% or more change from one day to the next.

Reporting time

- Results are available within 2-6 days depending on the mycobacterial species

- Result should be reported when clear trend is observed

- If GI is unchanged, identification as *M. tuberculosis* complex should not be interpreted in less than 4 days

- Average time for differentiation is 4 days

Quality control:

- A panel of mycobacteria strains including NAP positive and negative strains must be included in each batch lot of the NAP kit received

- *tuberculosis* $H_{37}Rv$, *M. kansasii*, *M. intracellulare* and *M. avium* can be used as QC

Limitations

- Differentiation of *M. tuberculosis* from NTM can be achieved only if pure culture is used

- In case of mixed culture with NTM & *M. tuberculosis* the increase in GI is due to NTM

- Presence of contamination also increases the GI and visual examination of such cultures is required (A smear from NAP vial in case of increasing GI may be helpful in confirming the presence of AFB)

Disposal of the used medium: Refer primary isolation procedure for BACTEC 460 TB system

DST FOR FIRST LINE & SECOND LINE DRUGS BY BACTEC 460

Principle

DST by BACTEC is based on the same principle employed in the conventional method except that a liquid medium is used. Comparison of growth between drug free and drug containing media aid in the determination of drug susceptibilities. With the liquid medium, growth is monitored daily and results are reported within 4 to 12 days. Suppression of growth occurs in the drug containing medium if the test organism is susceptible to the drug tested. This can be detected by either a decline or a very small increase of the daily GI as compared to the control. If the organisms are resistant, little or no suppression occurs. The critical proportion for resistance is taken as 1% for all anti tuberculosis drugs.

To determine the 1% proportion of resistance, the bacterial inoculum used in the control vial is 100 fold less than that used for the drug containing vial. The rate of increase in the GI (change in GI over that of the previous day), is designated as ***delta (Δ) GI***. This value is compared between control vial and the drug containing vials. If the daily GI increase in drug vial is ≥ control vial, the organism is considered as resistant and vice-versa is considered susceptible.

SIRE – First line drugs (KITS **available)**

Second line – *in-house* preparation

Storage and reconstitution:

- Store the drug vials in a cool (2-8^{o}C), dry place out of direct sunlight.
- Reconstitute the lyophilized drugs in 5ml of sterile distilled water (Can be stored in smaller aliquots at -80^{o}C).
- Perform 1:3 dilution for Streptomycin and Ethambutol and add into the vial as final drug concentration.
- Use the reconstituted medium directly for H and R.

Preparation of drug medium:

- Pre test the BACTEC 12B vials before proceeding to DST to screen out contamination and to induce 10% CO_2 into the medium to enhance growth.

- Use freshly prepared drug solution or thaw the frozen stock.
- Add 0. 1ml of the drug into the BACTEC 12B vial.
- Use separate syringe for each drug.
- Ensure that there are no air bubbles in the syringe.

Drug concentrations

DRUG	Final conc. (µg/ml)
STR	2. 0
INH	0. 1
RIF	2. 0
EMB	2. 5
KAN	4. 0
ETH	2. 5
OF	2. 0
AMI	1. 0
CAP	1. 25

Preparation of Bacterial suspension

- Actively growing *M. tuberculosis* cultures in 12B medium or on solid medium are used for setting up susceptibility testing.
- If susceptibility testing is delayed, refrigerate the primary isolate vial, but set up DST within 1 week.

a. From BACTEC 12B vial

Daily reading (GI)	Action
300-499	Incubate one additional day and then set up test
500-799	Set up test on same day
800	Dilute 1:2 with 1ml diluting fluid and set up test

b. from solid (LJ) medium

- Make a homogenized suspension of representative colonies from solid medium. The culture should not be more than 4-5 weeks old.
- Adjust the suspension to McFarland 0. 5.
- If viability or age of the culture is uncertain, subculture into a 12B vials to obtain fresh growth.

Inoculation

- Arrange the 12B vial in a rack and label them as control and drugs used.
- Swab the septum of the vial before and after inoculation (use separate cotton swab with appropriate disinfectant)
- Inoculate 0. 1ml of the bacterial suspension into each of drug containing 12B vial after thorough mixing using syringe.
- Inoculate 0. 1ml of the suspension into vial containing 9. 9ml of diluting fluid (provided by manufacturer) to get 1: 100 dilution and mix thoroughly.
- Inoculate 0. 1ml of the diluted suspension into the control vial.
- Incubate the vials at 37^{o}C.
- **Reading schedule**
- The first day of reading is taken on the third day after DST is set up.
- Test the vials daily then (including holidays and weekends) on the instrument till the GI reaches 30 or more in the control vial.

Interpretation

When the control vial reaches a GI of 30, the results can be interpreted as follows:

ΔGI (Control) > ΔGI (drug) = **susceptible**

ΔGI (Control) < ΔGI (drug) = **resistant**

ΔGI (Control) = ΔGI (drug) = **borderline** (repeat test)

Example

Day /GI						
	1	**2**	**3**	**4**	**ΔGI**	**Results**
Control	3	10	23	48	+25	
Strept	52	82	79	76	-3	S
INH	41	92	215	581	+266	R
RIF	29	78	181	476	+295	R
Eth	75	82	68	72	+4	S

Limitations

- Variations in preparation of inoculum affect the outcome of susceptibility test results; presence of large clumps and improper dilution of control vial can affect the test result.
- If growth in the control vial shows unsatisfactory increase in GI, or when the drug vials show extensive increase in GI from day 1, test should be repeated.

Quality Control

- Standard laboratory strain ($H_{37}Rv$) should be tested on a daily basis; whenever a new batch of drugs is prepared; after servicing of the instrument; after long gap of setting up DST.
- Standard ATCC strains (resistant & susceptible) for each drug as reference control.
- Duplicate specimens can be set up as IQC.

Documentation

- Note down the lab numbers on the daily result sheet in-order to avoid confusion.
- Enter the readings in the specific register maintained.
- After finalizing the results the print outs can be discarded.

Disposal

- Follow the precautions and disposal procedure as for primary isolation.
- Discard the vial with diluting fluid (used for control vial inoculation) after autoclaving (as this vial does not contain any radioactive material).

MYCOBACTERIUM BY MGIT 960

The purpose is to detect the growth of culture (viable tubercle bacilli) in the sputum and extra pulmonary specimen. Quantification of growth is expressed in growth unit (GU).

Principle

Blood and bloody specimens are not suitable for the MGIT system. Sputum samples are processed by NALC-NaOH and inoculated in to 7 ml MGIT tubes, which are supplemented with oleic acid albumin dextrose catalase and a cocktail of antibiotics (PANTA). The MGIT tubes contain a fluorescent sensor in the base of the tube, which binds with $O_{2\ and}$ quenches fluorescence. Microorganisms present in these specimens metabolize nutrients and O_2 in the culture releasing the fluorescent compound and fluorescence begins to appear.

The instruments photo detectors, light emitting diodes (LEDS) measure the level of fluorescence which corresponds to the amount of O_2 consumed by organisms.

Detection of the presence of microorganisms growing in the culture medium results from these fluorescence dye in the base measurements.

How the MGIT works?

The instruments automatically test all the tubes continuously. A single MGIT 960 instruments is capable of monitoring a total of 960 MGIT 7 ml tubes. The tubes are arranged in three drawers (A, B and C), each of which holds up to 320 tubes.

The capacity is 154 samples per week with 6 week protocol.

Each drawer contains a sample measurement model consisting of

Stations – wells in the rack into which tubes are inserted

The detector assembly – This sits below the rack and has 16 detectors 1 for each row of station. The assembly moves from left to right and back, taking test readings for each of the 20 station columns and the calibration tube.

Drawer status indicators lamps on the front of each drawer, indication a positive, negative and a station ERROR.

Barcode scanner at the front of the instrument to scan tube levels for specimen identification. The scanner turns on automatically.

LCD display and keypad – present information about the system status and function key definitions

Floppy disk is provided to enable you to update system software and to save date to disc for troubleshooting and system diagnostic purposes.

Computer – located in the top section of the instrument, is responsible for tube positivity analysis, instrument control, and the built in tube data source and the user interface.

System software presents a simplified user interface on the LCD display, with picture icons to represent all the functions, operations, setup parameters and status condition.

There are three basic type of displays

Main status screen – when all the instrument drawers are closed, this screen appears. A summary area shows the number of tubes that are in error or anonymous. The current date and time is also shown.

Configuration /maintenance screens - allows you to set the protocol length, time date and their formats, audible alarm volume, instrument identification number, block and anti block stations and verify all the indicator lamps.

Activity screen when the drawer is opened, software key definitions appear that enable you to enter new tubes remove positive, negative and on going tubes, identify anonymous tubes and resolve station error conditions.

A row of LEDs below the tubes illuminates activating their fluorescent sensors. Photo detectors take the readings. A test cycle of all the drawers is completed every 60 min. Positive cultures are immediately flagged by an indicator light on the front of the drawer, an optional audible alarm and are displayed on the LCD screen (liquid

crystal display) when positive tubes are identified, the technician removes them from the instrument for confirmation of results for isolation and detection of the organism.

Definition of growth unit (GU)

The GU is an algorithmic measure of sensor fluorescence derived from the row fluorescence voltage signal produced by optical integration of a MGIT tube in the BACTEC 960 instrument.

The machine takes reading every an hour. If the GU reaches or exceeds the cut off of the 75 then the instrument flags this as a positive. This would be a regular true positive and the technician would perform the routine tests to confirm positivity and then proceed to run an ID and antimicrobial susceptibility testing (AST).

If the instrument flags the tube as positive and the MGIT tube records a GU of **'0' or higher before 5 hours,** Then this is a threshold (T) positive, meaning that the growth has explored passed the 75 cut off.

Growth curve of such **growth would be every step whereas** the regular positive curve would be gradual up to and beyond the 75 cut off. If explosive growth has occurred the software records 'T' in the growth column. 'T' indicates that the MGIT tube is contaminated and these tubes will show confirmatory organisms either in ZN /AP or on the culture media (BA) or on both.

The GU is not an indication of biomass within the vial. At positivity, biomass is approximately 10^5 to 10^6 CFU/ml. An instrument positive tube is flagged as a positive when it reaches at least GU of 75. There is no direct correlation of biomass and GU at the time of instrument positivity.

Sputum processing for MGIT (Qualitative method)

Objectives

- Decontamination of bacteria other than mycobacteria and
- Liquefaction of organic debris in the specimen

Principle

NALC, is used for the rapid digestion. NaOH is used at a lower concentration of 1% Sodium citrate exerts a stabilizing effects on the NALC by chelating heavy metal ions present in the specimen. The PBS neutralizes the NaOH and dilutes the homogenate to loosen viscosity and specific gravity prior to centrifugation. A relative centrifugal force about 3000 g is adequate to sediment mycobacteria. The rate of mycobacteria sediment is critically dependent on time of centrifugation and relative centrifugal force applied to the specimen.

Materials required for processing the specimen

BSC, 0. 5% Lysol, waste disposal bin, conical flask, polypropylene centrifuge tube with tight screw cap, NALC powder, 2% NaOH and sodium citrate, PBS (pH 6. 8), vortex mixer, test tube racks for 50 ml tubes, pipettes and aerosol resistant tips.

Procedure

- Place a cotton towel soaked in 5% Lysol on the work area of BSC.
- Add equal volume of NALC-NaOH mixture to the specimen volume to the centrifuge tube. Total volume cannot exceed 20 ml.
- Close the tubes tightly and vortex until liquefied. Keep aside for 15 minutes
- After 15 minutes, add PBS to the 50 ml mark on the centrifuge tube.
- Mix by inversion or vortexing.
- Balance the tubes and insert in centrifuge bucket.
- Centrifuge at 3000 g for 15 minutes.
- Decant the supernatant in 5% Lysol bin.
- Add 2 ml of PBS in the deposit using sterile transfer pipette.

- Recap and vortex to re suspend the pellet.
- Inoculate (0. 5 ml) in to MGIT 960 tubes.

Procedure for BACTEC MGIT 960

Materials required

- Reconstitute PANTA in 15 ml of growth supplement. This mixture is stable for 7 days if stored between 2^0C and 8^0C.
- Label the tube with lab number and bar code label on side of tube for each specimen.
- Add 0. 8 ml of liquid PANTA to each bottle using a sterile plastic pipette, taking care not to contaminate them.
- Add 0. 5 ml of the re suspended pellet (specimen) in to the MGIT tube.
- Tightly recap the MGIT tube and mix well.

Common procedures for entering bottles in to MGIT 960

- Scan barcodes and assign stations through tube entry function.
- Accession bar coding disabled (default setting)
- Open the desired drawer.
- Press the tube entry soft key.
- Place the tube in the alignment block in front of the scanner with the bar code label facing the scanner.
- Use a spare bar code label if the tubes label is damaged.
- System – beeps once if scan is successful.
- Carefully place the tube in the assigned position.
- Row and column position is indicated on main body of display.
- Assigned station LED's illuminate green in the drawer.
- Repeat steps 3 and 4 for each tube.
- Close the drawer or press exit to continue with next tube.

Caution

- Do not turn tubes after placing them in the station.
- Do not remove tubes unless they are positives.

Instructions for removing positive bottles from the machine

Remove positives

- Press silence alarm to switch quick audible alarm
- Open the drawer where the 'red positive' light is lit.
- Press 'remove positive tubes' key.
- Remove tube from the station with alternating flashing green/red indicator lights. Remove one positive tube at a time.
- Scan the positive tubes barcode label by placing the tube in the alignment block in front of the scanner with the barcode label passing the scanner.
- Station indicator will extinguish.
- Place the tube into a rack.
- When the rack is completed, the instrument will beep 3 times, the drawer indicator light extinguishes, the bar code scanner turns off and the OK icon appears on the display screen.
- Close the drawer or continue the next task.
- Take a printout.

Returning the positive tubes to the instrument for further testing

The system allows you to return a pulled positive tube to the instrument for further testing for up to 5 hours after removal.

The re-entry feature resets positivity routines, retains the start of protocol date and continues to test the tubes as an ongoing culture. Open the door; press the 'tube entry key'. Scan the tubes bar code label. Place the tube in the indicated station (this may differ from the original station).

Confirmation

Unscrew the MGIT tube lid (those gave positive red light) and use a 10 µl to BAP plate to check the contamination. Incubate BAP along with the tube in the incubator

at 37^0C for 48 hours. All removed tubes must be entered on to sheet kept in unloaded positive folder.

If there is growth on the plate retreat the deposit

- Spin at 3000 g for 15 minutes
- Pour off the supernatant into 5% Lysol bin and re-suspend the pellet by mixing

If there is no growth on the BAP

- Spin at 3000 g for 15 minutes.
- Pour off the supernatant into 5% Lysol bin and re-suspend the pellet by mixing.
- Label the slide and make a film by 5% BSA to fix the smear Examine for ZN staining for other organisms.
- Record the number of days and hours at the time of positivity for each sample.
- Examine the ZN for the presence of AFB's
- Describe AFB's i.e. typical, atypical and whether cording is seen or not
- If AFB are present, the broth is not contaminated with anything then subculture on to two LJ slopes and PNB slope.
- Definitive identification will be obtained.
- The rest of the deposit is transferred to the same MGIT tube and kept for storage.

Quality control

To be performed every morning, preferably before unloading or loading of tubes

- Maintenance button- tools select
- To check the lights on the drawers: select positive (+red) negative (- ve green) and exclamation (! Yellow) respectively – all should light up
- Open each drawer – select first light – all stations should be green. Select same switch to switch off
- Select light: all station light on red
- Close drawer and repeat for all drawers

- Press exit door
- Press temperature: each drawer temperature will be recorded. Record all readings on a log book
- Filter cleaning – once in a month – remove face plate at bottom of instrument, grip the bottom and pull firmly forward
- Remove the filter and place it in mild disinfectant
- Dry thoroughly and replace
- Replace the face plate, hold it in place – the cut out should surround the on/off switch and firmly press in towards the instrument
- The face plate will snap in to place
- 13 Calibration tube for quality assurance

Power failures

If power is lost for more than 4 hours, tube should be removed and incubate at 37^0C. Better connect the system to an UPS.

DRUG SUSCEPTIBILITY TESTING BY MGIT 960

Rationale

Drug resistant tuberculosis has become a serious public health problem. Resistance to first line anti-TB drugs makes the disease more difficult and expensive to treat. Rapid detection of these resistant isolates is critical to effective patient management. Antimycobacterial susceptibility testing is valuable for proper treatment of patients. WHO recommends the use of liquid culture systems for rapid detection of drug susceptibility. Both MGIT and BACTEC provide DST results approximately at the same time frame.

Principle

Susceptibility testing by MGIT is a 4-13 day qualitative test. The test is based on growth of the *M. tuberculosis* strain in a drug containing tube compared to the drug free tube (Growth control). The instrument continuously monitors the 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. Results are interpreted automatically and reported as susceptible or resistant.

Available DST kit

- **SIRE** kit – supplied by manufacturer (4 drug combined)
- **INH** and **STR**– available separately for (concentrated drug for customized use)
- **SIRE** supplement – supplied by manufacturer
- **PZA Kit –** supplied by manufacturer
- **PZA** supplement – supplied by manufacturer
- **Second line** drugs – *in-house* preparation

Storage and reconstitution

- Lyophilized drug vials and SIRE supplement are to be stored at 2-8^{o}C
- Once reconstituted, the antibiotic solutions may be stored at -20^{o}C or colder up to 6 months. Once thawed, drugs should be used immediately
- Reconstitute SIRE drug vial should be reconstituted with 4ml of sterile distilled water and PZA vial with 2. 5ml.
- Date of preparation should be marked on the vial and the Cryovials (during storage)

Kit components

SIRE kit

- One vial each of lyophilized first line drugs namely, STR, INH, RIF & EMB.
- 8 vials of SIRE supplement consisting of 20ml of OADC enrichment
- One kit can be used to perform 40 tests

PZA kit

- Two vials of lyophilized PZA drug and 6 vials of PZA supplement

Concentration of drugs used

Drug	**Concentration (µg/ml)**
STR	1. 0
INH	0. 1
RIF	1. 0
EMB	5. 0
PZA	100
KAN	2. 5
Eto	5. 0
OF	2. 0
AMI	1. 0
CAP	2. 5

DST preliminary setup

- Label 5 individual MGIT 7ml tubes (Used for isolation) for each test isolate
- In each set, label one tube as growth control (GC) and the set as S,H,R& E
- Label one tube each as growth control (GC), and the rest as STR, INH, RIF & EMB
- Use 2 tubes of 7ml PZA tubes (green labeled) one for GC and one for drug
- Aseptically add 0. 8ml of MGIT SIRE / PZA Supplement to tube
- Add 0. 1ml of the respective drugs into the corresponding labeled tubes

Types of cultures: Positive MGIT tubes and LJ

Preparation of cultures suspension

a. From positive MGIT tubes

General note

- It is important to prepare the inoculum using the time reference to obtain the appropriate organism concentration for DST
- The first day of an instrument positive is considered as "0" day and can be used for DST set up from the next day (Day1) till the Day 5
- DST can be set up without any dilution of cultures ranging between 0-2 days after positivity but should be diluted (1 in 5) in case of cultures ranging between 3-5 days
- Beyond 5 days from the day of positivity the culture should be sub cultured into one fresh MGIT tube (with / without PANTA if required) and DST should be carried out.

Inoculum preparation from Day1-2

- Vortex the MGIT tubes and let stand in the cabinet for allowing the coarse particles to settle (at least 20min).
- Place 3 sterile screw capped vials in the MGIT carrier rack and label as neat, 1:10 & 1:100.
- Add 4. 5ml of sterile saline (0. 85% - 0. 85gms in 100ml of distilled water, autoclaved and used) to each of 1:10 & 1: 100 vials.

- Carefully transfer 3-4ml of the culture from positive MGIT tube in to the neat vial using a sterile disposable transfer pipette.

- Add 0. 5ml of the culture from the neat vial into 1:10 vial and mix for at least 10 times for even distribution (care must be taken to avoid aerosols while mixing)

- Similarly transfer 0. 5ml from 1:10 vial to 1:100 vial and mix it thoroughly.

- Add 0. 5ml of the suspension from 1:100 vial to the MGIT tube labeled as Growth control for SIRE – GC

- Add 0. 5ml of suspension from 1:10 vial for PZA- GC

- Add 0. 5ml of the culture from neat vial to all the labeled drug containing tubes.

- Gently tilt the tubes 3-4 times for even mixing (take care to tighten the tubes completely to avoid any sort of leakage)

Inoculum preparation from Day 3-5

- Add 1ml of the well mixed inoculum into 4ml of the vial labeled as neat (1 in 5 diluted culture will be considered as neat in this case)

- Prepare 1 in 10 and 1 in 100 dilution from neat (1 in 5) as before in saline. (Final dilution for control tubes would be 1: 50 for PZA &1:500 for SIRE)

b. Inoculum preparation from LJ medium

- Prepare culture suspension in 4ml of Middlebrook 7H9 broth with 8-10 glass beads (preferably the whole growth)

- Vortex the suspension and adjust to 1. 0 McFarland standard

- Let the suspension STAND for 20min without disturbing

- Transfer the supernatant into another screw capped vial and let stand for 15min

- Again transfer the supernatant into another vial and match with 0. 5McFarland standard

- Dilute 1ml of the suspension in 4ml of sterile saline (1 in 5 dilution). This will be considering as neat.

- Prepare 1 in 10 and 1 in 100 dilution from neat (1 in 5) as before in saline. (Final dilution for control tubes would be 1: 50 for PZA &1:500 for SIRE)

Loading the MGIT DST set into the instrument

- Place the GC and drug tubes for a particular culture in a standard order of arrangement
- Select the appropriate DST carrier set (carrier sets are available as 2, 3, 4, 5 & 8 set) depending on the number of tubes tested for each culture
- Always place the GC tube behind the bar code of the DST carrier set as bar coding is done only for the carrier set and not for the tube
- Arrange the drug tubes adjacent to the growth control
- Use separate DST carrier set for PZA (preferably 2 carrier set)
- Note down the culture number and the drugs set up in the DST register
- Open the segment in the instrument (which is allocated for DST) and select the culture input option on the screen
- Pass the barcode of the DST carrier set through the barcode reader
- Place the carrier set in the position defined by the instrument
- Note down the position of the DST set in the register for each culture

Quality control

- QC can be tailor made according to the lab use
- A panel of QC can be prepared inclusive of laboratory standard strains, strains with known / confirmed susceptibility pattern; duplicates of the isolates tested can also be included
- QC can be set up on daily basis or whenever a new batch of drugs are prepared, after instrument servicing, after a long gap in setting up DST sets

Results

- The MGIT instrument monitors DST sets until a susceptible or resistant determination is made
- Whenever the GC tube reaches a GU of 400 units, the instrument finalizes the results of DST for the particular culture
- The strain is considered “susceptible” if the GU is <100 in the drug tube and resistant if >100 and subsequently it is represented in the result sheet
- The completed DST sets are indicated by ‘positive’ signal in the DST segment of the instrument

- Unload the DST set and then print the result
- Immediately cross check the culture number & its position in the register and mark it on the result sheet
- File the result sheet and enter the results in the register

Trouble Shooting: Repeat the entire test whenever E/92/200 (under inoculum) or E/92/400 (over inoculum) error codes appear

ANIMAL PASSAGE OF *M. TUBERCULOSIS* H_{37}RV

Objective

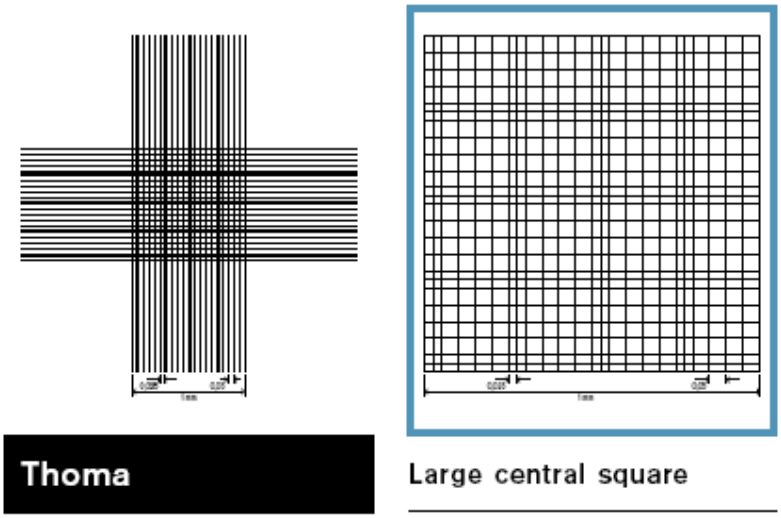
To passage standard strain of *M. tuberculosis* $H_{37}Rv$ in guinea pigs to maintain its virulence and phenotypic characters.

Materials required

- Guinea pigs - 3 month old
- Three week old cultures of *M. tuberculosis* $H_{37}Rv$ in LJ slopes
- Sterile phosphate buffered saline (PBS)
- Sterile Bijou bottles with 8-10 beads

Preparation of single cell suspension

- Prepare 2 – 3 ml of homogenised suspension of *M. tuberculosis* $H_{37}Rv$ in PBS by completely scrapping the colonies present in an LJ slope (3 + growth)
- Using 1ml tuberculin syringe aspirate and discharge the suspension for about 6 to 8 times, carefully within the same Bijou bottle to prepare single cell suspension
- Transfer 0. 5ml of the suspension into 1. 5 ml Eppendorf tube and seal it using parafilm.
- Heat kill the suspension in the eppendorf tube for 30 minutes at 80°C
- Load the heat killed suspension directly into the Thoma counter using the cover glass and count the number of bacilli in any of the 5 small squares



Total number of bacilli present in one ml is determined by the following formula: **Total number of bacilli counted / 0. 02 bacilli/ml**

Where, 0. 02 is the correction factor provided by the chamber manufacturer to derive the number of bacilli present in one ml

- The viable count should be 10^6 bacilli per ml

Inoculation

- After adjusting the required number of viable bacilli in one ml of PBS solution the vials containing the suspension should be sealed and maintained at 4°C and when ready for transporting to the animal facility it should be packed in an ice box with coolants
- Weight of the Guinea pigs are recorded and it should be approximately 300 g
- Inject 0. 1 ml o the suspension using 1ml tuberculin syringe intramuscularly in the thigh muscles of guinea pigs
- Inoculate the rest of the suspension on to an LJ slope and incubate it at 37°C
- The animals are sacrificed after 6 weeks after the inoculation
- The animals are sedated using 0. 1ml of Pentathol sodium (Reconstitute 1 g in 10ml to get 100mg/ml)
- Look for any lesions in lungs, spleen and liver after sacrificing the animals and collect the organs separately in 5 ml selective Kirchner's media
- Remove the organs in the laboratory from the medium

- Label the rest of the media as original Kirchner's and incubate along with the inoculated slopes.

- Lung samples are alone decontaminated with 5% sulphuric acid as done for the extrapulmonary biopsy specimens.

- Inoculate loopful of suspension onto 2 LJ slopes and the one Kirchner's media (0. 1 ml of inoculum)

- In case of liver and spleen the homogenize suspension and directly inoculate onto LJ slopes and kirchner's media. (0. 1 ml of inoculum)

- Examine the LJ slopes and subculture SK medium s standard procedures.

- Isolate the colonies and store the culture as per standard procedures (SOP : Storage of cultures)

MAINTENANCE OF *M. TUBERCULOSIS* STOCK CULTURES AT -80^0 C

Storage of Mycobacteria in 7H9 Liquid medium

- Add 0. 5 ml of double sterile distilled water in a Bijou bottle containing glass beads (3 mm. diameter)
- Add bacterial growth from one slope of LJ medium with 3+ growth, mix using vortex mixture for about 30 seconds.
- Allow the coarse particles to settle down
- Pipette out 100 µl of bacterial suspension into McCartney bottles containing five ml of 7H9 medium
- Incubate at 37˚ C for 10 days.
- After 10 days examine for any gross contamination (turbidity). If turbidity is observed, discard the subculture and a attempt a fresh subculture.
- Inoculate a loopful of media on a nutrient agar plate.
- Incubate at 37˚C for 24 hours and check for sterility.
- Record the observations are recorded in the Sterility check Register.
- Using 1ml tuberculin syringe, prepare uniform suspension by aspirating and discharging the suspension for about 10 times within the vial.
- Adjust the turbidity to McFarland No.1 standard.
- Pipette out aseptically 750 µl of bacterial suspension into a cryovial
- Add 750 µl of 40% sterile glycerol
- Label it with lab number & date and arrange in cryovial rack.
- Store Cryovials at –80^0 C freezer.
- Maintain a date wise list of all cultures stored with particulars of the culture in the storage register.

STORAGE AND DISCARDING OF CULTURES FROM COLD ROOM & INCUBATOR ROOM

Storage at 5- 8 °C is very important for the maintenance of culture.

Storage in Cold Room (5- 8 °C)

- The sputum & Extra pulmonary specimens from TRC, Madurai, Sub centers (S, E, B), Surveillance Study (BS),Tobacco study (TS),Referred culture samples(RF), until processed.
- LJ medium and Drug containing medium for 1 month after sterility check
- Hen's eggs for 3 days
- The Liquid medium (7 H9) & SKM for 1 month after sterility check
- Positive cultures in a storage box (steel box) according to Lab number & Center wise for 6 months
- Duplicate cultures from subcultures/ ID cultures
- Positive cultures pending for DST, Subculture, ID and at -80 ^{0}C

Modular Cold room (Blue Star)

- Store the Culture medium (12B, 13 A) vials, Drugs of BACTEC, Drugs S,H,R,E and MGIT.
- Store the Cyanogen Bromide
- Store the Blood agar, Muller Hinton agar plate and antibiotic disc used for (OI) Laboratory
- Discard the Plain medium and drug containing medium after 1 month.
- Discard the cultures after 6 months (check the culture registers and ensure the cultures are more than 6 months old)

Storage in Incubator Room (37^{0} C)

- After processing the samples (sputum) label the racks with particular study alphabets with increasing Lab number
- Incubate the MIC racks at 37^{0} C for 28 days
- Incubate PST racks at 37^{0}C for 6 weeks
- Incubate subculture & identification racks at 37 ^{0}C for 28 days

- Discard MIC racks and PST racks after DST reading

Arrangement of racks for culture reading

- First ,remove the racks which completed 8 weeks of incubation, arrange racks in descending order (8^{th} week, 7^{th} week and so on) for culture reading
- Keep the racks in the same order after culture reading (8^{th} week, 7^{th} week and so on)
- Ensure the number tags are intact in the rack (this applies to all the studies)

Discard

- The plain medium and drug containing medium after 1 month
- The cultures after 6 months (check the culture registers and ensure the cultures are more than 6 months old)
- MIC racks and PST racks after DST reading

Maintenance & Documentation

- Wipe the floor daily with 5%phenol
- Record the temperature shown in the electronic digital screen.
- If temperature fluctuates, inform to the Senior Technical officer (STO)

Inform HOD

- If the caps of the bottles are loose or broken.
- If the Liquid culture is contaminated with the flask.
- Expiry dates of chemicals & reagents
- The readings of manual thermometer and digital thermometer.
- Maintain AMC

SOP FOR FM: IQC AND EQA

IQC

- The unstained sputum smears will be prepared as per manufacturing quality control (MQC) slides preparation guidelines and stored in the laboratory at the ambient condition.

- The MQC slides should be 50 to 100 AFB per 100 oil immersion fields according to Ziehl Neelsen staining method.

- The laboratory technicians used to stain a set of smears (1 positive and 1 negative) by Auramine Phenol staining method for as per the guidelines.

- The IQC stained slides should be read by the Sr. Lab. Technician for 30 HPF and the result should be entered in the 100 box sheet.

- If the actual AFB load is reduced in the smear, calculate the % of AFB loss and note down for analysis.

EQA

- A set of unstained (5nos) MQC slides with different grades should be given to the readers in FM for staining and reading.

- All the QC results should be sent to HOD bacteriology, TRC for assessing the quality of reading.

- The QC activity for TRC and Madurai unit should be done for every 6 months periodically.

- The stained QC slides and the 100 box forms should be filed for later use in TRC and Madurai unit.

PANEL SLIDE PREPARATION

Objective

- To assess the External Quality Assurance of smear microscopy performed in Intermediate Reference Laboratory of District Tuberculosis Centre.

Materials Required

- 2% *N*-acetyl *L*- Cysteine (NALC) (Himedia -RM3142)
- 2. 9% Sodium citrate. 2H2O (Qualigens -14005)
- 10% Formalin solution (Qualigens -24005)
- McCartney bottles
- Pasteur Pipette
- Vortex mixer
- Shaker
- Hot plate
- Distilled water
- Slides

Preparation of Reagents

- *2 %* NALC*:* 2 g of NALC dissolved in 100 ml of distilled water
- *2. 9 % Sodium Citrate 2H_2O:* 2. 9 g of Sodium Citrate2H_2O dissolved in 100 ml of distilled water.
- *10% Formalin Solution:* 1ml of formalin mixed with 9 ml of distilled water

(Note: All the reagents stored in Refrigerator up to one week)

Selection of sputum specimens

- Specimen should be fresh, not more than 2 days old
- The color should be white to light green
- Avoid blood stained sputum
- Select positive **s**putum sample with known smear results (should have more than 60 AFB/ field).

- Select Negative **s**putum sample, (should have more than 20 pus cells/ field).
- Add one drop of 10% Formalin per ml of negative sputum.

Preparation of mucolytic solution

- Mix 2% of NALC with an equal amount of 2. 9% Sodium citrate right before use.
- Note: Mucolytic solution should not be stored

Positive stock preparation

- Mix Positive sputum with an equal amount of mucolytic solution
- Shake gently in a shaker for 10-15 minutes and vortex for 5 minutes.
- Keep it for 30 minutes
- Make two smears prepared from positive stock
- Stain by ZN method
- Read the slides by two different readers and record the average number of bacilli per field(eg. ,40+50=90/2=45AFB/ field)

Negative stock Preparation

- Mix Negative sputum with an equal amount of mucolytic solution
- Shake gently in a shaker for 10-15 minutes and vortex for 5 minutes.
- Keep it for 30 minutes
- Make two smears prepared from negative stock
- Stain by ZN method
- Read the slides by two different readers and record the average of pus cell

Calibration

- Pipette out one ml of positive sputum by using Pasteur pipette and count the drops (For example 20 drops/ ml)
- Pipette out one ml of negative sputum by using Pasteur pipette and count the drops (For example 18 drops/ ml)

Preparation of 3+ suspensions

- 3+smears can be directly taken from positive stock suspension (45AFB/Field)

- (ii)To achieve, the desired concentration of AFB bacilli is 36 AFB/Field

Calibration

- Pipette out one ml of positive sputum by using Pasteur pipette and count the drops (For example 20 drops/ ml)
- Using the following formula

$$\mathbf{N = DC / AC} \text{ X } \mathbf{A}$$

Where:

N - Volume of positive sputum to be added.

DC - Desired AFB concentration.

AC - Actual AFB concentration.

A - Total volume of the required suspension

- DC=36 bacilli/field; AC=45 bacilli/field; A=2ml
- N = DC / AC X A= 36/45x2=1. 6ml positive stock (2-1. 6=0. 4 ml of negative stock)
- Add 32 drops (1. 6 x 20=32) of 3+ Positive stock.
- Add 7 drops (0. 4 x 18=7) negative Stock to the above bottle
- Vortex thoroughly for 5 minutes
- Leave it for 30 minutes
- Prepare two smears, stain by ZN method ; read the slides by two different readers and record the; average number of bacilli per field(eg. 35+37=72/2=36AFB/ field)

Preparation of 2+ suspension

Calibration

- Pipette out one ml of 3+suspension by using Pasteur pipette and count the drops (For example 20 drops/ ml)

Preparation of 2+ suspension from 3+ suspension

- To achieve the desired concentration of AFB bacilli is 6AFB/F.
- Calculate the desired concentration as follows,

N = DC / AC X A

- DC=6 bacilli/field; AC=36 bacilli/field; A=2ml
- N = DC / AC X A=6/36x2=0. 33ml of 3+suspension (2-0. 33 =1. 67 ml of negative stock)
- Add 7 drops (0. 33 x 20=7) of 3+ Positive stock.
- Add 30 drops (1. 67x 18=30) negative Stock to the above bottle
- Vortex thoroughly for 5 minutes
- Leave it for 30 minutes
- Prepare two smears, stain by ZN method ; read the slides by two different readers and record the ; average number of bacilli per field (eg.5+7=12/2=6AFB/ field)

Preparation of 1+ suspension

Calibration

- Pipette out one ml of 2+suspension by using Pasteur pipette and count the drops (For example 20 drops/ ml)

Preparation of 1+ suspension from 2+ suspension

- For 1+ grade, the desired concentration of AFB bacilli is 60 AFB/100F.
- Calculate the desired concentration as follows,

N = DC / AC X A

- DC=0. 6/Field or 60 bacilli/100 field ,AC=6 bacilli/field; A=2ml
- N = DC / AC X A=0. 6/6x2=0. 2ml of 2+suspension (2-0. 2 =1. 8 ml of negative stock)
- Add 4 drops (0. 2 x 20=4) of 2+ suspension
- Add 32 drops (1. 8x 18=32) negative Stock to the above bottle
- Vortex thoroughly for 5 minutes
- Leave it for 30 minutes
- Prepare two smears, stain by ZN ; method read the slides by two different readers and record the ; average number of bacilli per field (eg. 55+65=120/2=60AFB/100 field

Preparation of scanty suspension

Calibration

- Pipette out one ml of 1+suspension by using Pasteur pipette and count the drops (For example 20 drops/ ml)

Preparation of 1+ suspension from scanty suspension

- For scanty grade, the desired concentration of AFB bacilli is 6AFB/100F.
- Calculate the desired concentration as follows,

N = DC / AC X A

- DC=0. 06/Field or 6 bacilli/100 field, AC=0. 6 bacilli/field; A=2ml
- N = DC / AC X A=0. 06/0. 6x2=0. 2ml of 2+suspension (2-0. 2 =1. 8 ml of negative stock)
- Add 4 drops (0. 2 x 20=4) of 1+ suspension
- Add 32 drops (1. 8x 18=32) negative Stock to the above bottle
- Vortex thoroughly for 5 minutes
- Leave it for 30 minutes
- Prepare two smears, stain by ZN; method read the slides by two different readers and record the; average number of bacilli per field (eg. , 7+5=12/2=6AFB/100 field
- (Note: All the suspension can be stored in refrigerator up to one week)

Smear Preparation

- Prepare Smears by using 5 mm 27 SWG
- 75 to 100 smears can be prepared per batch from each grade of positive suspension and negative stock.
- After smear preparation, air dry and heat fix all slides
- Store each grade in separate boxes.

Validation and Documentation

- Randomly select 6 slides from each grade
- Stain by ZN method.
- Give for reading to different readers.

AFB COUNT SHEET

Method : NALC **Readers Name:**

Batch No. :

Date of Preparation:

MQC Panel Slide Validation Sheet

Method **Date:** **Batch No. :**

Grade	Slide No	Random No	Reader I	Reader II	Reader III	Reader IV	Reader V	Reader VI	Average Per 100 field (SD)
Negative	1								
	2								
	3								
	4								
	5								
	6								
Scanty	1								
	2								
	3								
	4								
	5								
	6								
1+	1								
	2								
	3								
	4								
	5								
	6								
2+	1								
	2								
	3								
	4								
	5								
	6								

Tabulate the Mean - 2 SD & Mean + 2 SD in the table shown below.

Consistency

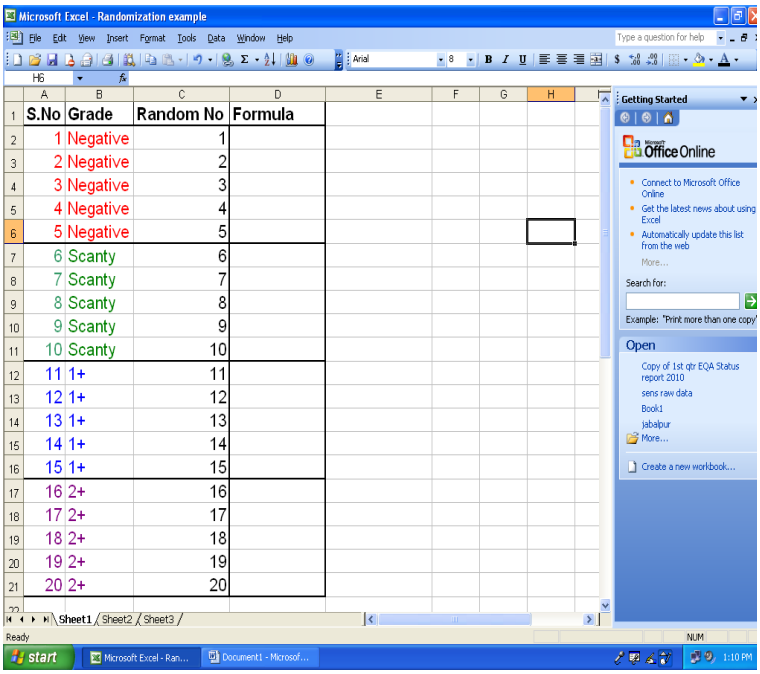
Grade	M – 2SD	M +2SD	Consistency
3+	> 11 field		True (sufficient)
2+	> 1 field	<10 field	
1+	>10/100 fields	<99/100 fields	
Scanty	>1/100 fields	<9/100 fields	

- **The batch is ready for panel testing.**

ARRANGEMENT OF THE PANEL SLIDE SETS FOR NRL OSE (Random Number generation)

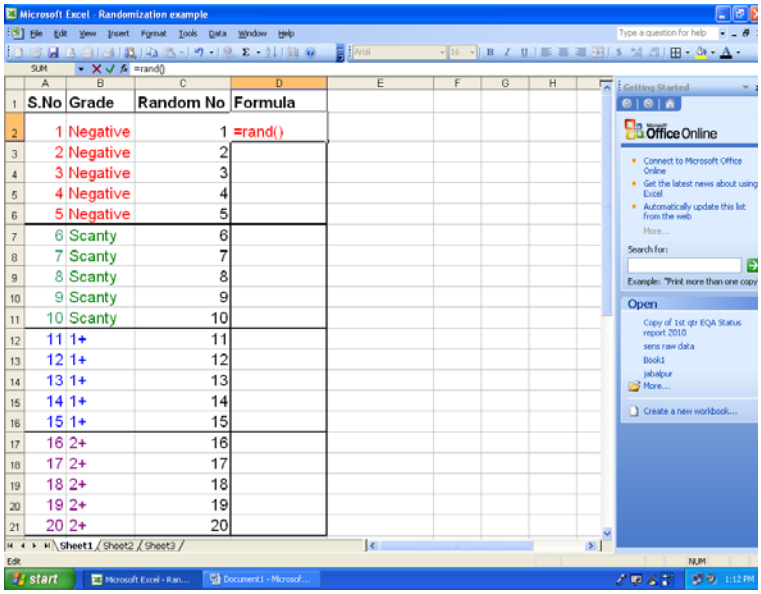
- Decide number of panel slides for each grade to be prepared
- Prepare panel smear according to SOP of panel slide preparation
- Store the heat fixed slides in slide storage box grade wise
- Generate random number using MS-excel sheet as follows

- Open MS-Office Excel Sheet.
- Enter S. No (in Column A), Grade (in Column B) and Random No(in Column C) as seen in picture
- [e. g. , Total No Slides=20(Neg:5;Scanty:5; 1+:5 and 2+:5)]

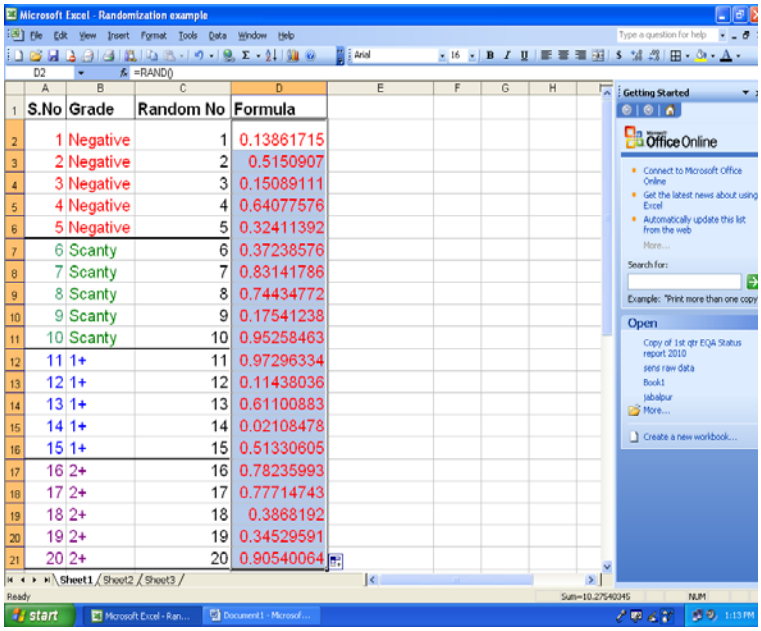


S.No	Grade	Random No	Formula
1	Negative	1	
2	Negative	2	
3	Negative	3	
4	Negative	4	
5	Negative	5	
6	Scanty	6	
7	Scanty	7	
8	Scanty	8	
9	Scanty	9	
10	Scanty	10	
11	1+	11	
12	1+	12	
13	1+	13	
14	1+	14	
15	1+	15	
16	2+	16	
17	2+	17	
18	2+	18	
19	2+	19	
20	2+	20	

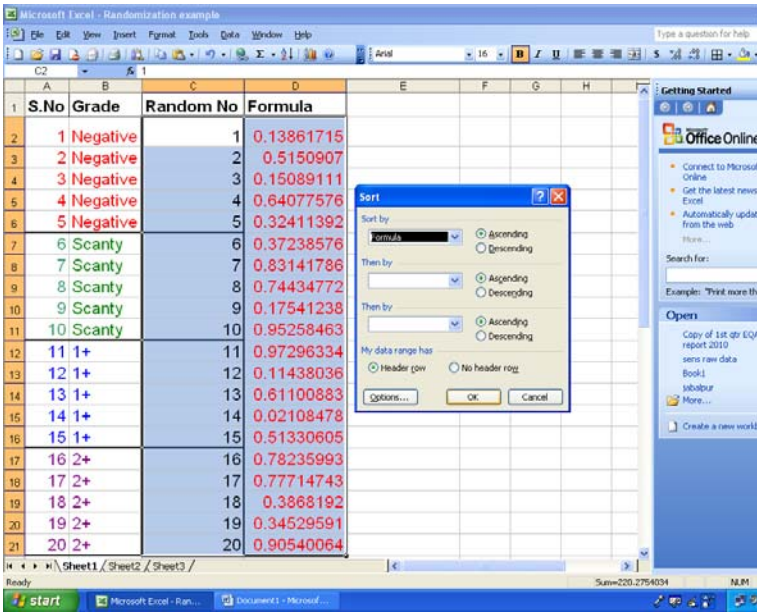
- Type *=rand()* under formula column(in Column D) and click enter



- Drag the formula for the entire column

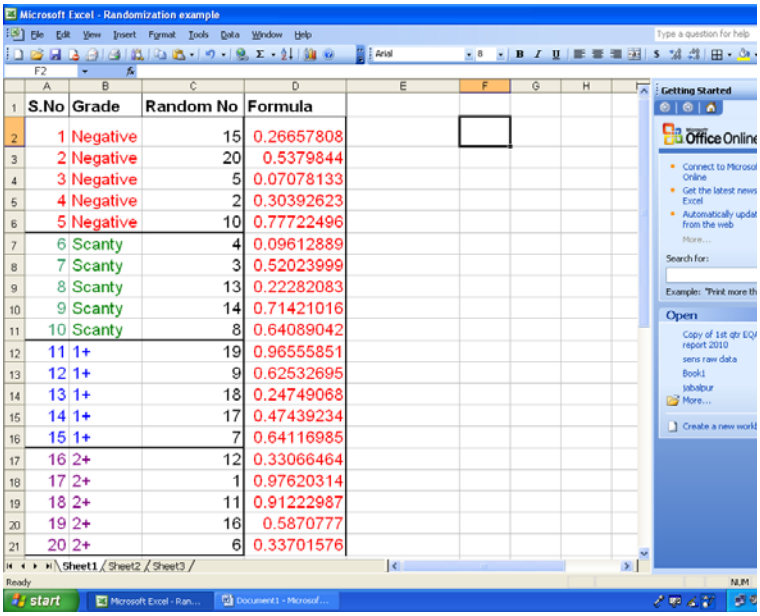


- Select Random No Column and Formula Column
- Click sort (A to Z) in Data in Menu bar
- In sort window, select formula in *sort by* drop down menu option then click ok



S.No	Grade	Random No	Formula
1	Negative	1	0.13861715
2	Negative	2	0.5150907
3	Negative	3	0.15089111
4	Negative	4	0.64077576
5	Negative	5	0.32411392
6	Scanty	6	0.37238576
7	Scanty	7	0.83141786
8	Scanty	8	0.74434772
9	Scanty	9	0.17541238
10	Scanty	10	0.95258463
11	1+	11	0.97296334
12	1+	12	0.11438036
13	1+	13	0.61100883
14	1+	14	0.02108478
15	1+	15	0.51330605
16	2+	16	0.78235993
17	2+	17	0.77714743
18	2+	18	0.3868192
19	2+	19	0.34529591
20	2+	20	0.90540064

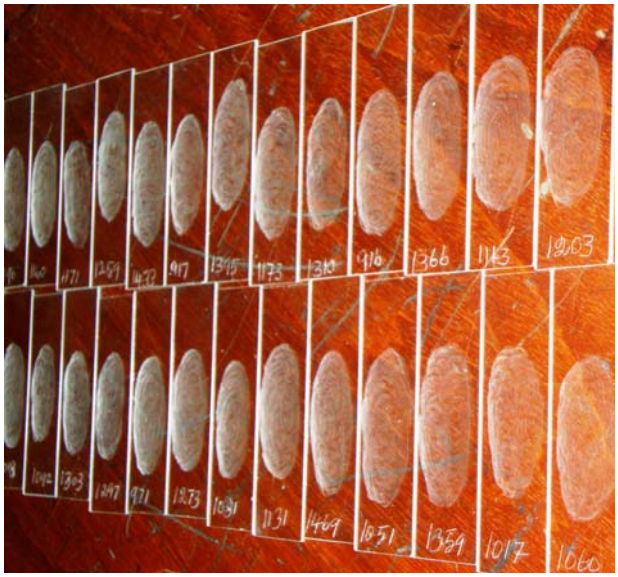
- Now you can get randomized number for each grade



S.No	Grade	Random No	Formula
1	Negative	15	0.26657808
2	Negative	20	0.5379844
3	Negative	5	0.07078133
4	Negative	2	0.30392623
5	Negative	10	0.77722496
6	Scanty	4	0.09612889
7	Scanty	3	0.52023999
8	Scanty	13	0.22282083
9	Scanty	14	0.71421016
10	Scanty	8	0.64089042
11	1+	19	0.96555851
12	1+	9	0.62532695
13	1+	18	0.24749068
14	1+	17	0.47439234
15	1+	7	0.64116985
16	2+	12	0.33066464
17	2+	1	0.97620314
18	2+	11	0.91222987
19	2+	16	0.5870777
20	2+	6	0.33701576

- Check the smear quality and take one grade for numbering at a time.
- Write random number on the slides (for each grade) using diamond marker without disturbing smears (avoid finger print).
- Take six slides from each grade for validation as per guidelines

Randomized panel slides



- Arrange five slides for each set with different grades (e. g. Neg (2);Sc (1); 1+ (2)).
- Record the slide numbers of each set and make the table.
- Pack the slide set with tissue paper (wrap individually to avoid false positive)
- Write the set number on the pack (tissue paper)

Choice of slides for set preparation

SET No.	1	2	3	4	5	6	7	8
Neg	270	226	304 392	323 389	361	380	212	382
Scanty	243	325	215	313	217	388 310	377	236
1+	279 272	329	220	219	358 248	286	202 368	309
2+	203	397 221	278	245	240	274	252	256 320

- Pack five sets in polythene cover and seal.
- Take slide sets and Panel slide result entry form for NRL OSE

PANEL SLIDES RESULT ENTRY FORM

(For NRL –OSE)

Name : **State** :

Designation : **District** :

Date : **TU** :

Set No : **MC** :

Tick appropriate column or write letter as indicated below table

Sl. No.	Slide No.	AFB result / Grade by		Specimen Quality		Staining		Size		Thickness		Evenness	
		MC	NRL (TRC)	≥10 WBC/ field	. < 10 WBC/ field	Good	Poor (U/O)	Good	Poor (B/S)	Good	Poor (K/N)	Good(E)	Poor (UE)
		1		2		3		4		5		6	
1													
2													
3													
4													
5													

- Write smear and grade
- Tick appropriate column
- Tick if **good;** write '**U'** if under-decolourized, '**O**' if over-decolourized
- Tick if **good**; write '**B'** if too big, **'S'** if too small
- Tick if **good**; write '**K'** if too thick, **'N'** if too thin
- Tick if **good**; write '**E'** if even, '**UE'** if uneven

Signature of examiner

Remarks

Signature of the NRL Consultant with date

PANEL CULTURE PREPARATION FOR EQA

Objective & Scope

To prepare a panel of 30 *M. tuberculosis* strains of different susceptibility pattern in Middlebrook 7H9 liquid media for External quality assessment programme.

Materials required

- *M. tuberculosis* strains of different susceptibility pattern in LJ slopes
- Middlebrook 7H9 liquid media with Bovine serum albumin Dextrose complex
- Cryovials
- Cryovial racks
- Sterile Bijou bottles with 8-10 glass beads (3 mm) containing 0. 3 ml distilled water
- Micropipettes with sterile aerosol barrier tips (200 µ l)
- Packing materials

Procedure (for ten centers)

Selection and subculture of *M. tuberculosis* strains

Select *M. tuberculosis* strains of different susceptibility pattern in consultation with HOD, statistician using EQA cultures from Antwerp, Belgium (25 + 5 duplicates)

Pattern	No of panel culture	Total
SHRE sensitive	5 + H37Rv+ 1 Duplicate	7
SHRE resistance	5 + 2 Duplicate	7
HRE resistance	2+ 1 Duplicate	3
HR resistance	2	2
HE resistance	3 + 1 Duplicate	4
SR resistance	3	3
SH resistance	2	2
S resistance	2	2
Total		30

- Subculture these selected cultures onto 3 LJ slopes
- Incubate at 37°C
- Wait for sufficient growth

- Look for pure growth and check for contamination ; if found do not use that slope

- Allot serial numbers on to the slopes and maintain the list with the allotted serial numbers for the corresponding lab numbers carefully

Inoculation

- Distribute 0. 5 ml of sterile 7H9 complete media to ten sets of cryovials of 30 numbers each using 5 ml sterile pipette

- Incubate them for 48 hours at 37°C for sterility checking

- Check for any turbidity, if any turbidity is visible discard the vial

- Label the vials with serial numbers 1 to 30 on each of the ten sets

- Separately label 1 to 30 numbers on 30 Bijou bottle containing 0. 7 ml of distilled water along with beads

- Deliver a loop full of colonies from LJ slopes into these Bijou bottles

- Vortex for 40 seconds

- Add 0. 3 ml of distilled water to the suspensions, mix well

- Deliver 50 µl of the suspension each onto the corresponding vials with due sterile precautions

- Incubate the vials at 37°C for sufficient growth (for 2 – 3 weeks)

- After 48 hours of incubation pick out spot one of the vials from each of the strain on to Nutrient agar plate for sterility checking

- Incubate the plate overnight and look for any contamination, if found discard that particular strain and redo the whole work for that particular strain

- After sufficient growth is visible, give the list containing the serial numbers with the culture number for randomization to a statistician.

- Notify the number and the details of the centers for whom the panel cultures are to be sent to the statistician and accordingly random numbers are to be generated

- Arrange the cryovials from 1 to 30 serially in separate cryovial boxes and check for the correctness

- Give Random numbers in the presence of statistician (after removing the serial numbers from the vials using cotton soaked with absolute alcohol)

- Seal the vials using parafilm
- Pack the boxes in a cardboard boxes using packing materials and wrap them up using brown sheets of paper
- Affix Receiver and sender address over the package and dispatch for the speed post section along with the covering letter from the HOD, Bacteriology.
- File a copy of the communication sent in corresponding folders.

Training Protocol for Laboratory Technicians & Senior Tuberculosis Laboratory Supervisor in Revised National Tuberculosis Control Programme (RNTCP)

- The World Health Organization (WHO) recommends the Directly Observed Treatment Short Course DOTS strategy for tuberculosis (TB) Control which has been adopted by many National tuberculosis Programme.

- An effective TB control programme depends upon laboratories providing accurate, reliable and timely detection of TB cases.

- Majority of smear positive cases are detected with direct smear microscopy. Therefore, assuring high quality direct smear microscopy is the responsibility of TB control programme and Ministry of Health working in partnership to provide training, support and ongoing monitoring of testing in all levels of laboratory.

- Acid Fast Microscopy involves procedures that should follow standard methods developed by the International organizations.

- Effective training in AFB direct smear microscopy requires a laboratory work shop that includes practical exercise with presentations and discussions of the challenges solution for common problems.

- Therefore these training material are designed as a "workshop in a box" that includes all the presentations, exercises, and step by step guidance for convening and conducting 10-15 days practical laboratory

- This training package is designed for laboratory workers who perform direct smear microscopy. It is important to have an understanding of the participant's baseline knowledge and fears so that these can be adequately addressed during the workshop.

- For optimum learning experience and management of the workshop it is recommended that the number of the participants should not exceed 4 to 6 participants per facilitator.

- The overall numbers of participants depends upon the laboratory facility for the practical sessions and participants experience in performing AFB microscopy.

RNTCP Training Schedule for STLS

	8. 30 A. M TO 1. 30 P. M	2. 00P. M TO 4. 30 P. M
DAY 1	Registration Introduction by Director, TRC Pre test theory (KAP Questionnaires) Modular reading (LT)	Pre training Smear Reading- 20 Slides.
DAY 2	Re-reading discrepant slides Computer slide show Modular reading (LT)	Pre training- Assessment of smear making, staining & reading – 10nos
DAY 3	Re-reading discrepant slides Computer slide show Modular reading (LT)	ZN stained smears reading - 20 Test 1.
DAY 4	Smear reading Modular reading (STLS)	Re-reading Discrepant slides Computer slide show Maintenance of microscope Test 2. ZN stained smears reading- 20
DAY 5	Checking Positive slides- 10 (Have to stain the smear and check) Modular reading (STLS)	Re-reading discrepant slides. Individual smear practice- 25 Smear making, Staining & reading –10
DAY 6	ZN stained slides reading - 20 Modular reading (STLS)	Smear reading Re-reading Discrepant slides Test 3
DAY 7	Checking Negative slides –10 (Have to stain the smear and check) Computer slide show Modular reading (STLS)	Re-reading discrepant slides ZN stained smears reading-20 Test 4
DAY	Revised Quality Assurance Protocol.	Reagents preparation for ZN

8	Modular Reading	staining Film show - Sputum AFB Microscopy (CDC Atlanta)
DAY 9	Smear reading (Thick, thin, big, small, even, uneven, under decolorized& over decolorized) Quality Assurance Modular reading	Quality Assurance practical (Role play) "Group photograph" Test 5
DAY 10	Quality assurance smear reading Quality assurance Modular reading	Quality Assurance practical (Role play) Revision test
DAY 11	Quality assurance (Role play)	Quality assurance practical (Role play)
DAY 12	**FIELD VISIT**	
DAY 13	Discussion about Field visit Scanty smears reading Quiz programme	Post training Assessment of Smear making, staining – 10
DAY 14	Computer programme Theory Post test	Post training slide reading -20 Computer slide show.
DAY 15	Discussion about training program Review of the performance with Quality Assurance table	Pledge, Feedback, Evaluation, Valedictory Function List of participants with details.

Total slides to be examined: 120. Total smears to be made: 40

COURSE MATERIALS: STLS Module, Manual for LT, LT Module, RNTCP at a glance, Revised Quality Assurance Protocol, Schedule of the training. Kit containing scribbling pad, pen, pencil, scale and erase

RNTCP Training Schedule for LT

	8. 30 A. M TO 1. 30 P. M	**2. 00 P. M TO 4. 30 P. M**
DAY 1	Registration - Introduction by Director, TRC Pre test theory (LT, KAP Questionnaires) Modular reading (LT)	Pre training Smear Reading -20 Slides.
DAY 2	Smear reading (Re-reading Discrepant slides) Computer slide show Modular reading (LT)	Pre training Assessment of smear making 10 slides staining & reading
DAY 3	Re-reading Discrepant slides. Computer slides show Modular reading contd. (LT)	25 stained slides reading Test 1
DAY 4	Smear reading contd. Modular reading (LT)	Re-reading Discrepant slides. Computer slide show Maintenance of microscope Test 2.
DAY 5	Checking Positive slides. Modular reading (LT)	Re-reading discrepant slides Smear making- 10 slide each Staining & reading Test 3.
DAY 6	Checking negative slides	Re-reading discrepant slides Computer slide show Test 4.
DAY 7	Revision test Scanty smears reading	Reagent preparation for ZN staining Film show- Kanne Kanmani Sputum AFB Microscopy (CDC Atlanta, USA)

DAY 8	**FIELD VISIT** Visit to Microscopic Centre Tuberculosis Unit Discussion with STLS & MO -TC	
DAY 9	Discussion on field visit Quiz programme Post training Assessment of smear making & staining Post test theory	Post training smear reading
DAY10	Discussion about training programme Review of performance	Pledge Feedback Evaluation Form Valedictory Function List of participants with details.

Total slides to be examined: 100

Total smears to be made, stained and examined: 40

COURSE MATERIALS

Manual for LT, LT Module, RNTCP at a glance, Schedule of the training.

Kit containing scribbling pad, pen, pencil, scale and eraser.

TEN DAYS TRAINING PROTOCOL FOR LAB TECHNICIAN AND MICROBIOLOGIST IN CULTURE & DST

	Section	Techniques
DAY 1		Introduction to Bacteriology lab
	RNTCP room	Pre-test
	Visit to Clinic	Specimen collection
	RNTCP room	Module reading DST(5 – 18)
DAY 2	Wash up section	Bottle washing Preparation
	Media preparation	Plain media
	RNTCP room	Module reading DST(19 – 33)
	Main lab	Specimen processing and inoculation
DAY 3	Media preparation	Drug containing media
	Main lab	Culture suspension and various DST method
	RNTCP room	Module reading DST(34 – 41)
DAY 4	RNTCP room	Module reading DST (42-66)
		Identification test (Niacin & Catalase)

DAY 5	RNTCP room	Module reading DST(42 – 66)
	Main lab	Demonstration of DST by proportion method
DAY 6	Culture reading room	Culture reading
	Main lab	Perform DST by proportion method
DAY 7	Main lab	Discussion
		BACTEC& MGIT,HPLC
DAY 8	Main lab	DST reading & Panel slide preparation
DAY 9	RNTCP room	Post test
DAY 10		Review of training
		Valedictory function

Tea Break: 11. 00 am-11. 30 am & 3. 00 pm -3. 30 pm

Lunch break: 1. 00 pm -1. 30 pm

Course material: RNTCP training manual for Culture and Drug susceptibility

FIVE DAYS TRAINING PROTOCOL FOR MICROBIOLOGIST IN CULTURE &DST

	Section	Techniques
DAY 1	RNTCP room	Introduction to Bacteriology lab
		Pre-test
	Wash Up Room	Bottle washing and disinfectant Preparation
	Clinic	Lab Reception –Sample Collection
DAY 2	Media Room	Plain LJ media preparation
	Main lab	Smear preparation and Staining methods (FM and ZN) Microscopic Reading and reporting
	Main Lab	Specimen Process and Inoculation
	C. Reading Room	Culture & DST reading and reporting
DAY 3	Media Room	Drug containing media preparation
	Main Lab	DST by PST method
		Hands on training on DST
		Hands on training on second Line DST
DAY 4	Main Lab	Identification test -Catalase, Niacin and PNB
		Rapid Method- BACTEC and MGIT Species Identification -Capilia TB
DAY 5	RNTCP Room	Post Test
	RNTCP room	Review of training Feedback & Post test Valedictory Function

Tea Break: 11. 00 to 11. 15am and 3. 00 to 3. 15 pm

Lunch break: 1. 00 to 1. 30 pm

Course material: RNTCP training manual for Culture and Drug susceptibility

LABORATORY DIAGNOSIS OF OPPORTUNISTIC INFECTIONS (OI)

Opportunistic infections (OIs) are caused by organisms of low or no virulence and include bacteria, fungi, parasites and viruses. Diagnostic techniques in the laboratory include various microscopic methods and cultures including a variety of media and incubation environments.

SOPs for these diseases focus on specimen collection, storage and transportation, specimen processing, examination, identification, recording and reporting, interpretation of results, and safety precautions.

General Safety considerations

Specimen collection

- Universal precautions and aseptic techniques to be followed.

Specimen transport and Storage

- Sterile leak proof and appropriate container in a sealed plastic bag.

Specimen processing

- Universal precautions to be followed.
- At least biosafety level II with good laboratory practice.

Specimen collections

Optimum time of specimen collection

- Specimens such as sputum and urine are best collected as early morning samples.
- Blood is best collected when the patient is febrile, i. e. When the temperature begins to rise.
- Ideally before initiation of antimicrobial therapy.

Correct specimen type

- Depends upon the clinical criteria and the organism suspected *e. g.* Blood culture, sputum, stool or urine.
- Appropriate specimen from the site of infection in appropriate container and adequate amount.

Collection of Clinical Specimens for Bacteriological Investigations

- **Swabs sticks (sterile)** Specimens collected by this method include pus and exudates from suppurating wounds, necrotic lesions, boils, abscesses, etc. The swabs should be made with absorbent cotton wrapped tightly round wooden applicator sticks in appropriate sterile test tubes.

- Collect two swabs; one for smear studies and the other for culture studies. if only one swab is received, first culture and then make smears.

- The swabs may be moistened with a few drops of sterile Normal Saline or Distilled Water prior to swabbing to prevent drying out of the material swabbed.

- **Universal containers (McCartney bottles)** Specimens include pus, sputum, urine**,** cerebrospinal fluid (CSF), body fluids (pleural, peritoneal, ascitic), all drains, lavages, rinses. Ideally, sputum and urine should be collected in wide mouthed Uricol and Sputum containers.

- **Sputum:** Ideally early morning sputum should be collected; the patient should be instructed to cough from deep within his lungs and expectorate the sputum into the container. This should be done prior to teeth brushing or other method of oral hygiene.

- **Urine:** Instruct the patient to wash the urinogenital area well, pat dry and then collect a "clean catch" or mid-stream urine sample.

- **Faeces:** A small aliquot of the specimen should be collected in a suitable sterile container.

Specimen transport and storage

Every specimen must be accompanied by a requisition form which gives

- Patient's name

- Patient's gender with age: whether male/female infant, child or adult

- Patient's Hospitalization/Identity Number with Ward/ Health centre details

- Type of specimen and date, mode and time of collection

- Investigation required as also whether specific antimicrobial tests should be included

- Brief clinical history mentioning patient's illness, suspected diagnosis, any antimicrobial therapy started.

Time between specimen collection and processing

- Ideally the specimen should reach the laboratory within 30 minutes of collection.
- Urine which arrives at the laboratory more than two hours after collection is not suitable for culture and should be rejected.
- All specimens received at the specimen reception site should initially be placed in an appropriate disinfectant solution for 30 minutes prior to dispatch to the laboratory.
- Specimens should be processed promptly after receipt in the laboratory.

Storage

Any specimen that cannot be sent to the laboratory within 30 minutes or cannot be processed immediately should be stored in the refrigerator or freezer (except CSF body fluids and hemoculture should be kept at 37 ºC).

Specimen processing

Proper documentation upon receipt of specimen

- Record the correctness of the specimen on the requisition form.
- Maintenance of laboratory records with lab number.

Initial processing

- Depends upon the types of specimen collected and organism suspected.
- Blood culture should be incubated at 37ºC immediately.
- CSF and other body fluids should be centrifuged at 3000g for 15 minutes. The sediment is used for culture and staining.

Microscopy

Perform staining as required.

- Gram stain is the most common stain used for identification of bacteria.

Reporting procedure

- Written report
- Culture and susceptibility report: 3-5 days (Report susceptibility test as clinically indicated)

Disposal of specimens

- All specimens must be autoclaved or disinfected before being discarded.
- Applicator sticks and gauge pieces should be incinerated.
- All used glassware should be put into the disinfectant bins for at least one hour before washing.

Staining Methods for Identification of Bacteria

Gram Stain

This is a universal staining technique requiring extreme skill to identify bacteria and fungi on basis of

- Gram character of individual bacteria (Gram positive or Gram negative)
- Morphology (whether cocci, bacilli, spirilli)
- Arrangement (whether as singles, pairs, clusters or chains)

Reagents required

1. 2% Crystal violet or Methyl violet or Gentian violet : Primary Stain
2. Gram's Iodine : Mordant
3. Acetone : De-colorizer
4. 0. 5 % Safranin : Counter stain

Method

- Using a sterile nichrome wire loop make a thin smear in the centre of a glass slide
- Air dry the smear within the safety cabinet and heat fix the smear.
- Place the slides on a staining rack over the staining sink.
- Pour few drops of Crystal violet on the smear and leave for 2 minutes.
- Wash off the stain with tap water and cover the smear with Gram's iodine for 45 seconds.
- Wash off the iodine in a slow stream of running tap water.
- Add 2 to 3 drops of Acetone (3-4 sec) and immediately wash off with tap water.

- Cover the smear with safranine for 25 to 30 seconds.
- Wash the slide in tap water, dry and observe under oil immersion lens of the microscope.

Observations

Gram positive organisms stain as deep violet cells; Gram negative organisms appear pink to red.

Culture methods

Selection of media

The most commonly used media in the Bacteriology laboratory include Blood agar and MacConkey agar (for primary isolation), Cysteine-Lactose- Electrolyte deficient agar (for urine specimens) and Mueller-Hinton agar for antimicrobial susceptibility testing.

Blood agar (BA) is used for the isolation of pathogens from all types of specimens except urine and stool specimens.

MacConkey agar (MA) is used for the isolation and identification of enteric bacteria and also differentiates lactose fermenters from nonlactose fermenters. It is also used for isolation of faecal pathogens.

Cysteine-Lactose-Electrolyte-Deficient (CLED) agar is used for the isolation of urinary pathogens including yeasts and those bacteria inhibited by MA. It can also differentiate lactose fermenters from nonlactose fermenters and provides a good approximation of bacterial load in urine samples.

Mueller-Hinton agar (MHA) is used exclusively for performing antimicrobial sensitivity tests (AMST). It is not advised as a medium for sterility checking or as an isolation medium.

Isolation of Bacteria from various specimens

All swabs

1ST DAY

- Make a smear from one of the swabs and Gram stain.
- Record the findings.
- Inoculate one plate each of Blood agar, Chocolate agar and MacConkey agar following the **"Four quadrant Streak Plate Isolation technique.**
- Incubate the plates at 37ºC for 24 to 48 hrs.

2nd DAY

- Compare the colonies on each plate and record the various types of growth.
- On Blood agar plate, look for alpha, beta or gamma hemolytic colonies in a preliminary identification of possible pathogens.
- Make Gram stained smears of all suspect colonies.
- Perform identification and biochemical tests for Gram positive/negative coccoid and/or Gram negative bacillary pathogens.
- Set up antimicrobial tests.

3rd DAY

- Record the results of identification and biochemical tests and IDENTIFY the isolate.
- Record the results of antimicrobial susceptibility testing and compare with Standard Antimicrobial Susceptibility Interpretation Chart
- Report as (a) SUSCEPTIBLE, (b) INTERMEDIATE SUSCEPTIBLE or (c) RESISTANT.
- Finalize the results, report and dispatch to Clinic reception.

All Respiratory specimens, pus, drains, lavages, rinses

- Make a smear from the specimens (except sputum) and Gram stain.
- If the sample is pus, look for thin Gram positive filaments and fragmented bacilli and pus cells
- Record the findings.

- Process as for swabs from points 3 to 13.

Sterile Body fluids: CSF, pleural, peritoneal, Synovial fluids, etc.

CSF

- Using strict aseptic precautions transfer the fluid to a microfuge tube and centrifuge it at 3000rpm for 15 to 20mins.
- Decant the supernatant.
- Gently tap the deposit in the tube to facilitate proper mixing.
- Using a suitable micropipette transfer 10µl of deposit to each of 2 clean glass slides.
- Spread one of the drops into a small circle not more than 3mm.
- Air dry and perform Gram stain on this smear.
- Place 1 drop (~10µl) of Camlin India ink or 10% Nigrosine on the other drop and mix well.
- Place a cover slip over the preparation carefully to avoid air bubbles and observe microscopically for presence of capsulated *C. neoformans.*
- **Inform the clinician immediately the results of Microscopic examination.**
- Inoculate each of Blood agar and MacConkey agar plates with 10µl of deposit, **without sterilizing the loop between streaking**.
- Also inoculate 2 slants Sabouraud's Dextrose agar (SDA) for isolation of CSF pathogenic yeasts.
- Incubate the media at 37ºC for 24 to 48 hrs. (SDA slants should be incubated at 25ºC and 37°C for at least 2 weeks).
- Observe the media over the next 2 days for bacillary/ yeasts growth.
- Make a Gram smear from one representative colony from either BA or CA.
- Proceed as for swabs from points 8 to 13.

Urine

- **Urine specimens should never be centrifuged prior to bacteriology isolations.**
- Transfer 20µl of well mixed urine sample to a clean glass slide.

- Place a cover slip over the drop and view under 10X and the 40X objectives of the microscope.
- Record the number of the following structures per high power field:
- Pus cells
- Epithelial cells
- Bacteria (whether cocci or bacilli (motile or nonmotile)
- Yeasts
- Any other abnormal details
- Inoculate 10 µl specimen on CLED plate.
- Using the sterile loop spread the inoculum over a small surface area.

(Do not sterilize the loop at any point of time hereon, or else final bacterial colony counts will be affected).

- Using the four quadrant principle, continue streaking the plate.
- Incubate the plate at 37ºC for 24 to 48 hrs.
- Observe the plate next day for bacillary/ yeasts growth.
- Proceed as given for swabs from points 6 to 13.

DAY 1: Reading of colonies after 24/48 hours (Primary isolation)

Colony characters: Macroscopic

1. **Size:** pin point, pin head, small, large.
2. **Color:** white, cream, pink, golden yellow, citrus yellow, orange, red, grey, black.
3. **Opacity:** transparent, translucent, opaque.
4. **Consistency:** smooth, rough, butyrous, mucoid or dry.

Microscopic

1. **Gram character**
   - Gram positive or Gram negative
2. **Shape**
   - spherical, ovoid, lanceolate, coffee-bean : Cocci

- rod like/ Comma : Bacilli
- spiral like : Spirilli, Spirochaetes

3. Arrangement of cells

- singles, pairs, short chains, long chains, clusters (regular/irregular), Chinese letter forms.

BA: Report as Gram positive/Gram negative cocci (mention arrangement) / bacilli showing alpha/beta/no hemolysis

MA/CLED: Report as lactose/non-lactose fermenting Gram positive/Gram negative cocci (mention arrangement)/bacilli

Identification of Cocci

1. Hemolytic Reactions on Blood Agar

Hemolytic reactions are generally classified as **alpha, beta or gamma** according to the appearance of zones around isolated colonies growing on or in the medium.

Alpha hemolysis: The colony is surrounded by a zone of incomplete hemolysis due to intact but discolored erythrocytes that have a greenish color. This appearance is generally due to the action of peroxide produced by the bacteria.

Beta hemolysis: The colony is surrounded by a white or clear zone in which few or no intact erythrocytes are found. Beta hemolysis is caused by one or more erythrocyte-lysing enzymes called hemolysins.

Gamma hemolysis is simply a synonym for negative hemolysis in which there is no change in the medium surrounding the colony.

2. Catalase test

Demonstrates the presence of enzyme catalase that catalyses the breakdown of H_2O_2 to oxygen and water.

- Label a clean grease free glass slide with the Lab No.
- Transfer 1 drop of 3% H_2O_2 to the slide using a sterile Pasteur pipette.
- Using a straight nichrome wire, touch the suspect colony and emulsify in the drop.

Positive: Effervescence (production of gas bubbles) on addition of isolate to a drop of H_2O_2. *Staphylococcus* spp.

Negative: No effervescence. All other cocci**.**

3. Coagulase test (slide test)

Detects the enzyme coagulase in the bacterial cell wall. It acts directly on the fibrinogen present in human or rabbit plasma. Fibrin precipitates on the cell surfaces causing them to stick together.

- Label a clean grease free glass slide with the Lab No.
- Transfer 1 drop of sterile human or rabbit plasma to the slide using a sterile Pasteur pipette.
- Using a straight nichrome wire, touch the suspect colony and emulsify in the drop.

Positive: Plasma coagulates within 20 seconds. *S. aureus*.

Negative: *S. epidermidis, S. saprophyticus,* all *Streptococci, Enterococci*.

CLED agar: Colony count of organisms grown

Count the single type of colonies appearing on CLED plate (Count applies to either 1µl or 10 µl)

Calculate the Colony Forming Units (CFU) / ml of urine using the formula

$$\text{No. of CFU / ml} = \frac{\text{No. Counted x 1000}}{\text{Vol. of urine inoculated}}$$

Interpretation

- Check that only one morphological type of colony grows on the agar.

If 2 or more types are seen, report as CONTAMINANTS ISOLATED REQUEST REPEAT.

- Colony counts should be interpreted taking into account the age of the patient, the mode of collection and whether chemotherapy has been initiated.
- Any count significant: Infants below 2 years, suprapubic collections.
- 2000 to 8000 significant: Children from 2 to 12 years, catheterized specimens
- 10,000: Significant
- Antimicrobial sensitivity tests are warranted.

- Counts >10,000: Highly significant

Identification of Gram negative Bacilli

Colonies on MacConkey agar:

Pink, nonmucoid small colonies: Lactose fermenter ***E. coli***

Pink, large mucoid colnies: Lactose fermenter ***Klebsiella*** spp.

Pale nonmucoid colonies: Nonlactose fermenters

Pale, spreading or swarming growth with fishy odour: ***Proteus*** spp.

Pale greyish colonies with greenish diffusable pigmentation and fruity or musty odour:

P. aeruginosa.

Oxidase test

Done only for nonlactose fermenters

Detects the presence of oxidase enzymes that will change the color of 1% tetra-methyl-p-phenylenediamine to a deep purple color. The dye is impregnated into sterile filter paper disks or strips.

- Label a clean grease free glass slide with the Lab No.
- Transfer an oxidase disk to the slide using sterile forceps and moisten with 1 drop of sterile distilled water.
- Using a straight nichrome wire, touch the suspect colony and rub it into the moistened disk.

Positive : Development of deep purple color within 20 seconds. *N. meningitidis,*

N. gonnorhoeae, P. aeruginosa, Vibrio spp.

Negative: No change in color of reagent strip/ disk. All other cocci, all enterobacteria

Day 2 (Contd): Setting up Antimicrobial Susceptibility Tests

Kirby Bauer Disk Diffusion test method

This is a disk diffusion method that is a rigorously standardized method as recommended by the Clinical and Laboratory Standards Institute (CLSI), formerly known as The National Committee for Clinical Laboratory Standards.

This test uses antibiotic-impregnated filter paper disks (6mm diameter) to test whether particular bacteria are susceptible to specific antibiotics. A known quantity of bacteria is grown on agar plates in the presence of thin disks containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the disk where bacteria are not capable of growing (called a zone of inhibition).

Reagents for the test

1. Mueller-Hinton Agar Medium
2. Standard Bacterial strains for antimicrobial susceptibility testing

1. *S. aureus* ATCC 25923 for testing Gram positive cocci other than *S. pneumoniae*
2. S. pneumoniae ATCC 49619 for testing *S. pneumoniae* isolates
3. *E. coli* ATCC 25922 for testing Gram negative bacilli
4. *P. aeruginosa* ATCC 27853 for testing *P. aeruginosa* isolates

Storage of commercial antimicrobial disks

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Discs should be stored as follows:

- Refrigerate the containers at 8°C or below, or freeze at -14°C or below, in a nonfrost-free freezer until needed.
- Sealed packages of disks that contain drugs from the ß-lactam class should be stored frozen, except for a small working supply, which may be refrigerated for at most one week.
- The unopened disk containers should be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening.
- Only those disks that have not reached the manufacturer's expiry date stated on the label may be used. Disks should be discarded on the expiration date.

Turbidity standard for inoculum preparation

0. 5 McFarland standard should be used.

Direct colony suspension method

Inoculum preparation

1. Using nichrome loop pick up 5 to 6 isolated colonies from the isolation plate.

2. Suspend the colonies in 2 to 3ml sterile nutrient broth/ normal saline/ Distilled water in a

Bijou bottle or Sterlin vials.

3. Adjust the turbidity of the suspension to match 0. 5 McFarland turbidity standard using sterile saline and vortex.

Inoculation of Test plates: Should be done within 15 mins of inoculum preparation.

- Dip a sterile cotton swab (absorbent) into the suspension. Rotate the swab several times in the suspension and then press firmly on the inside wall of the tube above suspension level to remove excess inoculum.

- Streak the swab over the entire pre-dried surface of MHA plate. Repeat the procedure 2 to 3 times by rotating the plate approximately 60º each time to ensure an even distribution of inoculum. Finally, swab the rim of the agar plate.

- Allow the plate to dry for 3 to 5mins to allow excess surface moisture to be absorbed before applying the drug impregnated disks.

Selection of Antibiotic impregnated disks for Isolates

Refer to Disk manufacturer instructions for details

For urinary isolates include Nalidixic acid and Nitrofurantoin.

Application of Antibacterial Disks to Inoculated agar Plates

Follow the guidelines for selecting the appropriate drug disks for Gram positive and Gram negative isolates.

- Bring the disks to be used to room temperature.

- Open each disk vial/cartridge at the time of dispensing the disks.

- With the help of a sterile forceps, apply the disks to the inoculated Mueller Hinton Agar plate. The disks should be distributed evenly so that they are at least 24 to 25mm from center to center.

- No more than 5 to 6 disks should be placed on a 100 mm plate.

- In case a disk falls on the medium by mistake, discard it, BUT do not place another disk on this spot. Instead place it in another location on the agar.

- Invert the plates and incubate at 37ºC for 18 to 24 hrs within 15 mins after the disks are placed.

- Replace the unused disks in the refrigerator.

Day 3: Finalizing of Results

A. Reading of all identification tests and their Interpretation

1. Record all the results of the identification tests put up and identify the isolate.

B. Reading of antibiotic sensitivity plates and their Interpretation

Refer Zone Size Interpretative Chart (HiMEDIA)

- Examine each plate for confluence of growth and zones of inhibition of growth around the disks.

- Measure the diameter of each zone of complete inhibition including the diameter of the disk.

- Measure the zones up to the nearest millimeter using a measuring ruler held to the back or underside of the plate. The petri plate is held above a black nonreflecting background and illuminated with reflected light.

- If blood agar plate is used for *Streptococci*, the zones are measured form the upper surface of the agar illuminated with reflected light with the cover removed.

- The zone margins should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye.

- The organisms are reported as susceptible, intermediate or resistant to the agents that have been tested.

Most antimicrobial disk manufactures supply the Interpretation Charts along with their products. These may be referred to in order to interpret the susceptibility results.

Filing of Final report (in register and then on report forms) followed by Despatch to Clinic Reception.

Patient Details

Nature of Specimen sent with Container No. and Date and Time of collection

Date of Reporting

Smear/ Wet mount Report: Not for respiratory and urine samples

Culture

Colony count (in case of urine)

Antibiogram

COMMON FUNGAL OPPORTUNISTIC INFECTIONS

Commonly encountered fungal infections in India

- Candidosis
- Cryptococcosis
- Pneumocystosis (*Pneumocystis carinii* Pneumonia or PCP)
- Aspergillosis
- Penicilliosis

Candidosis

Candidosis is a common endogenous opportunistic yeast infection mostly caused by *Candida albicans*.

C. albicans is part of normal flora in 25-50% normal healthy individuals and 70-90% carriage in HIV infected patients.

Cryptococcosis

Cryptococcal meningitis is considered as one of the AIDS defining infections caused by an encapsulated yeast *Cryptococcus neoformans.*

***Pneumocystis carinii* pneumonia or pneumocystosis**

This is a form of pneumonia caused by the yeast-like fungus *Pneumocystis carinii* (*jirovecii*).

Aspergillosis

This is caused by saprophytic *Aspergillus* spp.

Penicilliosis marneffei

This infection is caused by the dimorphic fungus *Penicillium marneffei*. It has recently been isolated from India (Manipur and Tamil Nadu) also.

Specimen collections

- Universal precautions and aseptic techniques should be strictly adhered to.

Storage

Specimens should be processed in the laboratory as soon as possible. Delay in processing of unrefrigerated specimens over 4 hours is undesirable.

Where there is a delay in processing, specimens should be refrigerated **(except CSF and specimens for isolation of *Cryptococcus*.)**

Specimen processing

- Universal precautions to be followed.
- At least biosafety Containment level II with good laboratory practice.

Specimen Disposal

All infected material should be treated as per WHO procedures.

General techniques used in Medical Mycology

The techniques used in clinical mycology are in general similar to those used in clinical

bacteriology.

Direct microscopy

Wet mount

- KOH preparation
- India ink/Nigrosin staining
- Lacto Phenol Cotton Blue

Stained smears

- Gram staining (for yeasts)
- Gomori's silver methenamine staining for *P. carinii* and mycelial fungi

Culture studies

The commonly used media is **Sabouraud's Dextrose agar (SDA) with and without antibacterial drugs (500mg/l each of Gentamicin and Chloramphenicol)**. Inoculated media are incubated at 37°C and 25°C respectively for at least 6 weeks before reporting as negative.

Identification of Yeasts

Candida and *Cryptococcus* species are readily isolated on SDA at 37°C or at room temperature (22 -25°C). Colonies are white and creamy with a soft consistency. Morphology is confirmed by Lactophenol cotton blue mounts and Gram staining.

Masses of globular, budding cells, pseudohyphae often with budding cells attached and true hyphae are indicative of the presence of yeasts.

Identification of Moulds or Mycelial fungi

Gross colony characteristics include growth rate, color (surface and obverse), texture, production of pigment (diffusible or nondiffusible), aerial and submerged hyphae.

Processing of Sputum for Detection of *Pneumocystis carinii*.

1. Only induced sputum or BAL should be processed.
2. Transfer sputum/ BAL to a sterile centrifuge tube/ McCartney bottle.
3. Add equal volume of sterile distilled water and mix well. Do not vortex.
4. Centrifuge at 300rpm for 15 mins. and discard the supernatant.
5. Repeat the distilled water washing twice more.
6. Discard supernatant and use the deposit for making smears.
7. Stain using Gomori's methenamine silver stain method.

Finalizing of Results

The same form used for Bacteriology specimens can be used for reporting Fungal culture results

Filing of Final report (in register and then on report forms) followed by Despatch to Clinic Reception.

Opportunistic Intestinal Parasites

Parasitic infections are caused by opportunistic intestinal coccidians such as

- *Cryptosporidium parvum*
- *Cyclospora cayetanensis*
- *Isospora belli*

General Safety considerations

Specimen collection

- Universal precautions and aseptic techniques to be followed.

Specimen transport and Storage

- Sterile leak proof and appropriate container in a sealed plastic bag.

Specimen processing

- Universal precautions to be followed.
- At least biosafety level II with good laboratory practice.

Specimen processing

Formalin-ethyl acetate sedimentation is the recommended stool concentration method for recovery of oocysts of coccidian parasites in clinical laboratories.
Staining Procedures

Modified Kinyoun's Acid-Fast Staining is the preferred method

Reporting of Results

The final reports can be made out on the same forms used for Bacteriology and Mycology reporting.

The report should include positive/ negative comments on the detection of intestinal Coccidians.

Discarding of all specimen containers, used plates, etc. in OI Laboratory

- All specimen containers, culture plates, used and other disposables must be discarded in the bins provided for proper autoclaving and disposal/ reuse.
- The biosafety cabinets and working areas of the laboratory should be kept clean, dust free and free from external sources of contamination.

Fumigation Procedures in Mycobacteriology Laboratory

Objective and Scope

- To Fumigate the Laboratory Rooms, Bio-safety cabinets and incubators as decontamination measure of the work environment.
- This document contains procedures that are required for fumigation of contaminated rooms and bio-safety cabinets in the TB Laboratory.
- The procedures, from start to finish, would take a minimum of 24 hours.
- Formaldehyde fumigation is a simple and easy procedure to perform but is inherently hazardous if practiced without appropriate precautions.
- Formaldehyde vapor is an extremely effective biocidal agent.
- It acts as an alkylating agent, inactivating micro-organisms by reacting with carboxyl, amino, hydroxyl and sulph-hydral groups of proteins as well as amino groups of nucleic acid bases.

Requirements

- Formalin (40% Formaldéhyde), commercial solution
- Potassium permanganate ($KMnO_4$) solid
- Wide mouthed glass containers (500ml capacity)

Procedure

- Inform all staff that fumigation needs to be conducted and ensure that no one is remaining back in the laboratory.

Fumigation of bio-safety cabinets for regular maintenance

- Switch-ON the cabinets
- Place 10 grams of potassium permanganate in a wide mouthed bottle and

  keep it in the cabinet.
- Add 25ml of formalin (Initially, bubbles start appearing in formalin and soon a violet froth is seen)
- After 2 minutes switch off and close the cabinets
- Vacate the place.

- Place a warning label on the door stating "DO NOT ENTER, FUMIGATION IS ON".

Fumigation of laboratories and rooms

There are two ways of fumigating the laboratory

- by using number of wide mouthed bottles placed at different locations in the lab so as to cover the entire area of the lab

- by using the vaporizer which consists of a fan and a trough for adding formalin solution. The quantity of formalin required is approximately, 2 ml per 0. 028m^3 of internal air space. This volume needs to be diluted in an equal volume of water.

- Lock the door and effectively seal around the edges with tape and turn on the instrument.

- The switch for the fumigator must be placed in such way that it could be turned on from outside the room.

- Allow the machine to run for 30 minutes and then switch it off.

- Display a notice in large type on the door such as the following:

WARNING, DO NOT ENTER – FUMIGATION ON

- After completion of fumigation (if overnight), exhaust out all the remaining fumes by opening the doors and windows of the room and leaving the BSCs 'ON' for 30 minutes.

- If the fumes are strong, neutralize with liquid ammonia placed in a petri-dish. Place 5-6 such dishes in different areas of the lab.

Note

Staff handling fumigation procedures should be conversant with the hazardous nature of the chemical, toxicity, safety procedures while handling the chemical and measures to be taken when there is a mishap. The Material Safety Data Sheet for formalin can be referred to for all information related to formalin. (http://cartwright. chem. ox. ac. uk/hsci/chemicals/formaldehyde. html; http://www. jtbaker. com/msds/englishhtml/f5522. htm)

- Normally, cabinets should be fumigated once in 15 days and additionally whenever there is a spill and when the HEPA filter /Pre filter is changed.

- Check levels of residual formaldehyde in the room with suitable air monitoring equipment, if available (formaldameter or air sampling tubes). Enter the place only if the level of formaldehyde is below 2ppm and wear a laboratory coat, and gloves.

- Check the room and all surfaces for formaldehyde residues and clean up as necessary.

- Allow other staff to enter the room only when formaldehyde levels are below 0. 5 ppm. (Preferably, levels should be as low as practicable before staff re-enter).

- Liquid ammonia can used for neutralization at an approximate concentration of 30 % or a quantity not to exceed one half of the quantity of formalin used in the fumigation. Ammonia can be released by sprinkling of spraying or by placing ammonia equal to 1/3rd the volume of formalin used originally in the trough of the vaporizer and leaving the instrument ON for sometime.

Documentation

- Keep records of all fumigations of rooms/laboratories/BSCs including date, personnel involved and the results of air monitoring on completion of the procedure.

Minor and major spills

Accidents in tuberculosis laboratories may be divided into two types

- Generate limited aerosols (eg.: a single culture tube of egg medium or spilling the contents of a sputum)

- Large volume of potentially infected aerosols (eg: bacterial suspension, during vortex liquid culture or unbalanced centrifuge tubes)

Plan of Action for Limited Aerosol Accident

Within the laboratory

- Cover the spill immediately to prevent further **aerosolisation.** Use any available material, eg. Paper towels, newspapers or even a laboratory coat.

- Soak the cover with appropriate disinfectant (5% Lysol) and completely wet the area.
- Let stand at least two hours, keeping the area wet during this time.
- Place the broken tubes/containers.
- In an appropriate container and discard by one of the waste disposal options.
- Mop the floor and lab benches with appropriate (5% Lysol) disinfectant**.**

Within BSC:

- Cover the spill immediately to prevent further aerosolization. Use any available material, eg. Cotton, Paper towels, newspapers or even a lab coat.
- Soak the cover with (5% Lysol) and completely wet the area.
- Switch on the BSC and leave the room for at least two hours.
- Place all broken tubes/containers in an appropriate container and discard by one of the waste disposal options.
- Clean the BSC with (5% Lysol) and mop the floor and bench top.

Plan of action for large volume aerosol accident (eg.: culture flask, large number of positive cultures in a rack, sputum rack, breakage in centrifuges) in room

Within the laboratory

- Evacuate the room immediately, except for the person who caused the accident.
- Shut off the air system, seal the exhaust and intake air ducts as quickly as possible.
- Turn the fumigator on to dispense the entire volume of formalin, allow the fog to settle and leave the room undisturbed for at least two hours.
- Put on protective clothing before re-entering the room.
- Soak the spill with 5% Lysol and leave for 30 minutes.
- Place all broken tubes/containers in the Lysol bin meant for the purpose.
- Mop the floor and bench top with Lysol.

Within the BSC

- Evacuate the room immediately.
- Leave the BSC switched ON and do not use for at least four hours.
- Fumigate the BSC using formalin as described earlier.

Decontamination of BSC before replacing filters

- Remove materials from the BSC.
- Fumigate (as per SOP) before changing the filters.
- Switch off the BSC and proceed with replacement of filters / repair.

Note

All the accidents have to be recorded in the register and report to Head of the Department

Maintenance of Laboratory Equipments in Mycobacteriology Laboratory Biological safety cabinets

Scope

This SOP describes optimal operation of the biological safety cabinet through regular servicing, certification and preventive maintenance. The biological safety cabinet is a fragile, precision piece of equipment intended for protecting the user (the TB laboratory worker) from airborne aerosols that may cause infection. Both class I and class II biological safety cabinets are adequate for TB laboratories.

Principle

- A class I BSC is an open-fronted ventilated cabinet with a unidirectional inward airflow away from the operator. Exhaust air is hard-ducted through HEPA filters (Figure 1).
- The HEPA filter traps 99. 97% of particles of 0. 3 μm in diameter
- The class I BSC provides protection for the worker and the environment but does not protect the product (specimens, strain cultures) against contamination.
- A class II BSC additionally provides protection of the product (specimens, cultures) against contamination by re circulating part of the HEPA-filtered air in a laminar vertical flow inside the cabinet so that a "curtain " of clean air descends across the whole working surface.
- A class II BSC should be vented to the outside through a thimble or with hard ducting (Figure 2).
- Both class I and II BSCs must be installed under the supervision of an engineer from (or authorized by) the manufacturing company.

Samples

- Specimens for isolation and culture of tubercle bacilli.
- Cultures of tubercle bacilli and other Mycobacterial species.

Equipment and materials (BSCs)

Class I and II BSCs should have a visible front display of current face velocity.
BSCs should be vented to the outside in accordance with Table 1.

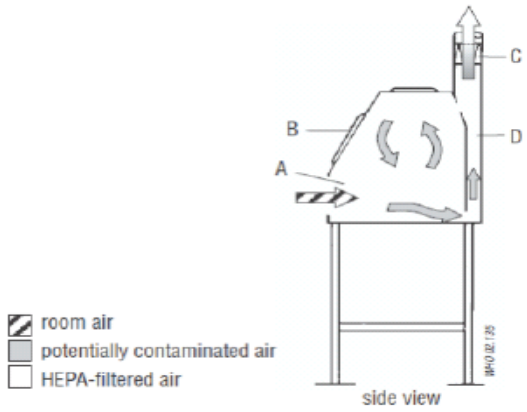


Figure 1. Schematic diagram of a Class I biological safety cabinet.
A, front opening; B, sash; C, exhaust HEPA filter; D, exhaust plenum
(Source: *Laboratory biosafety manual*, 3rd ed. Geneva, World Health Organization, 2004)

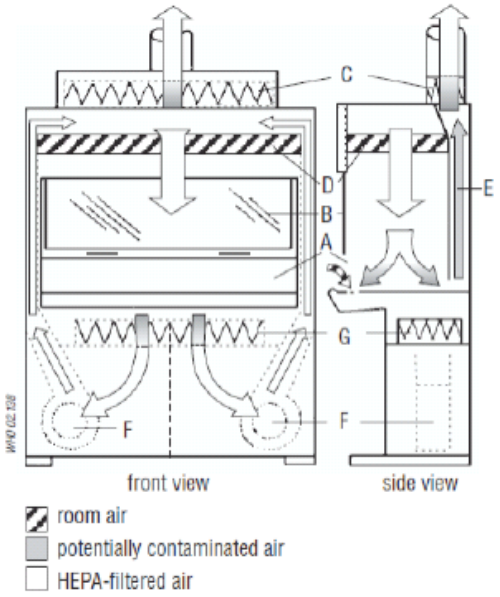


Figure 2. Schematic diagram of a class IIB1 biological safety cabinet

Where,

A, front opening; B, sash; C, exhaust HEPA filter; D, supply HEPA filter; E, negative-pressure

Exhaust plenum; F, blower; G, HEPA filter for supply air. Connection of the cabinet exhaust to the building exhaust air system is required.

(Source: Laboratory biosafety manual, 3rd ed. Geneva, World Health Organization, 2004)

Table 1. Differences between Class I and class II biological safety cabinets

BSC class	Face velocity (m/s)	Airflow (%)		Exhaust system
		Recirculated	Exhausted	
Ia	0. 36b	0	100	Hard duct
IIA1	0. 38–0. 51	70	30	Thimble connection
IIA2a	0. 51	70	30	Thimble connection
IIB1a	0. 51	30	70	Hard duct
IIB2a	0. 51	0	100	Hard duct

- All biologically contaminated ducts are under negative pressure or are surrounded by negative pressure ducts and plenums.
- Note that according to the standard EN 12469, face velocity should be within the range 0. 7 - 1 m/s.
- For safety reasons in dealing with risk group 3 pathogens (TB bacilli), exhaust to the room should be avoided.
- The BSC should be located away from sources of air disturbance such as doors, windows, air conditioning, ventilation supply or return outlets, exhaust fans, etc.
- Personnel traffic in front of the BSC should be minimized to avoid disrupting the airflow.
- The BSC must be connected to a suitable UPS system (with capacity for at least 15 minutes of extra running time) along with an inverter in any location where the electricity supply may be interrupted.

Warning: In case of power failure, infectious particles are no longer trapped to the HEPA filter and flow back to the open front of the BSC, constituting a major biohazard for personnel.

Anemometer

- Appropriate thermal or rotational anemometer, portable, with a telescopic extension for the probe, automatic temperature compensation up to 80 °C, and multiple range selectors (velocity range).

- The velocity resolution of the measurement should be at least 0. 01 m/s in the lowest velocity range.

- The housing of the instrument should be robust, sealed against dust and moisture and able to be disinfected with 70% alcohol.

- The keypad should be covered for protection.

- Anemometers must be calibrated in the horizontal and vertical planes according to the manufacturer's instructions.

Smoke generator

- Commercial airflow testers are recommended. They are small glass tubes, sealed at each end.

- Both ends are broken off with the tool provided and a rubber bulb is fitted to one end. Pressing the rubber bulb to pass air through the tube causes it to emit white smoke.

Reagents and solutions

5% Phenol

Certification

Certification must be done by a qualified service professional *before* the BSC is put into service and annually thereafter.

- The BSC must be re-certified whenever it has been relocated, serviced or repaired and after HEPA filters have been changed.

- Re-certification should include tests for cabinet integrity, HEPA filter leaks, downflow velocity profile, face velocity, negative pressure/ventilation rate, air-flow smoke pattern, and alarms and interlocks.

- Optional tests for electrical leaks, lighting intensity, ultraviolet light intensity, noiselevel and vibration may also be conducted.

- Special training, skills and equipment are required to perform these tests and it is highly recommended that they are undertaken by a qualified professional.

Daily use

- Switch on the BSC airflow 5–15 minutes before use (check the manufacturer's instructions).
- Check the airflow conditions on the display (models that indicate positive, negative or differential pressure, with an accuracy of 2%, may be available).
- Complete the check-off form (see annex 1). If the reading is below the threshold, do not use the BSC and immediately alert the head of the laboratory.
- If the airflow conditions are adequate, wipe the work surface, interior walls and interior of the glass window with 70% alcohol.
- Assemble all items needed for work in the BSC to avoid frequent displacements.
- Place items so that active work flow from clean to contaminated areas across the work surface.
- Organize the work to minimize arm movements. Arm movements in and out of the BSC should be horizontal so as to minimize turbulence and prevent air inside the BSC from flowing out.
- Conduct all manipulations within the BSC as far as possible towards the back of the unit.
- Do not overcrowd the working area as this disturbs the airflow.
- In class II BSCs, never allow the grille to be covered by anything.
- Have a small autoclavable waste bag and/or a container with disinfectant for liquid waste (and for sharps if needed) inside the BSC.
- Do not accumulate waste in the BSC – remove it when activities are finished.
- After use, allow the BSC to operate within 5 minutes to allow the cabinet to purge (to allow time for contaminated air to be removed from the interior of the BSC).
- Wipe the work surface, interior walls and interior of the glass window with 70% alcohol.
- If the BSC is equipped with UV lamps, switch on the UV light and leave for a minimum of 30 minutes.
- Switch off the BSC fan.

Weekly maintenance

- If the BSC is equipped with UV lamps, clean the UV lamp with a gauze pad moistened with 70% alcohol.
- Always wear gloves, never touch the lamp with bare hands.

Monthly maintenance

Qualitative check

- Perform a qualitative check with a smoke generator across the entire width of the BSC opening.
- The smoke test is an indicator of airflow direction, not of velocity.
- Airflow smoke patterns tests are performed to determine whether:
- The airflow along the entire perimeter of the work access opening is inward,
- There is refluxing to the outside at the window wiper gasket and side seals.
- In addition in class II BSCs, the smoke test allows to determine whether:
- Airflow within the work area is downward with no dead spots or refluxing,
- Ambient air passes onto or over the work surface.
- If the BSC is found to be defective, alert the head of the laboratory.

Air flow measurements

- Air flow measurements should be measured using an anemometer. User's tests are restricted to downward airflow measurement in a Class II BSC.
- To determine airflow velocity over a large cross-section, which is the case for a BSC, measurements have to be taken at several points distributed over the whole plane. The average of the measured values is assumed to represent the average airflow velocity.
- The instrument should be equilibrated to ambient temperature before the voltage difference is zeroed, because the temperature difference between the two thermistores (one protected and one cooled by airflow) is measured and converted into air velocity.
- The sensor at the telescope should be placed at the desired point, directed (most instruments have an arrow) according to the airflow; airflow over the sensor should be free and unhindered.

- The sensor tip must be kept stationary in a stable position while a measurement is taken.

- Measurement at any position should be made over ≥ 60 seconds.

- Positions and interpretation of measurements are indicated in Annex 1.

Recording and reporting

- Each use of the BSC should be recorded in the logbook (Annex 2). These records are used for maintenance/service schedules, specifically for checking airflow velocities, replacing the UV lamp.

- BSC servicing forms (Annexure 3) should be used for reporting incidents, accidents and/or mechanical problems. They should be kept on file for a minimum of 1 year, after which they may be archived.

- After re-certification, the service professional must issue a certificate indicating the performance characteristics of the BSC and safety compliance.

- The most recent inspection certificate must be displayed close to the BSC.

- Certificates should be retained in the archives for as long as the

- BSC is in use in the laboratory.

Quality control

- In case of any problem or malfunction, an "Out of Service" notice must be displayed on the BSC indicating that it is not to be used until the problem has been diagnosed and corrected.

- The BSC must be re-certified by a qualified service professional at least once a year and after every replacement of filters.

Safety precautions

Waste management

At the end of each day, collect autoclavable bags containing contaminated material and autoclave as soon as possible.

Personal protection

Refer to specific SOP for details of protective clothing for use in TB culture/DST laboratories.

Other

- Never work in the BSC with the UV lamp on.

- Replace UV lamps at the end of the period of use recommended by the manufacturer (cumulative duration of BSC use is recorded in the logbook). Use gloves to remove lamps.

- Wipe with a disinfectant and discard following national guidelines for special waste. Fumigation of the BSC is required *before*: replacement of HEPA filters;

- Release of the BSC for use after a major biohazardous spill;

- Repair work requiring access to the sealed plenum;

- Service or replacement of the circulation fan or components;

- Maintenance work in contaminated areas;

- Performance tests requiring entry into contaminated areas;

- Movement of the BSC cabinet to another laboratory;

- Changing work programmes, e. g. to non-TB work;

- Release of the BSC cabinet for resale or salvage.

- Fumigation must be carried out by a qualified service professional.

- Users are *not* to make any repairs. Repair and service of the BSC must be carried out by a qualified service professional.

- Any problem or malfunction detected must be reported to the laboratory manager, who will contact the maintenance contractor to correct the problem/malfunction as soon as possible.

- HEPA filters *must never be touched* by personnel and no attempt should be made to clean or disinfect filters using chemical or mechanical methods.

Annex 1: Measurements of downward airflow velocity in a class II BSC.

A. 1 BSC dimensions: 120 cm width, 60 cm depth

The anemometer is placed over the work area, 5 cm above the lower edge of the front screen.

0 15 45 75 105 120 cm

	1			

A.2 BSC dimensions: 150 cm width, 60 cm depth
The anemometer is placed over the work area, 5 cm above the lower edge of the front screen.

0 19 56 94 131 150 cm

	1			

A.3 BSC dimensions: 180 cm width, 60 cm depth
The anemometer is placed over the work area, 5 cm above the lower edge of the front screen.

0 22 67 112 157 180 cm

	1			

A.4 Interpretation of measurements
Measurements are made for each position indicated in the Annexes above.
The time for measurement is 60 seconds per measuring point.
Report measurements in the table

Position	Air flow velocity (m/s)
1	
2	
3	
4	
5	
6	
7	
8	
Total	
Average: (Total / 8)	

Interpretation

- According to EN 12469:2000

- Air flow velocity should be within the range 0. 25 - 0. 50 m/s. Additionally no individual measurement should differ more than 20% of the value indicated by the manufacturer

- Compare the average (determined)_____m/s to the reference of the manufacturer_____m/s +/- 20%

- According to NSF 49 Requires the compliance to the manufacturers' set points or down flow velocity with a deviation

- of 0. 025m/s from nominal set point.

- Compare the average (determined) _____m/s to the reference of the manufacturer_____m/s +/- 0. 025m/s

Annex 2: BSC logbook - daily/weekly maintenance form

Date	Time of use	Cumulative duration of use	Cumulative duration of use of UV lamps	Operator's name	Visual alarm	Sound alarm	Smoke test	Airflow m/s (for class II BSC)	Observations
			Change UV lamps after XXX hours of use (according to manufacturer's recommendations)						

INSPISSATOR

Scope

This SOP describes the optimal operation of an Inspissator through regular servicing and preventive maintenance.

Principle

- The Inspissator (coagulator) is intended for batch production of egg-based culture medium (Lowenstein–Jensen, Ogawa and other modified egg-based media) with or without the addition of drugs.

- Available Inspissators differ in size and in the type of heater: some keep the temperature homogeneous and stable by means of a water bath and others by forced air circulation.

Equipment and materials

- Inspissator properly maintained and installed according to the manufacturer's manual (specific to each inspissator).

- Calibrated thermometer

- Timer

- Removable tube racks with appropriate tubes or bottles for culture

- Thermo resistant gloves

Reagents and solutions

Distilled water, obtained from ion-exchange systems

Detailed instructions for use

Check before use

- Replace Inspissator water with fresh distilled water once a week to prevent corrosion of the stainless steel tank.

- Ensure that the heavy coil / element is submerged below the water level at all times, to prevent overheating and damage of the element.

Loading the Inspissator

- Heat the Inspissator to 80 °C.

- Place the bottles/tubes in a slanted position in the Inspissator. Cover with a blanket.

- Coagulate the medium for 60 minutes at 80–85 °C.
- The quality of egg-based media deteriorates if coagulation is performed at too high a temperature or for too long. Heating for a second or third time has a detrimental effect.
- Check the quality of the medium once cooled (see section 4. 7 below).

Cleaning

- Clean the Inspissator with a damp cloth after disconnecting from the electricity supply. Avoid the use of solvents for cleaning.
- Clean the immersed parts using proprietary heating element cleaners.

Note: Follow the manufacturer's instructions carefully, as these cleaners may be toxic.

Recording and reporting

- Record data in the logbook after preparation of each batch of medium.
- Record details of the loading and operation of the inspissator in the inspissator logbook (Annex 1).
- Incidents, accidents and/or mechanical problems must also be recorded

  (Annex 2).
- Visually check the quality of the medium once it has cooled. It should not be liquid,and should not release large volumes of water.
- Discolouration of the coagulated medium may be due to excessive heating.
- The appearance of small holes or bubbles on the surface of the medium also indicates faulty coagulation procedures, especially where the medium is in contact with the glass (internal wall of the tube).
- If the medium is liquid or disintegrates easily, the inspissation temperature might have been too low. This can be detected by tapping (on the hand) one or two tubes randomly chosen from the inspissated batch.
- Poor quality media should be discarded.

Sterility check

- After inspissation, the whole media batch should be incubated at 35–37 °C for 24 hours and tubes checked for the appearance of contamination.

- Alternatively, a randomly selected sample of tubes is incubated at 35–37 °C for 24–48 hours.

- The batch is used only after a negative test result (verifying the absence of contamination).

Safety precautions

- Use *thermoresistant gloves* to protect from heat. Be careful of steam when opening the inspissator.

- For service or repairs, only the control unit – not the tank or bridge plate – is removed. Service and repair should be performed by a qualified service technician.

- Refer to the manufacturer's manual for safe handling of the inspissator.

- Related documents

- Manufacturer's manual, specific to each inspissator

Laboratory services in tuberculosis control. Part III: Culture. Geneva, World Health

Organization, 1998 (WHO/TB/98. 258).

Annex 1. Inspissator logbook

Date	Time	Operator	Volume of load (no. of vials/ tubes)	Medium type						
				LJ	LJ+pyr	Ogawa	LJ INH	LJ RIF	LJ EMB	LJ SM

PERIODICITY:		
Date	Maintenance operation	Operator

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

CENTRIFUGE

Scope

This SOP describes the optimal operation of a centrifuge through regular servicing and preventive maintenance.

Definitions and abbreviations

The relative centrifugal force (RCF) is determined according to the formula

$$RCF = 1.\ 12 \times 10–6 \times R \times (rpm)^2$$

where:

RCF- relative centrifugal force

R = radius in millimeters from the centre of the rotating head to the bottom of the spinning centrifuge tube

rpm = number of revolutions per minute

Sedimentation efficiency must be determined in RCF (*g*, absolute value) and not in rpm, which is a measure of speed for a particular centrifuge head.

To generate an RCF of 3000*g*, the required rpm is calculated from the formula:

$rpm = 1000\sqrt{}\ (RCF/1.\ 12R)$

Principle

A centrifuge is intended to separate particles in a liquid by sedimentation. Dense particles sediment first, followed by lighter particles.

In a TB laboratory, centrifuges are used for the sedimentation and concentration of tubercle bacilli within liquefied sputum or body fluids

Note: To obtain a high sedimentation efficiency of 95%, i. e. 95% of bacilli recovered in the sediment and only 5% of bacilli still in suspension to be discarded with the supernatant, an RCF of 3000gmust be maintained for 15 minutes.

The centrifugation time and high speeds used to achieve effective sedimentation efficiency lead to heat build-up in the centrifuge and the specimen.

Samples

Decontaminated and liquefied specimens, watery specimens.

Equipment and materials

Centrifuge

The centrifuge must generate an RCF of 3000*g*

- The centrifuge lid must have a locking mechanism to prevent opening while the rotor is still rotating.
- Ensure that the centrifuge is located on a rigid, flat, level surface: any change in the surface may influence the centrifugation process. Because of the vibration they produce.
- Centrifuges must be kept apart from balances.
- Allow sufficient free space around the centrifuge for adequate ventilation to prevent overheating.

Aerosol-free swing buckets

- For use in a TB laboratory, the centrifuge must have aerosol-free (O-ring sealed) safety swing buckets that can be removed from the centrifuge and placed inside a BSC for the removal of individual centrifuge tubes.
- The sealed buckets protect operators from infectious particles in case of tube damage during centrifugation. (It is advisable to use transparent bucket covers so that leakage can be detected before opening.)

Centrifuge tubes

- Centrifuge tubes must withstand of at least 3000*g* and must be used with suitable rubber or plastic cushions matched to both the tubes and the bucket holder.
- Adaptors, if used, must be those recommended by the manufacturer.

Other items

- Cloth or paper towels for cleaning and disinfection.
- Balance with 0. 1 g accuracy for balancing centrifuge tubes.
- Tachometer for periodic calibration of centrifuge speed.

Reagents and solutions

Ethanol (70%) or Cedax

Detailed instructions for use

- Before use, check the inside of the centrifuge and the rotors to ensure that everything is dry.
- If there is any sign of corrosion, discontinue use until the corroded part has been repaired by a qualified service technician.
- Check that shock-absorbing pads are in the bottom of the centrifuge buckets.
- Balance the opposing buckets by weighing them with their tubes on an open two-pan balance. Add water to or an empty tube placed in the buckets to achieve final balance. NEVER add water to a specimen to balance tubes.
- Never fill centrifuge tubes to more than three-quarters capacity.
- Symmetrically distribute balanced tubes in opposing buckets. Always operate the centrifuge with all buckets in place, even if two opposing buckets are empty.
- Switch on and follow the manufacturer's instructions to set the centrifugation conditions: 3000*g*, 15minutes.
- Set the brake switch on if the brake is recommended.
- Close and lock the lid.
- Start the centrifuge cycle.
- While the centrifuge is reaching full speed, (stand with your hand) to check the vibration. If excessive vibration occurs, or if a crack is heard or tube breakage is suspected, switch off the unit.
- Open the centrifuge only after the signal for end of centrifugation is seen.
- Remove the sealed buckets (not tubes) slowly and carefully to prevent re suspension of the sediments.
- Place the buckets inside the BSC and carefully open the buckets; check for tube damage before removing tubes from the buckets.
- Sediments and supernatants should be visible after centrifugation.
- When the centrifuge is under refrigeration, leave the top closed to avoid condensation.

Reading and recording

Record details of operation of the centrifuge in the centrifuge logbook (Annex 1).

Quality control and maintenance

Initial installation

Initial calibration should be performed only by a qualified service technician.

Daily maintenance

Wipe the inside of the bowl with disinfectant solution and rinse thoroughly.

- The centrifuge must not be used if the interior is hot, if unusual vibrations or noises occur, or if deterioration (corrosion of parts) is detected. A qualified service technician should be contacted.

- Most vibrations are due to improper balancing and can be corrected by rebalancing the buckets and tubes.

Monthly maintenance

- Clean the centrifuge housing, rotor chamber, rotors and rotor accessories with a neutral cleaning agent.

- Clean plastic and non-metal parts with a 5% Lysol

Annual maintenance

- The centrifuge must be serviced annually by a qualified service technician who must ensure that the unit operates safely and properly.

- The service should include cleaning condenser coils, fans, screens and filters, checking the centrifuge brushes, bearings, timer, temperature and speed, and checking for electrical integrity.

- The service technician must issue an inspection certificate indicating compliance with safety and proper operation. The most recent inspection certificate must be displayed close to the centrifuge (Annex 2).

Waste management

- Tubes broken during centrifugation must be discarded immediately. Put them in a Metallic bucket container, and autoclave.

- Clean metal bowls and parts with 70% ethanol and plastic parts with 5% bleach.

Do not use bleach for metal parts as it causes corrosion.

Annex 1. Centrifuge logbook

Operator's name	Date	Time	RCF = 3000*g* (tick if correct)	Time = 15 min (tick if correct)	Sediment (tick if correct)	Specify other centrifuge conditions, if any Note observations, if any

Annex-2 Periodicity

Date	Date Maintenance operation	Operator

Failure Events

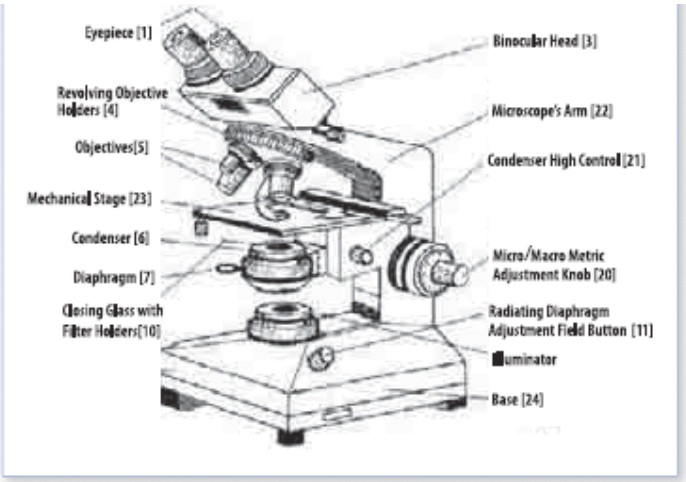
Date	Event	Corrective Action taken	Operator

Maintenance of a light microscope

Scope

This SOP describes the optimal operation of a light microscope through regular servicing and preventive maintenance.

Figure: Parts of microscope



Definitions and abbreviations

microscope magnification

Individual objective magnification x eyepiece magnification.

Principle

The light microscope is a precision instrument intended for microscopic detection of tubercle bacilli in specimens in the routine diagnostic TB laboratory (see Annex 1).

Equipment and materials

- Install the microscope on a rigid, flat, level surface.
- Do not place the microscope where it could be exposed to direct sunlight, dust, vibration (e. g. centrifuge tubes), water (sink, spray from a tap), chemical reagents, or humidity.
- If the microscope is to be used every day, try to keep it in the same place in the laboratory.
- In humid climates, however, where fungal growth is likely, the microscope is best kept overnight in a light box or cabinet equipped with a 20 W light bulb.

- Alternatively antifungal strips are obtainable from the manufacturers of some microscopes; replace strips every 18–24 months, according to the manufacturer's recommendations.

- Silica gel desiccant may be used instead of an antifungal strip.

- Silica gel is blue when dry but turns pink when wet. Regenerate the gel by heating until it turns blue again.

- Clean lens with tissue paper or silk cloth, without scratching.

- Plastic microscope cover is required in dusty areas and whenever the microscope is not in use.

- Procure storage cabinet or light box, for humid climates. The cabinet should have holes to permit circulation of air and should be fitted with a 20-W light bulb.

General Maintenance of Microscopy

- Synthetic, high-grade immersion oil (Heavy Liquid paraffin) of medium viscosity and with a refractive index >1. 5.

- Do not use cedar wood oil as it dries onto the oil immersion objective lens and will rapidly spoil the optical quality.

- Any cleaning with xylene or other organic solvents is absolutely forbidden since it will spoil the oil immersion objective.

- Cleaning fluid as recommended by the manufacturer, or an 80:20 mixture of ethyl ether: alcohol, or 70% Alcohol.

Detailed instructions for use

See SOP on Ziehl-Neelsen microscopy

Quality control and maintenance

Daily maintenance

- The modern light microscope needs no particular maintenance, but considerable care is required in its use, regular cleaning, and protection from dust, sand and fungus.

- Each day before use, check for broken or damaged parts and ensure that the lenses, mirrors and other light-conducting surfaces are clean.

- Check the lenses for dirt or grit; they may easily develops scratches if they are wiped without first blowing away dust and small sand particles.

- First blow the lens clean, using a blower brush if available, then clean the lenses with clean, dry tissue paper, If this does not produce a clear image, try again using the cleaning fluid provided by the manufacturer, or 80:20 ethyl ether: alcohol or 70% alcohol on the tissue paper

- Do not remove eyepieces or objectives from their fixation holes; clean only their external surfaces as needed.

- For proper cleaning of its lower lens, the condenser may have to be removed from its fixing.

- Ensure that moving slides over condenser do not create scratches.

- At the end of each day, use tissue paper to carefully remove immersion oil from the 100x lens.

- In humid climates, put the microscope into a light box or closed cabinet overnight to minimize fungal growth.

- If the microscope is stored in this way, do not place the plastic cover over it. About 25 g of silica gel should be placed in a dish in the bottom of the box to absorb moisture.

- If the microscope is not stored in a box, cover it completely with a suitable plastic cover.

Monthly maintenance

- Blow dust off the lenses, using a blower brush if available, before cleaning them.

- Apply cleaning fluid (not xylene) or the lens paper to clean the lenses.

- Remove the slide holder from the mechanical stage and clean it in the same way.

- Wipe dust off the body of the microscope with soft tissue paper moistened with water.

- Record maintenance in the logbook (Annex 2).

Quarterly maintenance

- In climates with relative humidity >80% for more than just a few weeks a year, fungal growth may damage the microscope.

- Fungal growth occurs almost exclusively on the prisms in the binocular tube, causing haziness and then dimness, and finally obscuring the view completely.

- Check the microscope for fungal growth from time to time and whenever the view gets hazy.

- With the light on and the 10x objective in place, fungal growth can be seen easily by removing the eyepieces and looking into the binocular tube.

- Fungal growth is best removed by a trained person.

- The binocular tube must not be opened ; the prisms must remain exactly as fixed by the factory; taking them out would destroy the microscope.

Troubleshooting

It may be possible to repair a faulty microscope by replacing easily removable parts (objectives, eyepieces, light bulbs, fuses); if this does not work, the microscope should be entrusted to a competent person for repair.

Never attempt to dismantle any part of the microscope for repair.

- Record troubleshooting and corrective action in the logbook (Annex 2).

- It is equally important to make sure that holes for the eyepieces and objectives are never left open for more than a few minutes.

- If a lens is missing, close the fixation hole using the plug provided or by sticking adhesive tape over it, otherwise dust will enter and cause haziness of the remaining objectives.

Problem: the viewing field is too dim

Solution:

- Condenser is too low; raise condenser to correct its position

- Open the diaphragm properly

Problem: there are dark shadows in the field that move as you turn the eyepiece

Solution

- If, Surface of eyepiece is scratched replace the eyepiece
- If, Eyepiece is dirty clean the eyepiece

Problem: the image with the high power objective is not clear

Solution

- Slide is upside down; Turn the slide over
- There is an air bubble in the oil; Move 100x lens quickly from side to side
- There is dirt on the objective; Clean the lens
- The oil is too sticky Use heavy paraffin oil. (Immersion oil)

Problem: the image with the low-power objective is not clear

Solution:

- There is oil on the lens; Clean the lens
- **There is a layer of dust on the upper Clean the lens surface of the objective**

Problem: the viewing field is still dim and cloudy

Consider the following possible causes:

- Massive growth of fungus on the lenses or prisms due to storage in a high humidity environment.
- Penetration of immersion oil between the lenses of the objective because of damaged lens cement (the result of using poor-quality oil such as cedarwood oil or the misuse of xylene).
- This is almost certainly the cause if a completely hazy field becomes clear after changing the objective.
- A damaged objective (as a result of careless focusing, dropping, rough changing of slides).

FLUORESCENCE MICROSCOPE

Scope

This SOP describes the optimal operation of a fluorescence microscope through regular servicing and preventive maintenance.

Principle

- The fluorescence microscope is a precision instrument intended for microscopic detection of tubercle bacilli in specimens in the routine diagnostic TB laboratory.

- The use of fluorochromes for staining allows smear examination at lower magnification than is used for light microscopy.

- Because of the lower magnification, each field examined under fluorescence microscopy is larger in area than that seen with light microscopy, thus reducing the time needed to examine a slide.

- High-volume laboratories, dealing with more than 25 slides per day, could use a fluorescence microscope.

Equipment and materials

- A binocular microscope equipped with a fluorescent light source and suitable filter set for auramine-stained smears. Fluorescent light is provided by a mercury vapour lamp, a halogen lamp or a light-emitting diode (LED).

- The mercury vapour lamp provides the strongest light but it has a limited life of about 100-200 hours, which must be monitored with a timer.

- Do not place the microscope where it could be exposed to direct sunlight, dust, vibration water (sink, spray from a tap), chemical reagents, or humidity.

- Install the microscope on a rigid, flat, level surface. It is too large and sensitive to be moved regularly; thus, any protection from humidity or dust that is needed should be provided for the microscope in situ.

Reagents and solutions

- Cleaning fluid, as recommended by the manufacturer, or 80:20 ethylether:alcohol or 70% alcohol.

- Lens paper, or silk cloth, or fine-quality tissue paper to clean lenses without scratching.

- Microscope cover

- Immersion oil, if needed (does not have to be special non-fluorescing quality).

Quality control and maintenance:

General maintenance

- The fluorescence microscope requires careful maintenance from both optical and mechanical points of view.
- Laboratory workers must be familiar with its general mechanical and optical principles.
- Record maintenance in the logbook (Annex 1).
- A mercury vapour lamp has a life of 100–200 hours and should be replaced after damage or **100 hours of use (whenever required).**
- This time can slightly exceed, but the risk of the lamp exploding increases.
- Lamps from different manufacturers will have different lamp life spans.
- They are expensive, so it is important to check the lifespan of each lamp and ensure that it is not exceeded (Annex 2).
- It may be possible to repair a faulty microscope by replacing easily removable parts (objectives, eyepieces, light bulbs, fuses); if this does not work, the microscope should be entrusted to a competent person for repair.
- *Never dismantle the microscope* – its operational maintenance efficiency and accuracy may be severely impaired.
- In climates with relative humidity in excess of 70% for more than just a few weeks a year, fungal growth may damage the microscope.
- Fungal growth occurs almost exclusively on the prisms in the binocular tube, causing haziness and then dimness, and finally obscuring the view completely.
- Check the microscope for fungal growth from time to time and whenever the view gets hazy.
- With the light on and the 10x objective in place, fungal growth can be seen easily by removing the eye pieces and looking into the binocular tube.
- Fungal growth is best removed by a trained person.
- The binocular tube must be opened but the prisms must remain exactly as fixed by the factory: taking them out would destroy the microscope.
- The correct procedure for changing mercury lamps must be used, by trained technicians
- Do not touch the lamp with the fingers

- Check which end should point downwards (described on package insert) and respect this way of mounting

- Adjust the position (horizontal and vertical) of the new lamp and of the lamp house mirror by using the adjustment knobs or refer to the microscope manual; replacement of halogen lamps does not require these adjustments.

- If the blue light remains weak after a new lamp has been fitted and properly adjusted, there may be another problem such as a blackened heat filter.

- Discontinue use of the microscope and request repair by a specialist.

Daily maintenance

- In a humid climate without continuous air conditioning and if the microscope has no dedicated antifungal protection (a special device inserted under the binocular tube), stand a dish of silica gel (25g) on the microscope stage and cover the microscope with a plastic cover.

- Renew the silica gel whenever it turns pink, which may be daily; regenerate the gel by heating until it turns blue again.

- Check for broken or damaged parts

- Check the counter to ensure that the lifespan of the lamp has not been exceeded.

- Check the lenses for dirt or grit; they may easily becomes scratched if they are wiped without first blowing away dust and small sand particles.

- First blow the lens clean, using a blower brush if available, then clean the lenses with dry lens paper

- If this does not produce a clear image, try again using the cleaning fluid provided by the manufacturer, or 80:20 ethyl ether: alcohol or 70% alcohol on the tissue paper.

- It is best not to remove eyepieces or objectives from their fixation holes but to clean only their external surfaces as needed.

- For proper cleaning of its lower lens, remove the condenser from its fixing.

- When replacing the condenser, ensure that slides moving over it cannot scratch its upper surface with the condenser in the uppermost position.

- It is equally important to make sure that holes for the eyepieces and objectives are never left open for more than a few minutes.

- If a lens is missing lens, close the fixation hole using the plug provided or by sticking adhesive tape over it, otherwise

- dust will enter and cause haziness of the remaining objectives.

Monthly maintenance

- Blow dust off the lenses, using a blower brush if available, before cleaning them.
- Then apply cleaning fluid (or suitable equivalent), NOT xylene) to the lens paper (or suitable equivalent) and clean the lenses.
- Remove the slide holder from the mechanical stage and clean it in the same way.
- Wipe dust off the body of the microscope with soft tissue paper moistened with water.

Yearly maintenance

- Thorough inspection and service by a qualified service technician.

Waste management and other safety precautions

- Worn or broken mercury vapour lamps should be disposed off as toxic waste.
- In case of explosion, leave the room immediately and arrange for thorough air change before entering again –*mercury vapour is toxic*.

Annex 1. Maintenance logbook

ITEM IDENTIFICATION			
Equipment:	MICROSCOPE	Brand name:	
Purchase date:		Model/type:	
Location within the laboratory:		Serial no.	
Warranty expiry date:			
Manufacturer:			Tel:
Address:.			
Contact person:			
Technical service representative:			Tel:

PERIODICITY:		
Date	Maintenance operation	Operator

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

Annex 2. Fluorescence microscope log-sheet

The mercury vapour lamp has a life of approximately 100-200 hours. Replace the lamp after 100 hours of use

Operator's name	Date	Time	Duration of use (hours)	Cumulative duration of use (hours)

INCUBATOR

Scope

This SOP describes the use and maintenance of an incubator through proper installation, regular monitoring and maintenance

Principle

The incubator is intended to ensure optimum growth conditions for the culture of *M. tuberculosis*.

Incubators are available in various sizes, from small (bench top) models to large incubator rooms equipped with fans to achieve a homogeneous temperature.

Since *M. tuberculosis* growth is inhibited above 37 °C, the incubator temperature should be set at 36 ± 1 °C.

Samples

Mycobacterial specimens and cultures.

Equipment and materials

- The incubator should be installed and operated according to the instructions in the manufacturer's manual (specific to each incubator).
- It should be located on a level surface in a dry, well-ventilated area, away from heat sources.

Detailed instructions for use and disinfection

General

- Keep door(s) closed to prevent heat loss to the environment.
- Ensure that rack positions are clearly marked.
- Switch off the incubator when it is not in use.
- Do not overload.

Procedure for disinfection

Every 6 months or in case of spillage of infectious material within the incubator:

- Disconnect the incubator from the electrical supply.
- Identify an adequate volume of available safe space for temporary storage of biohazardous material.

- Ensure that materials kept in the incubator are clearly marked.
- Clean metallic surfaces (racks, floor, walls and doors) with 70% ethanol.
- Clean any materials located in the incubator that may have been contaminated by spillage.
- Reconnect the incubator to the electricity supply and switch it on.
- Replace incubator contents once the temperature has reached 36 °C.
- Notify staff of the replacement of the contents.

Reading and reporting

Use a record from to keep a daily record of the incubator temperature (Annex 1).

Record regular maintenance of the incubator on the maintenance logbook (Annex 2).

Quality control and maintenance

- Perform monthly temperature spot checks by placing(maximum/minimum thermometers at various positions in the incubator to verify that the internal incubator temperature is constant at 36 °C.
- Repairs should be performed by a qualified service technician.

Calibration

- The temperature should be calibrated: Before use;
- After temperature changes have been detected and corrected;
- Following a power failure;
- After cleaning of spillages.

Calibration process

- Ensure that the door is closed and that the incubator is switched on.
- Set the required temperature using the temperature control and leave the incubator to run for 1 hour.
- Place a thermometer in the centre of the incubator with the probe away from the heating element.
- Take the temperature reading after 30 minutes; if the temperature is not 36 ± 1 °C, adjust the control.
- Repeat the process every 30 minutes until the required temperature is reached.

- Continue to take readings until two consecutive readings (30 minutes apart) are 36 ± 1 °C.

- Record readings in the incubator logbook. The incubator is ready for use only at the calibrated temperature.

Waste management

All inoculated tubes and vials, whether negative or contaminated, should be autoclaved as potentially infectious material.

Annex 1. Incubator logbook

Temperature required:	36 ± 1 °C (acceptable variation)

Trimester:				Year:				
Month	Temp. °C	Operator (initials)	Month	Temp. °C	Operator (initials)	Month	Temp. °C	Operator (initials)
1			1			1		
2			2			2		
3			3			3		
4			4			4		
5			5			5		
6			6			6		
7			7			7		
8			8			8		
9			9			9		
10			10			10		
11			11			11		
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29			29			29		
30			30			30		
31			31			31		

Annex 2. Incubator maintenance logbook

ITEM IDENTIFICATION			
Equipment:	INCUBATOR	Brand name:	
Purchase date:		Model/type:	
Location within laboratory:		Serial no.	
Warranty expiry date:			
Manufacturer:			Tel:
Address:.			
Contact person:			
Technical service representative:			Tel:

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

Disinfection date	
Disinfection reason (regular, spillage)	
Operator's name	
Calibration date	
Calibration reason (temperature correction, power failure, disinfection)	
Operator's name	

Disinfection date	
Disinfection reason (regular, spillage)	
Operator's name	
Calibration date	
Calibration reason (temperature correction, power failure, disinfection)	
Operator's name	

Disinfection date	
Disinfection reason (regular, spillage)	
Operator's name	

Calibration date	
Calibration reason (temperature correction, power failure, disinfection)	
Operator's name	

FREEZER

Scope

This SOP describes the optimal operation of the freezer (or deep freezer) – achieved through proper installation and maintenance (regular defrosting and cleaning).

Definitions and abbreviations

°C: degrees Celsius

UPS: uninterrupted power supply.

Principle

The freezer is intended to ensure a suitable environment for material that requires preservation at upto–80°C.

Samples

Typically, a freezer is used to preserve

- Mycobacterial cultures and specimens stored for specific purposes;
- Specific reagents and solutions used for drug-susceptibility testing.
- Never store flammable solutions in a freezer that is not approved and certified for this purpose.
- Use separate freezers for clean and infectious materials.

Equipment and materials

- The freezer should be installed and operated in accordance with the specific manufacturer's manual. Specifically:
- After transportation, leave the freezer in the upright position for some hours before connecting it to the electricity supply.
- Do not install the freezer close to heat sources.
- Locate the freezer in a dry, well-ventilated area.
- Ensure that the freezer is placed on a level surface.
- To prevent loss of valuable cultures, chemicals, etc. in case of a power failure, the freezer should ideally be connected to a suitable UPS system.

Procedure

Defrosting

- Identify an adequate volume of available space in another freezer to store materials during the defrosting procedure. Ensure that materials kept in the freezer are clearly marked.
- Switch the freezer off and disconnect it from the electrical supply. Open the freezer door and leave it open.
- Position a container to catch the melted ice.
- Never use sharp tools to chip off the ice.
- Sponge up any melted ice.

Cleaning

- Clean the interior of the freezer with a disinfectant solution (see SOP on disinfectants).
- Clean the outside of the freezer with a cleaning solution, and dry with a soft cloth.
- Reconnect the freezer to the main power supply and switch it on.
- Replace the original freezer contents once the temperature has reached –80 °C.
- Do not overcrowd.
- Notify staff of the replacement of the freezer contents.
- Note the replacement of freezer contents in the freezer logbook.

Reading and reporting

- Use a record form to keep a daily record of the freezer temperature (Annex 1).
- Record the regular maintenance of the freezer on a maintenance card (Annex 2).

Maintenance

Daily

Check the compressor for any unusual sound and for overheating.

Monthly

Clean the filters and screens of the ventilator system with a brush or vacuum cleaner.

Every six months

- This can be done more frequently if necessary, particularly in the event of leakage of biological materials onto the internal surface of the freezer or when the ice build-up on the internal wall reaches a thickness of 5–6 mm.
- Clean the condenser coils and fan with a brush or vacuum cleaner.
- Repairs should be performed only by a qualified service technician.

Waste management

- Freezer contents may be disposed of *only* with the prior consent of the laboratory manager.
- Details of disposal must be noted in the freezer logbook.
- Infectious cultures or contaminated materials must be autoclaved before disposal.

Related documents

Manufacturer's manual, specific to each freezer.

Maintenance and repair of laboratory, diagnostic imaging and hospital equipment.

Geneva, World Health Organization, 1994.

Maintenance manual for laboratory equipment, 2nd ed. Geneva, World Health Organization, 2008.

Annex 1. Temperature record form

Equipment: FREEZER	Reference:
Location:	Installation date:
Temperature required: –18 °C ± 2 °C (acceptable variation)	

Trimester:				Year:				
Month	Temp. °C	Operator (initials)	Month	Temp. °C	Operator (initials)	Month	Temp. °C	Operator (initials)
1			1			1		
2			2			2		
3			3			3		
4			4			4		
5			5			5		
6			6			6		
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31			31			31		

Annex 2. Maintenance logbook

ITEM IDENTIFICATION			
Equipment:	FREEZER	Brand name:	
Purchase date:		Model/type:	
Location within laboratory:		Serial no.	
Warranty expiry date:			
Manufacturer:			Tel:
Address:.			
Contact person:			
Technical service representative:			Tel:

Maintenance card

PERIODICITY: Every six months or when needed		
Date	Maintenance operation	Operator
	Defrosting: Relocation: What/Where: What/Where: What/Where: Refilling:	
Remarks:		
Date	Maintenance operation	Operator
	Defrosting: Relocation: What/Where: What/Where: What/Where: Refilling:	
Remarks:		
Date	Maintenance operation	Operator
	Defrosting: Relocation: What/Where: What/Where: What/Where: Refilling:	
Remarks:		

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

AUTOCLAVE

Scope

The autoclave using saturated steam under pressure is the most efficient means of sterilizing instruments, glassware and media solutions in the general TB diagnostic laboratory, and of decontaminating biological material consisting of infectious waste

The current SOP describes the procedure for use of a pressure cooker autoclave with fully manual operation. It must be adapted for other autoclave types with automatic air and condensate discharge.

Disinfection

A physical or chemical means of killing microorganisms, but not necessarily spores.

Decontamination

Any process for removing and/or killing microorganisms. The same term is also used for removing or neutralizing hazardous chemicals and radioactive materials.

Sterilization

A process that kills and/or removes all classes of microorganisms and spores.

Inactivation

A process rendering an organism inert by application of heat, or other means.

Units

°C: degrees Celsius

1 kPa (kilopascal) = 0. 01 bar

1 kg/cm2 = 98 kPa

1 psi (pounds per square inch or lb/in2) = 6. 8947 kPa

At sea level, atmospheric pressure is 101 kPa or 1. 013 bar or 1 atm or 760 mmHg or

1. 03 kg/cm2 or 14. 7 psi

Principle

The autoclave is a sealing chamber that can contain steam at more than 115kPa (1. 06 kg/cm^2 or 15 psi). At this pressure, steam is most saturated and temperature is 121°C at sea level

Note:

Any air remaining in the chamber will reduce the efficiency of the autoclave. Pans of material to be sterilized or decontaminated must have some water added that will turn to steam as the pans are heated and prevent air pockets from forming in the pan

Equipment and materials

Autoclave

- Each autoclave should be installed according to the specific instructions supplied by its manufacturer.
- Autoclaves should be located away from the main working area as they are noisy and hot, release steam and may be a source of major hazards.
- Autoclave used to decontaminate infectious material, the valve for exhaust air should be equipped with a bacterial filter.
- The autoclavable sterile filter should consist of a filter cartridge with a membrane, pore size 0. 2 μm, incorporated in a pressure-resistant housing and easily replaced.
- The filter is automatically sterilized during each sterilization process.
- Cloth (linen or cotton) or steam-permeable paper or parchment for items that must be wrapped.
- Incubator for spore test, calibrated at 55–60 °C; a dry-block incubator is suitable for this purpose
- Chart or paper if autoclave is equipped with a recorder
- Adhesive autoclave indicator tape; thermo resistant gloves; eye protection such as safety glasses.

Reagents and solutions

Heat-resistant *Geobacillus stearothermophilus* spores (previously *Bacillus stearothermophilus*)

Detailed instructions for use

The manufacturer's instructions for operation and cleaning of the autoclave must be followed at all times.

WARNING: Improper use of the autoclave can be very dangerous. It can explode if the pressure is too great, if a part is defective, or if the door or cover is opened while under pressure.

The caps of containers of liquid must be left loose during autoclaving: if they are tight, containers may explode when the autoclave is opened.

- Fill the bottom of the autoclave with distilled water, up to the autoclave basket support or to the level marked by the manufacturer. If the water reaches the basket, drain off excess water by opening the drainage tap.

- Apply strips of autoclave tape to containers.

- Place the autoclavable containers containing the material to be sterilized or decontaminated.

- Separate autoclaves should be used for sterilization of solutions or glassware (clean materials) and for decontamination of infectious materials (infected material).

- Do not mix material to be sterilized with material to be decontaminated.

- Do not overload.

- Close the lid and make sure that the rubber gasket is in its groove. Screw down the clamps firmly.

- Open the air outlet valve.

- Turn on the heating.

- When a jet of steam is released from the outlet valve, wait 3 or 4 minutes until the jet of steam is uniform and continuous, indicating that all the air has been driven out of the autoclave, then close the outlet valve.

- Check and re-tighten the lid clamps.

- Start timing when the needle on the dial remains at the desired temperature.

- Solid material for sterilization: 121 °C (appropriate pressure 115 kPa), 20 minutes; containers of infected material:121 °C (appropriate pressure 115 kPa), 30 minutes.

- The time required for sterilization of liquids, reagent solutions or culture media is dependent on the volume.

- Turn off the heat when the required time is up.

- When the temperature falls below 80 °C, open the air outlet valve slowly to equalize pressures inside and outside the autoclave.

- When the hissing sound stops, unscrew the lid clamps and open the lid.

- Leave the material to cool before removing it from the autoclave.

- Check that the autoclave tape has turned colour before removing the material.

Recording and reporting

- Record details of the loading and operation of the autoclave in the autoclave logbook(Annex 1).
- Incidents, accidents and/or mechanical problems must be recorded and reported to a supervisor immediately.
- Most types of non-manual autoclaves have a paper print-out for monitoring temperature and air pressure during the different steps of operating cycles.
- These records must be stored with the appropriate log-sheet.

Quality control

Chemical indicator

- A visible chemical indicator, such as an autoclave tape, must be used with each load to be sterilized.
- Remember that tapes for ovens (dry heat) are not adequate for autoclaves.

Note: Temperature-sensitive autoclave tape alone is not sufficient to indicate that the sterilization temperature (121 ºC) reached the heart of the load.

Biological indicator

- Even if the autoclave has a paper print-out for monitoring effectiveness (temperature and air pressure), a biological indicator must be used periodically (after every 40 hours of use)
- The results must be documented (Annex 1) and kept for at least 1 year.
- Heat-resistant *Geobacillus stearothermophilus* spores are used for testing autoclave efficiency.
- A spore vial is placed in a waste bag located in the centre of a load of maximum allowable size and exposed to 121 °C for at least 15 minutes.
- The autoclaved spore vial is incubated along with a non-autoclaved control vial at 56–60 °C.
- Negative growth in the autoclaved vial demonstrates proper autoclave efficiency. If the autoclaved vial shows positive growth the procedure is repeated with another test vial.
- Consistent positive growth indicates a problem that must be corrected before the autoclave can continue to be used.

Safety precautions

Personal protection

- Use thermo resistant gloves to protect the hands from heat.
- Wear eye protection such as safety glasses or a visor when removing materials from
- *Maintenance*
- Preventive maintenance procedures for the autoclave must be carried out as detailed below.
- Any problem or malfunction detected must be reported to the laboratory manager, who will contact the maintenance contractor to correct the problem/malfunction as soon as possible.
- Users are *not* permitted to make any repairs: repair and service of the autoclave must be done by a qualified service professional.

Daily preventive maintenance.

Check that the lid/door gasket is clean. not protruding.

- Check for leaks (e. g. bubbles) while the autoclave is running.
- Clean the autoclave and work area after every episode of use.

Weekly preventive maintenance

Remove and clean the drain strainer if necessary.

- Check that the pressure release safety valve is functioning properly. Keep away from the release valve exhaust during this check to prevent a burn injury.

- Register action and date in the logbook (Annex 2).

Monthly preventive maintenance

- Inspect autoclave gaskets, lid/doors, and internal walls for residue built-up or wear.

- Notify the laboratory manager if any deterioration is observed.

Annual preventive maintenance

- All autoclaves must be inspected and certified annually by a qualified service technician.

- At the minimum, pressure gauges and thermometers should be tested.

- The service technician must issue an inspection certificate indicating compliance with safety and proper operation. The most recent inspection certificate must be displayed close to the autoclave.

Three-yearly preventive maintenance

- Pressure testing and inspection must be done at three-year intervals on all pressure vessels.

- Test certificates must be kept on record.

Annex 1. Autoclave logbook

Date: Time : Operator's name :

Type of load : media ☐ glassware ☐ waste products ☐

Presence of biohazardous material: YES ☐ NO ☐

Load size (specify):

Autoclaving conditions : 121 °C (115 kPa) Time at 121 °C: min

Check of autoclave tape: white ☐ black ☐

BIOLOGICAL INDICATOR

(test the biological indicator after every 40 hours of autoclave use, to be scheduled according to the autoclave logbook records, e.g. every 1, 2 or 3 months)

Operator's name:

Autoclave cycle: Date: Time :

Autoclaving conditions : 121 °C (115 kPa) Time at 121 °C: min

Growth of spores in the autoclaved vial: Yes ☐ No ☐

Annex 2. Autoclave maintenance logbook

ITEM IDENTIFICATION			
Equipment:	AUTOCLAVE	Brand name:	
Purchase date:		Model/type:	
Location within laboratory:		Serial no.	
Warranty expiry date:			
Manufacturer:			Tel:
Address:.			
Contact person:			
Technical service representative:			Tel:

PERIODICITY:		
Date	Maintenance operation	Operator

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

Identification of Mycobacteria by HPLC

Principle

- The objective of this method is to identify mycobacteria by analysis of mycolic acids, using high performance liquid chromatography (HPLC).
- A suspension of acid-fast bacteria is saponified to cleave the mycolic acids bound to the cell wall.
- Mycolic acids are then separated by acidification and extraction into chloroform.
- After conversion to ultraviolet (UV)-absorbing *p*-bromophenacyl esters the mycolic acids are analyzed on a reverse-phase C18 column using HPLC.
- A gradient of methanol and dichloromethane (Methylene chloride) generated by microprocessor-controlled pumps is used to separate the mycolic acid esters, which are detected with a UV spectrophotometer.
- Reproducible chromatographic patterns containing combinations of different diagnostic peaks are formed.
- Pattern recognition is by visual comparison of sample results with mycolic acid patterns from reference species of known mycobacteria.
- Correct pattern interpretation requires training.
- Computer-assisted pattern recognition technology and high-sensitivity fluorescence detection are being evaluated.

Materials

A. Chemicals

Chemicals and solvents must be AR grade

Additionally, solvents must be HPLC grade (recommend filtered reagent-grade water (Millipore H_2O).

The following chemicals are required:

1. Hydrochloric acid
2. Potassium hydroxide

Derivatization reagent

p-bromophenylacyl bromide (0. 1 mmol), and dicyclohexyl- 18-crown- 6 ether (0. 005 mmol) in acetonitrile.

Chloroform

UV cutoff 245 nm, stabilized and packaged under nitrogen in a

dark amber bottle or other opaque container

Dichloromethane (Methylene chloride)

UV cutoff 233 nm, stabilized and packaged under nitrogen in a

dark amber bottle or other opaque container.

Methanol

UV cutoff 210 nm.

B. Reagents for saponification, extraction, and derivatization of mycolic acids

1. Saponification reagent

- Potassium hydroxide (KOH) 200 g Millipore water 400 ml
- Methanol 400 ml

While stirring, slowly

- Add the KOH to the water in a glass beaker, using an ice bath to cool the mixture.
- Continue stirring until the KOH has dissolved. When cooled, add the methanol.
- Store in a convenient container at ambient temperature until Use (Expiration: 1 year)

2. Acidification reagent

- Millipore water 400 ml
- Conc. hydrochloric acid (HCl) 400 ml

While stirring, slowly

- Add 400 ml of conc. HCl to the water in a 1 L beaker.

- Store in a convenient container at ambient temperature until used (Expiration: 1 year).

3. Potassium bicarbonate reagent

- Potassium bicarbonate (KHCO3) 4 g
- Millipore water 98 ml
- Methanol 98 ml
- Add the KHCO3to the 98 ml water in a suitable container.
- Stir until dissolved, then add the methanol and stir.
- Store at ambient temperature in a convenient container (Expiration: 1 year)
- . If precipitation occurs, heat to 35°C±1°C to re-solubilize before use.

4. Derivatization reagent

p-bromophenylacyl bromide (0. 1 mmol), and dicyclohexyl- 18-crown-6 ether (0. 005 mmol) in acetonitrile.

Preparation

1. P-Bromophenyl acyl –Bromide 0. 1 mmol

    Mol wt -277. 9g in 1000ml =1mol

    1m mol=27. 79mg/ml

    The required amount is 138. 95 mg in 5ml

2. D-Cyclo hexyl-18 –crown 6 ether in 0. 005 mm (Mol wt -375. 5)

    37. 25 mg /ml =0. 1mmol

    The required concentration is 0. 005 mmol

    37. 25/20=1. 8625mg/ml

    The required amount is 9. 3mg/mg in 5ml

Expiration: Two years from the date of manufacture of the oldest component.

5. Clarification reagent

Mix 100 ml acidification reagent with 100 ml methanol.

Store at ambient temperature in a convenient container.

Expiration: 1 year

C. Sample diluent with internal standards

Add 4 mg of LMW-ISTD and 2 mg of HMW-ISTD to 50 ml dichloromethane in a 50 ml volumetric flask (8 and 4mg/100ml, respectively).

Store at 4°C in a tightly capped dark amber bottle.

Expiration: 3 months

D. Equipment

1. Biological safety cabinet (FOR CULTURE TRANSFER)
2. Chemical fume cabinet
3. Agilent 2000HPLC equipped with a C18 end capped column with integral guard column, packed with 3mm silica (4. 6 mm x 7. 5 cm) (Merck or an equivalent column); auto sampler and a column oven. An inline filter and vacuum degasser
4. Autoclave
5. Water bath (85-100°C)
6. Vortex-type mixer (multiple tube-type recommended)
7. Adjustable micropipette (20-200 ml)
8. Safety pipetting device

E. Supplies

1. Pasteur pipettes, 5 3/4", disposable glass
2. Borosilicate glass culture tubes (13 x 100 mm, new, defect-free, unwashed) with new, teflon-lined screw caps
3. Inoculation loops (3mm)
4. Dark amber glass reagent dispensing bottles; or 1. 0 and 5. 0 ml

   glass pipettes
5. Volumetric flasks, 50, 100 and 250 ml
6. Dark amber glass bottles with caps, 1 L, 50 and 100 ml
7. 200µl tips for adjustable micropipette
8. Autosampler vials and caps

Sample preparation

Procedure

Cell harvesting procedure

- Add 2. 0 ml saponification reagent to a new 13 x 100 mm tube.
- Take one / two loopful of culture and transfer into the tube containing saponification reagent and cap tightly If it is not autoclave change the cap (new cap)
- Mix vigorously for at least 20 sec.

Saponification and extraction procedure

- Autoclave the tubes for a minimum of 1 h at 121°C, 15 psi
- Cool to ambient temperature
- Add 2. 0 ml of chloroform.
- Add 1. 5 ml of acidification reagent. Cap tightly.
- Vigorously mix the tubes for a minimum of 20 sec. Allow the layers to separate for 20-30 sec.
- If the bottom layer remains turbid, mix again for 30-60 sec. If still turbid, proceed.
- Using a glass Pasteur pipette, remove the bottom (chloroform) layer (containing mycolic acids) and transfer to a new tube.
- Be careful not to transfer any of the upper (aqueous) layer. If necessary, samples may be capped and stored at 4-6°C overnight.
- Evaporate the chloroform at 85-105°C in water bath until the sample is thoroughly dry.

C. Derivatization to *p*-bromophenacyl esters

- Add 50 µl of potassium bicarbonate reagent to the dry sample.
- Evaporate at 85-105° C in water bath until thoroughly dry
- Cool the sample to ambient temperature or below and add 1. 0 ml of chloroform, followed by 50 ml of derivatization reagent.
- Cap the tube tightly and mix vigorously for 30 sec.

- Heat the sample at 85-100°C for a minimum of 20 min. After the first 30-60 sec of heating, check the volumes.
- If the volume in any tube appears to be less than 1 ml, cool the tube to ambient temperature or below.
- Adjust the chloroform to 1. 0 ml, recap with a new cap and reheat the sample.

D. Clarification by liquid-liquid extraction

- Cool the samples to ambient temperature or below and add 1ml of clarification reagent. Recap.
- Mix each tube vigorously for a minimum of 20 sec and allow the layers to separate (5-10 sec, minimum).
- Remove the bottom (chloroform) layer with a glass Pasteur
- Pipette and transfer to a new tube.

E. Completion and storage

- Evaporate to dryness at 85-105°C. Cap tightly.
- Store the sample at 4-6°C in the dark until ready for analysis.

CHROMATOGRAPHIC SETUP

Gradient conditions

- Gradient conditions are defined for a C-18, reverse phase analytical cartridge column, 4. 6 mm x 7. 5 cm, packed with 3 mm silica.
- Setup the instrument to reproduce the following solvent flow conditions.
- For specific instrument settings see the manufacturer's instruction manual
- The solvent flow rate must be maintained at a constant flow of 2 ml/min until all samples have been analyzed.
- The initial solvent mixture is 98% methanol (solvent A) and 2% Methylene chloride (solvent B) (98:2, v/v).
- During the first minute following injection the solvent mixture is changed to 80% A and 20% B using a linear gradient (elapsed time = 15 min). It is programmed in the HPLC system.
- During the next 14 min, using a linear gradient, the solvent mixture is changed to 35% A and 65% B (elapsed time = 15 min).

- During the next 0. 5 min the gradient is returned to 98% A and 2% B (elapsed time = 15. 5 min).
- Finally, the solvent mixture is held at 98% A and 2% B to equilibrate the column for a duration of 4. 5 min (total elapsed time =15 +5 =20min)

Start up instructions

- Turn on the chromatograph and the detector lamp 30 min
- before use or as recommended by the manufacturer.
- Insure that the amount of solvent is adequate and that the waste
- collection container is in place.
- Verify the column temperature is 35°C + 1°C.
- Prime the pumps.
- Run the analysis method with or without the injection of a Methylene chloride blank. Check for leaks, bubbles, and confirm the pump pressure to be within limits.

HPLC ANALYSIS

A. Sample preparation

- Dissolve the sample in approximately (50-500) 10-300 µl **(??)** of sample diluent.
- The optimum amount of sample diluent is sample and system
- Dependent. (Rapidly-growing mycobacteria may require more sample diluent).
- Slow -growing mycobacteria may require less sample diluent.
- Transfer the sample to an auto-sampler vial, and label appropriately.

B. Analysis

- Load the samples into an auto sampler tray.
- **The order of controls and samples should be as follows:**
- Begin with a control set containing the negative and positive controls.
- Next, load the sample set.

- A positive control is added every 12-15 samples, and at the end of the sample set.

- Inject and analyze the control set.

- Verify that the negative control pattern does not have any peaks (in the region between the LMW-ISTD and HMW- ISTD)

- Verify that the positive control pattern matches the pattern for *M. intracellulare*, ATCC 13950

- When the controls are correct, inject the samples. Samples that yield unacceptable results must be rerun or reanalyzed after corrective measures are taken. (as described in the section on Quality Control).

- Record and compare the beginning pressure of each run. If the pressure varies by more than approximately 10% of the previous day's starting pressure then take the appropriate corrective action

- Record separately the daily number of runs for the guard column, inline filter, and column.

- Record any adjustments or repairs on the sample log and on the equipment maintenance log.

INTERPRETATION OF RESULTS AND REPORTING

A. Sample specifications

- If any sample produces a chromatogram with weak or off scale peaks, adjust the concentration and repeat the run (HPLC)
- If a positive control result is not satisfactory, correct the problem and rerun all samples following the last satisfactory positive control.

B. Visual interpretation

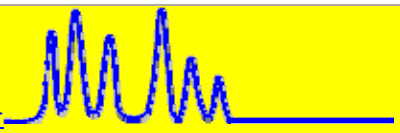
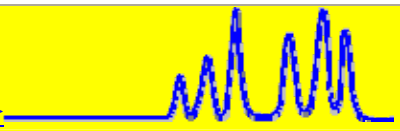
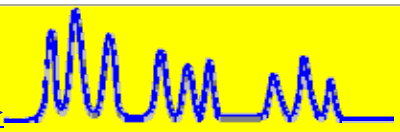
Visual interpretation of chromatographic patterns has demonstrated an accuracy level of > 91%.

Note: Unknown strains must be analyzed with the same method and conditions as the reference samples and controls.

- Results are interpreted visually by manually comparing the sample chromatogram with the laboratory reference pattern to determine a match.
- Suggested approach for visual identification.
- Determine relative retention times (RRT) for all peaks in the chromatograms.
- Chromatographs can be setup to calculate RRT values automatically.
- Separate chromatograms into groups of single, double, distinct triple cluster and multi-peak cluster patterns.

These groups can contain, but are not limited to the following organisms:

Single	Double	Triple	Multi
M. asiaticum	*M. avium*	*M. simiae*	Other *Mycobacterium* species
M. bovis	*M. celatum*		
M. bovis var. BCG	*M. chelonae/ M. abscessus*		
M. gastri	*M. fortuitum/ M. peregrinum* group		
M. gordonae	*M. gordonae* (Chromatotype II)		
M. kansasii	*M. intracellulare*		
M. malmoense	*M. mucogenicum*		
M. marinum	*M. scrofulaceum*		
M. szulgai	*M. terrae* complex		
M. tuberculosis	*M. xenopi*		

FREE SERACH on the basis of the species name:		M. fluoranthenivorans-> M. saskatchewanense-> - M.bovis BCG
SPECIFIC SEARCH on the basis of the peaks arrangement:		
	one, early, cluster of peaks	< span> 
	one, late, cluster of peaks	< span>< span> 
	two early clusters of peaks	< span>< span> 
	two clusters of peaks, one early and one late	< span>< span> 
	two late clusters of peaks	< span>< span> 
	three scattered clusters of peaks	< span>< span> 
	three late clusters of peaks	< span>< span> 

SOURCE: http://www. mycobactoscana. it/page4. htm

Documentation

Storage Culture Processing and HPLC analysis

S. No	Lab. No	Tube. No	Date		Culture Morphology	Specimen Identification	MHA/SC/ZN smear	Storage date/Number	Remarks
			Extraction	Analysis					

MAINTENANCE OF BACTEC 460

Scope

This SOP describes the optimal operation of a BACTEC 460 through regular servicing and preventive maintenance.

Principle

- The BACTEC 460 is a precision instrument intended for isolation, identification and drug sensitivity of tubercle bacilli in specimens in the routine diagnostic TB laboratory.
- Uses The system radio labeled $^{14}CO_2$ (fatty acid) in the medium.
- When the inoculated BACTEC vial are tested the liberated $^{14}CO_2$ is measured in terms of 0-999 GI (Growth Index)
- BACTEC 460 introduce fresh 5% C in to the medium every time a vial is tested.
- It is has a special TB hood.
- The TB hood provides HEPA filtered exhaust air and negative pressure in the test area.
- In addition UV light for extra protection.

Equipment and materials

- BACTEC 460, is installed and maintained according to the manufacturer's manual.
- CO2 cylinder
- Removable BACTEC tube racks

Reagents and solutions

- BACTEC 12B, PANTA Supplement vial, NAP vial, SIRE Drugs,
- Diluting Fluid.
- Cleaning fluid, (cedex)

Quality control and maintenance

Initial installation

- Initial calibration should be performed only by a qualified service technician

Daily maintenance

- Check the display lights
- Make sure the aerobic switch is on.

Change the needles and tubing

- Carefully remove the needle with tube and immerse the needle into the large container of cedex solution and Autoclave.
- Check any block in the needle. If any block is noticed clean the needle with the cleaning wire.

When new batch is introduced Do a performance test

- Using 1 ml syringe, inject 0. 2 ml of standard 14 C solution into acid vial.
- Shake vigorously for 10 seconds to liberate a known amount of 14 CO_2 in the vial.
- Test the acid vial and GI (Growth Index) should be 50-60
- <50—Probably needle block / Instrument malfunctioning.
- >60 –Over standard 14 C solution/ Instrument malfunctioning
- **Monthly maintenance**
- Change the filters and the media trap
- Check the color of the CO_2 absorber.
- Clean the underneath the needle heater.

6-Month maintenance

- Change the dust filter

Yearly Maintenance

- Clean the brake on the drive motor

As required

- Replace the needle heater (if average 31 to 45 samples per day , 3 times per year)
- Change the printer paper
- Replace the individual needles (service person)
- Chang UV bulb and HEPA filter

Documentation:

BACTEC 460 Maintenance log book

Month /Year ---

Date/Signature	Display Lights/Printer	Needles and Tubing	Perform Test

1. **Day (1) of each month.**

   Change filter and media trap--- Date / Sign:

   Change Color of CO2 absorber

   Clean underneath needle heater.

2. **Every 6 months:**

Change dust filter----Date / Sign.

3. **Yearly**

   Clean brake on drive motor----Date / Sign.

3. **As required**

   Replace needle heater---Date / Sign

   Miscellaneous maintenance notes*:*

pH METER

Scope

This SOP describes how to use and maintain a pH meter. The pH meter is intended for accurate measurement of the pH of buffer solutions and/or culture media solution used in the general diagnostic TB laboratory

Principle

Optimal operation of the pH meter is achieved through regular, two-point calibration

(Standards at pH 4 and pH 7) and proper probe storage

Reagents and solutions

Standard buffers may be commercially available or prepared on site.

Reference buffer at pH 4, pH 7, pH8 and pH 10

Detailed instructions for use

Calibration

- Calibration must be carried out, in accordance with the manufacturer's instructions, once daily or, in case of infrequent use, at least on the day of pH testing.
- It must be performed *before* the first measurement of the day. Record in the logbook.
- Temperature variation affects pH measurement.
- Calibration and testing of the sample must therefore be done at the same temperature ± 2 °C.
- Select for calibration two buffer solutions that are within 3 pH units of the solution to be tested.
- Discard contaminated or cloudy standard buffers.
- Calibration results are acceptable if the pH of the buffer solution is within 0. 1 pH units of the expected value.

pH measurement

- Before use, rinse the electrode with deionized water and blot dry with a soft, clean paper towel.
- Transfer the electrode to the test solution.
- Compensate for the temperature if necessary.
- Record the pH when the reading is stable (5–20 seconds after insertion of the electrode into the solution)
- Rinse the electrode with deionized water and store according to the manufacturer's instructions.

Cleaning and maintenance

- Clean the pH meter with a soft, clean, damp paper towel after use.
- No solvents should be used.
- Replace the electrode filling solution on a regular basis, according to the manufacturer's instructions. Record in the logbook (see Annex).
- Repair and service of the pH meter should be done by a qualified service technician.

Quality control and maintenance

- Discard contaminated or cloudy standard buffers.
- Calibration results are acceptable if the pH values of the reference buffer solutions are within 0. 1 pH units of the expected values.

Calibration and Record sheet

Date	Temp °C	Electrode Serial no.	pH4	pH7	pH8	pH10	Solution/ buffer	pH read	signature

Periodicity

Date	Maintenance operation	Operator

Failure Events

Date	Event	Corrective action taken	Operator

REFRIGERATOR

This SOP describes the optimal operation of the refrigerator – achieved through proper installation and maintenance (defrosting / cleaning) – and relates to equipment in an infectious area of the laboratory.

Principle

The refrigerator is intended for ensuring an environment for material that requires preservation at 2–8 °C.

This equipment can be placed in a clean area of the laboratory or in an infectious area.

Samples

Refrigerator placed in a clean area of the laboratory

- Culture media prepared for use.
- Antibiotics that require cold storage at 2–8 °C, preferably in a desiccator.
- Reagents/solutions to be stored at 2–8 °C.
- Never store flammable solutions in a refrigerator that is not approved and certified for this purpose.

Refrigerator should placed in the mycobacteriological laboratory

Specimens and mycobacterial cultures.

Use separate freezers for clean and infectious materials.

Equipment and materials

The refrigerator should be installed and operated in accordance with the specific manufacturer's manual. Specifically:

After transportation, leave the refrigerator in the upright position for some hours before connecting it to the electricity supply.

- Do not install the refrigerator close to heat sources.
- Locate the refrigerator in a dry, well-ventilated area.
- Ensure that the refrigerator is placed on a level surface.
- To prevent loss of valuable cultures, chemicals, etc. in case of a power failure,the refrigerator should ideally be connected to a suitable UPS system.

Reagents and solutions

Disinfectant solution

Procedure for cleaning / defrosting

Defrosting

- Identify an adequate volume of available space in another refrigerator to store materials during the defrosting procedure.
- Ensure that materials kept in the refrigerator are clearly marked.
- Mycobacterial cultures stored in the refrigerator may stand at room temperature with no loss of viability.
- Switch the refrigerator off and disconnect it from the electrical supply.
- Open the refrigerator door and leave it open.

Note: Turning the thermostat to "0" does not switch the power off

- Open the refrigerator door and leave it open.
- Remove the drain cap (if there is one) from the low temperature compartment,
- Place a container under the drain hole to catch the melted ice.
- Never use sharp tools to chip the ice off during the defrosting process.
- Sponge up any melted ice.

Cleaning

- Once all the ice has melted clean the interior of the refrigerator with a disinfectant solution (see SOP on disinfectants).
- Replace the drain cap, if necessary.
- Clean the outside of the refrigerator with a cleaning solution, and dry with a soft cloth.
- Reconnect the refrigerator to the electricity supply and switch it on.
- Replace the refrigerator contents once the temperature has reached 2–8 °C.
- Do not overcrowd.
- The whole process should be completed in a few hours.

Reading and reporting

- Use a record form to keep a daily record of the refrigerator temperature (Annex 1).
- Record the regular maintenance of the refrigerator on a maintenance logbook (Annex 2)

Maintenance

Daily

- Check the compressor for any unusual sound and for overheating.

Every six months

- Defrost the refrigerator
- Clean the condenser coils and fan with a brush or vacuum cleaner.
- Check the compressor for any unusual sound and for overheating.
- Repairs should be performed only by a qualified service technician.

Waste management

- Refrigerator contents may be disposed of only with the prior consent of the laboratory manager.
- Details of disposal must be noted in the refrigerator logbook (Annex 2).
- Contaminated materials must be autoclaved before disposal.

Annex 1. Temperature record form

Equipment: REFRIGERATOR	Reference:
Location:	Installation date:
Temperature required: +6 °C ± 2 °C (acceptable variation)	

Trimester:				Year:				
Month	Temp. °C	Opeartor (initials)	Month	Temp. °C	Operator (initials)	Month	Temp. °C	Operator (initials)
1			1			1		
2			2			2		
3			3			3		
4			4			4		
5			5			5		
6			6			6		
7			7			7		
8			8			8		
9			9			9		
10			10			10		
11			11			11		
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21			21			21		
22			22			22		
23			23			23		
24			24			24		
25			25			25		
26			26			26		
27			27			27		
28			28			28		
29			29			29		
30			30			30		

Annex 2. Maintenance logbook

ITEM IDENTIFICATION			
Equipment:	REFRIGERATOR	Brand name:	
Purchase date:		Model/type:	
Location within laboratory:		Serial no.	
Warranty expiry date:			
Manufacturer:			Tel:
Address:.			
Contact person:			
Technical service representative:			Tel:

PERIODICITY: Every six months or when needed		
Date	Maintenance operation	Operator
	Defrosting: Relocation: What/Where: What/Where: What/Where: Refilling:	
Remarks:		
Date	Maintenance operation	Operator
	Defrosting: Relocation: What/Where: What/Where: What/Where: Refilling:	
Remarks:		
Date	Maintenance operation	Operator
	Defrosting: Relocation: What/Where: What/Where: What/Where: Refilling:	
Remarks:		

FAILURE EVENTS			
Date	Event	Corrective action taken	Operator

WATER DISTILLATION PLANT

Scope

This SOP describes the use and maintenance of a water distiller capable of producing at least 4 litres of distilled water per hour.

Principle

The water distiller purifies water by evaporation and condensation of the steam. It removes organic as well as inorganic material. However, it does not produce sterile water because spores may not be destroyed during the process and because the equipment does not guarantee sterile conditions of collection and storage.

Detailed instructions for use

Follow the manufacturer's instructions for operation of the water distillation apparatus. Before switching on, always check that:

- There no water leaks from the hose connection;
- The drain water flows freely;
- The flow rate of the water supply is sufficient;
- The reservoir is empty; a safety cut-out should switch off both the electricity and the water supply when the reservoir is full

Quality control and maintenance

- Lime scale will eventually accumulate in the boiler and in the heating element and must be removed on a regular basis to obtain optimal performance of the distiller
- The interval between cleaning operations depends upon the hardness of the water supply.
- Descaling is performed with concentrated hydrochloric acid.
- Record in the logbook Protective equipment (cotton gowns, goggles, thick rubber gloves) should be worn during the operation.
- The chemical reaction should be allowed to continue until all the limescale deposits have been removed.
- At the end of the descaling operation, the acid may have not been completely neutralized and the liquid flowing to the drain may be strongly acid.
- Necessary safety precautions should be observed and control of effluent be followed till acid is totally eliminated.

- The connection to the electricity supply should be made by a qualified technician. If the quality of the feed water is poor, a pre-filter unit should be fitted.

Periodicity

Date	Maintenance operation	Operator

Failure Events

Date	Event	Corrective action taken	Operator

ELECTRONIC BALANCE

Scope

This SOP describes the optimal use and maintenance of a laboratory balance, i. e. a balance used to measure mass to a high degree of precision.

There are many types of laboratory balance but this procedure focuses on electromagnetic balances.

Principle

- Electromagnetic balances are fragile, precision instruments intended for the accurate weighing of chemicals in the routine TB diagnostic laboratory.
- They are analytical balances, with a maximum weighing capacity of 20–200 g and a sensitivity of 0. 01mg / 0. 001mg.
- For other purposes, such as balancing loads for the centrifuge, use an open two pan balance (sensitivity of 0. 5 g, and capacity of up to several kilograms).
- Use a balance with a sensitivity appropriate for the desired weight.
- Optimal operation of the balance is achieved through regular calibration, verification and proper maintenance.
- Initial calibration and verification services are usually provided by the manufacturer

Equipment and materials

- The balance should be installed on a solid, vibration-free surface, away from direct sunlight and at an even temperature.
- It must be precisely levelled, using the built-in spirit level: the levelling foots of the balance are turned until the air bubble is correctly positioned in the centre of the spirit level.
- Protect the balance from drafts of air. Air moving across the pans will cause inaccurate readings.
- Always keep the balance and weights clean and dry to protect them from corrosion.

Other items:

Weighing paper, Set of calibrating weights, Dust brush.

Reagents and solutions:

Silica blue desiccant

Detailed instructions for use

- Check whether the sensitivity of the balance is appropriate for the amount of material to be weighed.
- Zero the balance before use.
- Put material to be weighed in a suitable container or on weighing paper, never directly on the pan of the balance.
- Determine the mass of the weighing container or paper.
- Place the material to be weighed in the container or on the weighing paper in the middle of the pan to avoid corner-load error.
- To prevent contamination of stock material, do not return unused substance to the stock bottle.
- Clean the balance with a soft, clean brush after use. Refer to manufacturer's manual for other instructions on cleaning.
- Balance pans and the working area can be disinfected with 70% ethanol.
- Always keep the work area clean.
- When the balance is not in use, keep it under an airtight plastic cover for protection against dust.
- Place a dish containing blue silica under the cover to remove humidity from the air. (When the silica desiccant has turned red, it must be regenerated by heating).

Reading and recording

The mass of the substance being weighed is read directly from the screen of the balance.

Quality control

Daily

- Using an appropriate milligram/gram weight, check daily that the weight registered by the balance agrees with the calibrating weights.
- Record this check in the logbook (see Annex).

Annually

- Calibration of the balance should be carried out annually or after any repair or resetting by a qualified service technician and registered in the logbook.
- Repair of the balance should be carried out by a qualified service technician.

Date	Date Maintenance operation	Operator

WATER BATH

Introduction

- The water bath is an instrument used in the laboratory for carrying out agglutination, inactivation, biomedical, and pharmaceutical tests and even for industrial incubation procedures.
- In our laboratory specifically used for performing Catalase test and Sample preparation in HPLC.
- The temperature range at which water baths are normally used is between room temperature and 60 °C.
- Temperatures of 100 °C can be selected, using a cover with special characteristics.
- Water baths are manufactured with chambers of a capacity ranging from 2 to 30 litres.

Installation

- Install the water bath close to an electrical outlet.
- The outlet must have its respective ground pole in order to guarantee the protection and safety of the operator and the equipment.
- Water baths generally operate at 120 V/60 Hz or 230 V/60Hz. Its installation and use is facilitated by a sink close by for supplying and draining of water.
- Verify that the location selected is leveled and has the necessary resistance to safely support the weight of the water bath when it is full of liquid
- Ensure that the location has a suitable amount of space for putting the samples and the accessories required for the normal operation of the water bath.
- Avoid placing the water bath where there are strong air currents which can interfere with its normal operation.

Operation Principles

Water baths are made of steel and are generally covered with electrostatic paint with high adherence and resistance to environmental laboratory conditions.

- Water baths have an external panel on which the controls can be found.
- Water bath have a tank made of rust proof material with a collection of electrical resistors mounted on their lower part. By means of these, heat is transferred to the medium (water) until reaching the temperature selected with a control device.

The resistor types

- ***Immersion type:*** The resistor are installed inside a sealed tube and located on the lower part of the container in direct contact with heating medium.

- ***External:*** These resistors are located on the lower part but on the outside of the tank. These are protected by an isolating material which prevents heat loss. This type of resistor transfers the heat to the bottom of the tank through thermal conduction.

Types of water bath

Class	Temperature range
Low temperature	Room temperature up to 60 °C
	Room temperature up to 100 °C
High temperature	Room temperature up to 275 °C. When it needs to reach temperatures above 100 °C, it is necessary to use fluids other than water as the boiling point of water is 100 °C under normal conditions
	This type of bath generally uses oils which have much higher boiling points.
Insulated	Room temperature up to 100 °C with accessories and/ or agitation systems (with water).

The control panel has these elements:

- The on and off control switch
- A Menu button for selecting the Operation temperature
- One button for temperature to set
- Screw for temperature adjustment
- A screen

6. A pilot light

7. Pilots (2) for identifying the temperature scale (°C, °F).

Procedure for using the water bath

- Before using the water bath, verify that it is clean and that accessories needed are installed. Follow The steps:
- Fill the water bath with fluid to keep the temperature constant (water).
- Verify that once the containers to be heated are placed, the fluid level should be between 4 and 5 cm from the top of the tank.
- Install the control instruments needed, such as thermometers and circulators.
- Use additional mounts provided for this purpose.
- Verify the position of the thermometer's bulb or thermal probe to ensure that the readings are correct.
- If water is used as the warming fluid, verify that it is clean.
- Select the operation temperature mode using the button 1 or 2 or 3 for adjusting the parameters. (This is a safety control which cuts off the supply of electricity if it exceeds the selected temperature).
- Press button to set the temperature.
- Rotate the screw to increase or decrease the temperature.

Avoid using the water bath with the substances indicated below:

- Bleach.
- Liquids with high chlorine content.
- Weak saline solutions such as sodium chloride, calcium chloride or chromium compounds.
- Strong concentrations of any acid.
- Strong concentrations of any salt.
- Weak concentrations of hydrochloric, hydrobromic, hydroiodic, sulphuric or chromic acids.
- Deionised water, as it causes corrosion and

Cleaning

Frequency: Monthly

- Turn off and disconnect the equipment. Wait until it cools to avoid the risk of burns and accidents.
- Remove the fluid used for heating. If it is water, it can be poured through a tap or drain off.
- Remove the thermal diffusion grid located at the bottom of the tank.
- Disassemble the circulator and clean to remove scale and potential algae present.
- Clean the interior of the tank with a mild detergent. Rub lightly with synthetic sponges or equivalent.
- Avoid using steel wool to remove rust stains as these leave particles of steel which could accelerate corrosion.
- Avoid bending or striking the temperature control capillary tube generally located at the bottom of the tank.
- Clean the exterior and interior of the water bath with clean water.

Trouble shooting

TROUBLESHOOTING TABLE		
PROBLEM	PROBABLE CAUSE	SOLUTION
There is no power to the instrument.	The water bath is disconnected.	Connect the water bath.
	The switch is defective.	Change the switch.
	The fuse is defective.	Substitute the fuse.
The water bath is not getting hot.	The temperature control not set.	Set the temperature control.
	The resistor(s) is/are defective.	Change resistor(s).
	The limit control is not set	Set the limit control.
The temperature is higher than that selected.	The temperature control is defective.	Change the temperature control if required.
	Verify the selection of the parameters.	
The samples are warmed slowly.	The tank is empty or contains very little fluid.	Fill the tank up to the recommended level.
The temperature is increasing very slowly.	The resistor(s) is/are defective.	Change the resistor(s).
	The temperature control is defective.	Substitute temperature control.

DRYING OVEN

Introduction

The drying oven is used for sterilizing / drying glassware and metal materials used for examinations performed in the laboratory.

Dry heat sterilization of clean material is conducted at 160 °C for one hour in the oven.

Upon being heated by high temperature dry air, humidity is evaporated from glassware and thus the possibility of any remaining biological activity is eliminated.

Operating Principles

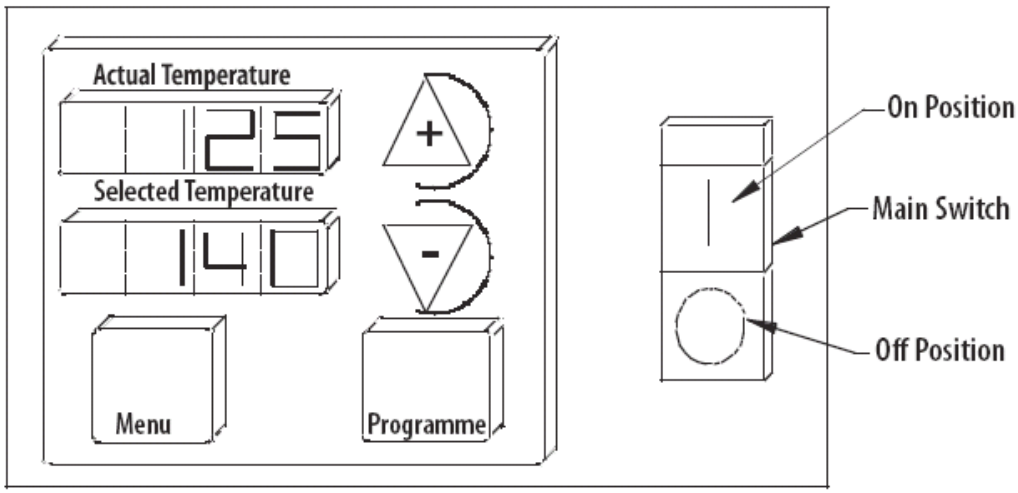
- Generally, drying ovens have an internal and an external chamber.
- The internal chamber is made of aluminum or stainless steel material with very good heat transference properties.
- It has a set of shelves made of stainless steel grids so that air circulates freely around objects requiring drying or dry heat sterilization.
- It is (shelves) isolated from the external chamber by insulating material which maintains high temperature conditions internally and delays the transference of heat to the exterior.
- The external chamber is made of steel laminate, covered with a protective film of electrostatic paint
- Heat is generated through sets of electrical resistors transferring this thermal energy to the chamber.
- These resistors are located in the lower part of the oven and heat is transferred and distributed by natural or forced convection (in oven with internal ventilators).
- The oven has a metallic door with its own thermal insulation equipped with a similarly insulated handle to prevent burns on hands.
- The door is installed on the front part of the oven by a set of hinges which allow it to open at a 180° angle.

Oven Controls

A diagram of controls regulating modern drying ovens is shown.

- The main switch.
- Screens for controlling the current and selected temperatures.

- The parameter selection button (menu).
- The button for programming operation cycles.
- Buttons for increasing and decreasing the temperatures.



Operation routine

- In general, the following procedure is performed:
- Activate the main switch, pressing the button usually identified by the symbol [I].

Press the key identified as Program.

- Select the operational temperature by pressing the key marked by the sign (+) until the selected temperature appears on the screen. The oven will start the heating process until reaching the selected temperature.
- For programmable ovens, instructions must be followed as defined by the manufacturer for setting additional parameters such as time, types of warming and alarms.

Precaution

- Do not use flammable or explosive materials in the oven.
- Avoid spills of acid solutions or corrosive vapours inside the oven to prevent corrosion of the surfaces and interior shelves.
- Use personal protection elements (insulated gloves, safety glasses and tongs for placing or removing substances or materials inside the drying oven).

Routine Maintenance

- Maintenance is simple and no complex routine maintenance is necessary.
- The procedures vary depending on the type of oven and designs from different manufacturers.

Frequency: Whenever necessary

- Change to electric components.
- Change the heating resistors.
- Change the cooling ventilator.
- Change the door gasket.
- Change of thermocouple.
- Change of door hinges

TROUBLESHOOTING TABLE		
PROBLEM	PROBABLE CAUSE	SOLUTION
There is no power to the oven.	The oven is not connected.	Connect the oven to the electrical outlet.
	The main switch is off.	Activate the start switch.
	The circuit breaker is defective.	Change the circuit breaker.
	The control card is defective.	Substitute the control card.
	The connector cable is defective.	Check/repair connector cables.
Erratic elevated temperature.	The thermocouple is defective.	Substitute the thermocouple.
	The control is defective.	Substitute the control.
The oven shows heating errors.	A temperature lower than that selected.	Change the temperature selection. Wait until it reaches the selected temperature.
	The thermocouple is defective.	Substitute the thermocouple.
	The heating resistor is defective.	Substitute the heating resistor.
	The relay is defective.	Substitute the relay.
	The control is defective.	Replace the control.
The screen displays the message "open".	The thermocouple circuit is open.	Verify the thermocouple connection or substitute the thermocouple.

Documentation

The daily register was maintained:

Date	ON Time	Actual Temperature	Time of Temperature at 160 C	OFF Time	Signature

Source: WHO Maintenance Laboratory equipment 2008

APPENDIX -1

List of Chemicals, Reagents and Drugs used in Mycobacteriology Laboratory

S. No.	Chemical name	Company	Cat. No.	Quant.	Test Used
1	Acetonitrile	Qualigens	271310	500 gm	Culture
2	Agar-agar	Difco	262710	500 gm	Culture
3	Amikacin	Sigma	283810	500 gm	Culture
4	Ammonium sulphate (Excel R)	Qualigens	340953-J	100 gm	FM
5	Amphotericin B	Sigma	340454/S	25gm	LJ
6	Auramine	Gurrr BDH	340484 B	26gm	ZN
7	Basic fuchsin (ExcelR)	Qualigens	29361	500gm	Identification
8	Benzene (Excel R)	Qualigens	MM081-500G	500 gm	Identification
9	Bovin albumin fraction V	Sigma	RM961-25G	25gm	Gram stain
10	4-Bromo phenacyl bromide	Sigma	0142-01	500 gm	Routine
11	Calcium chloride dihydrate (Excel R)	Qualigens	RM 1073-25G	25 gm	Nitrate reduction
12	Capreomycin	Sigma	M001A	100 gm	Routine
13	Carbenicillin	Sigma	M002	100 gm	Routine
14	Casein hydrolysate (Bacto casitone)	Sigma	RM1852-100G	100 gm	Identification
15	Catalase (from bovine liver)	Sigma	RM 110-25G	25gm	Phage lyophilisation
16	Cetrimide – SQ	Qualigens	RM027-500G	500 gm	LB broth
17	Citric acid – SQ	Qualigens	44006	1000 ml	HPLC
18	Cupric sulphate. 5H2O (Excel R)	Qualigens	11535	500gm	Medium
19	Cyanogien bromide- pure	SRL	39402	25 gm	ZN
20	D-Glucose (Dextrose) (Excel R)	Qualigens	32015	500ml	
21	Dichlro methane	Qualigens	12135	500 gm	7H9

22	Dicyclohexano-18-crown-6	Sigma	22274	100 gm	Decontaminatio n
23	Dihydro streptomycin	Sigma	22585	500 gm	Buffer
24	Disodium hydrogen phosphate (Excel R)	Qualigens	12515	500 gm	7H9
25	Ethambutol	Sigma	15405	500 gm	Medium
26	Ethionamide	Sigma	43506	1 litre	HPLC
27	Fetal calf serum	Sigma	15825	500 gm	Culture
28	Glycerol (Exel R)	Qualigens	15455	500 ml	Medium
29	HPLC-chlroform	Qualigens	43316	1 litre	HPLC
30	Hydrochloric acid (ExcelR)	Qualigens	29505	500ml	FM
31	Hydrogen peroxide 30% (ExcelR)	Qualigens	15465	500ml	Catalase
32	Iodine (Excel R)	Qualigens	15474	100 gm	Gram stain
33	Iso propanol (Excel R)	Qualigens	13825	500ml	BACTEC
34	Isoniazid	Sigma	13005	500 gm	Reagent
35	Kanamycin	Sigma	32405	500 ml	
36	L-Asparagine	Sigma	39702	25 gm	Identification
37	Magnesium citrate	Sigma	5615	500 gm	Disnfectant
38	Magnesium suhphate (Excel R)	Qualigens	13405	500gm	LJ
39	Magnesium sulphate	Sigma	15724	250 gm	Gram stain
40	Malachite green	Gurrr BDH	26785	500 gm	FM
41	McConkey agar	Hi media	15955	500gm	Identification
42	Methanol	Qualigens	15915	500 gm	FM/identifiactio n
43	Methyl violet	Hi media	15895	500gm	Culture
44	Methylene blue	Gurrr BDH	27855	500 gm	Nitrate reduction
45	Middlebrook 7H 9 Dehydrated powder	Difco	27745	500 gm	Identification
46	Middlebrook 7H10 Dehydrated powder	Difco	14575	500 gm	Nitrate reduction
47	Middlebrook 7H11 Dehydrated powder	Difco	29997	2. 5 litre	ZN
48	MTT	Sigma	14005	500 gm	Medium
49	N (1-Napthyl ethylene diamine di-HCl)- AR	Hi media	28555	500 gm	LB Medium
50	Neutral red	Qualigens	15995	500 gm	Nitrate reduction
51	Nutrient agar	Hi media	B-2006	10 gm	HPLC
52	Nutrient broth	Hi media	A1774	250mg	DST

53	Ofloxacin	Sigma	A9528	100mg	PACT
54	Oleic acid	Sigma-Aldrich (Fluka)	A4503	500g	Medium
55	Ortho toluidine	Hi media	C4142	5 gm	Drug
56	Para Nitrobenzoic Acid (PNB)	Gurr BDH	C9231	1gm	DST
57	Phenol (Excel R)	Qualigens	22090-100G	100gm	Medium
58	Phenol red	Sigma	C1345-100G	100 gm	Supplement
59	Polymyxin B	Sigma	D-2637	1 gm	HPLC
60	Potassium dihydrogen orthophosphate	Qualigens	D-7253	5gm	DST
61	Potassium iodide (Excel R)	Qualigens	E4630	25gm	DST
62	Potassium permanganate - SQ	Qualigens	E6005	5gm	DST
63	Propylene glycol (extra pure - AR)	SRL	C 8056	100ml	Supplement
64	Pyrazinamide	Sigma	I3377	50g	DST
65	Rifampicin	Sigma	K1377	5g	DST
66	Sodium Carbonate anhydrous (ExcelR)	Qualigens	A 8381-1KG	1Kg	LJ
67	Sodium Chloride (Excel R)	Qualigens	63067-100G-F	100g	LJ
68	Sodium Hydroxide pellets (ExcelR)	Qualigens	M7506	500gm	LJ
69	Sodium nitrate-SQ	Qualigens	M2003-1G	1 gm	Identification
70	Sodium pyruvate	Qualigens	O8757	10gm	DST
71	Streptomycin Sulphate	Sigma	P5530	25 gm	Identification
72	Sulphanilamide (Excel R)	Qualigens	P4932	50 Lacs unit	PACT
73	Sulphuric acid (Excel R)	Qualigens	P7136	10gm	DST
74	Trehalose (D+)	Hi media	R-3501	5 gm	Drug
75	Tri Sodium citrate (ExcelR)	Qualigens	S9137	25gm	DST
76	Trimethoprim	Sigma	T7883	100gm	PACT
77	Tryptone (Bacteriological)	Qualigens	1754	500ml	Catalase
78	Tween 80	Sigma	Pr. No: 75090	100 ml	Supplement
79	Yeast Extract	Hi media	34779	100 gm	Niacin
80	Zinc dust (Excel R)	Qualigens	1629111	500 ml	PNB