PREPARATION OF STAINING SOLUTION

**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 (KMnO₄)

• Write the date of preparation.

**Carbol fuchsin**

**Chemicals required**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin (Hi media)</td>
<td>10g</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>100 ml</td>
</tr>
<tr>
<td>Phenol</td>
<td>50 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>900 ml</td>
</tr>
</tbody>
</table>

• 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 (H₂SO₄)

Chemicals required

Conc. H₂SO₄ : 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.
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 at 121°C
- Store at 37°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 H₂SO₄ 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$_2$PO$_4$ : 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.
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°-90°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°C for 24 hours select 2 bottles of plain LJ for sterility check and record in the Media Sterility register.
Procedure for Mycobacteria Culture using Löwenstein Jensen (acid buffered) medium from Sputum (Simple Method)

1. Label the specimen collection tube (plastic) with patient’s name and/or other reference number.
2. Collect about 2 mL of sputum positive specimen specimen and label a laboratory number on the tube.
3. Add 1 volume NAOH to this sputum, but 2 volumes to thick and viscous sputum.
5. Place the specimen on rack and stand at room temperature for 15 min. If there are large number of specimen, handle in batches so that the NAOH treatment time does not exceed 20 min.
6. Label the back of the modified LJ medium (acid buffered) in plastic container with laboratory number and date of inoculation. Drain water from culture bottle before inoculation.
7. Use a sterilized Pasteur pipette to pipette out the NAOH treated sputum specimen. Use separate sterilized pipette for each specimen. Do not reuse before sterilization.
8. Incubate the NAOH treated sputum onto LJ slants, until with (2 to 3 days) 0.1 mL, cover the surface of the slant as much as possible. Incubate an additional LJ containing routine if necessary.
9. Loosen cap slightly and inoculate the inoculated LJ slants in horizontal position at 37°C for 24 hours.
10. After 24 hours, tighten the cap and continue the incubation with the LJ tubes in upright position.
11. After 8 weeks incubation, report “culture negative” if no growth is observed.
12. After 8 weeks incubation, report “culture positive” if growth is observed.
13. Mycobacterium tuberculosis appears as dry, smooth, nonmotile colonies with irregular edges and ivory-white. DO NOT OPENS positive cultures tubes, and send to reference laboratory.
14. For non-tuberculosis mycobacteria, some are MTB like, but some are smooth, wet and yellow. DO NOT OPENS positive cultures tubes, and send to reference laboratory.

# For packaging and transportation details, please refer to the WHO document, which is available online at www.who.int/iris/research-publications/handbook/WHO_CDS_16PA_2015_eng.pdf
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.
- Sterilise 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° 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**  
Streptomycin (S)  
Isoniazid (I)  
Rifampicin (R)  
Ethambutol (E)

**Second line drugs**  
Kanamycin (K)  
Ethionamide (TH)  
Ofloxacin (OF)

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.

<table>
<thead>
<tr>
<th>ML of stock (µg/ml)</th>
<th>L-J fluid</th>
<th>Final Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 ml of 100 µg/ml</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>4.0 ml of 100 µg/ml</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>2.4 ml of 2000 µg/ml</td>
<td>600</td>
<td>8</td>
</tr>
<tr>
<td>4.8 ml of 2000 µg/ml</td>
<td>600</td>
<td>16</td>
</tr>
<tr>
<td>9.6 ml of 2000 µg/ml</td>
<td>600</td>
<td>32</td>
</tr>
<tr>
<td>3.84 ml of 10,000 µg/ml</td>
<td>600</td>
<td>64</td>
</tr>
</tbody>
</table>
SD₄ 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

<table>
<thead>
<tr>
<th>ML of stock solution (µg/ml)</th>
<th>L-J fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 ml of 2000 µg/ml</td>
<td>600</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>ML of stock solution (µg/ml)</th>
<th>Distilled Water(ml)</th>
<th>Final Conc(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml of 10,000</td>
<td>9</td>
<td>1000</td>
</tr>
<tr>
<td>2 ml of 1000</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>1 ml of 100</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>2 ml of 5</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

**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.

<table>
<thead>
<tr>
<th>ML of Stock solution (µg/ml)</th>
<th>L-J Fluid (ml)</th>
<th>Fluid Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 ml. (1 µg/ml)</td>
<td>100</td>
<td>0.025</td>
</tr>
<tr>
<td>5 ml. (1 µg/ml)</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>2 ml. (5 µg/ml)</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>1.2 ml. (100 µg/ml)</td>
<td>600</td>
<td>0.2</td>
</tr>
<tr>
<td>6 ml. (100 µg/ml)</td>
<td>600</td>
<td>1.0</td>
</tr>
<tr>
<td>3 ml. (1000 µg/ml)</td>
<td>600</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**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:**

<table>
<thead>
<tr>
<th>Stock solution (µg/ml)</th>
<th>Distilled Water</th>
<th>µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 ml of 10,000</td>
<td>16 ml</td>
<td>2000</td>
</tr>
<tr>
<td>2 ml of 2000</td>
<td>18 ml</td>
<td>200</td>
</tr>
</tbody>
</table>

**Media preparation:**

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

<table>
<thead>
<tr>
<th>ML of stock solution (µg/ml)</th>
<th>LJ fluid (ml)</th>
<th>Final Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 ml of 200 µg/ml</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>4 ml of 200 µg/ml</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>8 ml of 200 µg/ml</td>
<td>100</td>
<td>16</td>
</tr>
<tr>
<td>9.6 ml of 2000 µg/ml</td>
<td>600</td>
<td>32</td>
</tr>
<tr>
<td>3.84 ml of 10,000 µg/ml</td>
<td>600</td>
<td>64</td>
</tr>
<tr>
<td>7.68 ml of 10,000 µg/ml</td>
<td>600</td>
<td>128</td>
</tr>
</tbody>
</table>

**Proportion sensitivity testing (PST) (R 40)**

<table>
<thead>
<tr>
<th>ML of stock solution (µg/ml)</th>
<th>LJ fluid (ml)</th>
<th>Final Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4 ml of 10,000 µg/ml</td>
<td>600</td>
<td>40</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>ML of stock solution (µg/ml)</th>
<th>L-J fluid (ml)</th>
<th>Fluid Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ml. (50 µg/ml)</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td>2.0 ml. (50 µg/ml)</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>2.4 ml. (500 µg/ml)</td>
<td>600</td>
<td>2.0</td>
</tr>
<tr>
<td>4.8 ml. (500 µg/ml)</td>
<td>600</td>
<td>4.0</td>
</tr>
<tr>
<td>4.8 ml. (1000 µg/ml)</td>
<td>600</td>
<td>8.0</td>
</tr>
</tbody>
</table>
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

\[
\begin{align*}
4 \text{ ml of } 10,000 \, \mu \text{g/ml} + 6 \text{ ml water} & = 4000 \, \mu \text{g/ml} \\
2 \text{ ml of } 10,000 \, \mu \text{g/ml} + 18 \text{ ml water} & = 1000 \, \mu \text{g/ml} \\
1 \text{ ml of } 1000 \, \mu \text{g/ml} + 19 \text{ ml water} & = 50 \, \mu \text{g/ml}
\end{align*}
\]

Media preparation

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

<table>
<thead>
<tr>
<th>ML of Stock solution (µg/ml)</th>
<th>LJ fluid (ml)</th>
<th>Final Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 ml of 50 µg/ml</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>4.0 ml of 50 µg/ml</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>2.4 ml of 1000 µg/ml</td>
<td>300</td>
<td>8</td>
</tr>
<tr>
<td>4.8 ml of 1000 µg/ml</td>
<td>300</td>
<td>16</td>
</tr>
<tr>
<td>2.4 ml of 4000 µg/ml</td>
<td>300</td>
<td>32</td>
</tr>
<tr>
<td>4.8 ml of 4000 µg/ml</td>
<td>300</td>
<td>64</td>
</tr>
</tbody>
</table>

Proportion sensitivity testing (PST) (K 30)

<table>
<thead>
<tr>
<th>ML of stock solution (µg/ml)</th>
<th>LJ fluid (ml)</th>
<th>Final Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 ml of 4000 µg/ml</td>
<td>600</td>
<td>30</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>ML stock solution</th>
<th>water (ml)</th>
<th>µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0 (10000 µg/ml)</td>
<td>14</td>
<td>3000</td>
</tr>
<tr>
<td>8.55 (10000 µg/ml)</td>
<td>11.45</td>
<td>4275</td>
</tr>
</tbody>
</table>

Media preparation:

For approximately 50 slopes of each concentration.

<table>
<thead>
<tr>
<th>Solution (ml )</th>
<th>LJ fluid (ml)</th>
<th>Fluid Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0ml. (3000µg/ml)</td>
<td>300</td>
<td>20</td>
</tr>
<tr>
<td>2.0 ml. (4275µg/ml)</td>
<td>300</td>
<td>28.5</td>
</tr>
<tr>
<td>4.0 ml. (3000µg/ml)</td>
<td>300</td>
<td>40</td>
</tr>
<tr>
<td>4.0 ml. (4275µg/ml)</td>
<td>300</td>
<td>57</td>
</tr>
<tr>
<td>8.0 ml. (3000µg/ml)</td>
<td>300</td>
<td>80</td>
</tr>
<tr>
<td>8.0 ml. (4275µg/ml)</td>
<td>300</td>
<td>114</td>
</tr>
</tbody>
</table>
OFLOXACIN (OF)

Stock solution

- Weigh accurately 100 mg of Ofloxacine 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

<table>
<thead>
<tr>
<th>Stock (µg/ml)</th>
<th>Water (ml)</th>
<th>Final Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ml of 10,000</td>
<td>18</td>
<td>1000</td>
</tr>
<tr>
<td>1 ml of 1000</td>
<td>19</td>
<td>50</td>
</tr>
</tbody>
</table>

Media preparation

For 100 slopes each of 2, 4 & 8 µg/ml and 16 slopes of each 0.5 & 1.0 µg/ml

<table>
<thead>
<tr>
<th>ML of stock (µg/ml)</th>
<th>LJ (ml)</th>
<th>Final Conc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ml of 50 µg/ml</td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td>2.0 ml of 50 µg/ml</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>1.2 ml of 1000 µg/ml</td>
<td>600</td>
<td>2.0</td>
</tr>
<tr>
<td>2.4 ml of 1000 µg/ml</td>
<td>600</td>
<td>4.0</td>
</tr>
<tr>
<td>4.8 ml of 1000 µg/ml</td>
<td>600</td>
<td>8.0</td>
</tr>
</tbody>
</table>
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°C for 24 hours.
- The media bottles are randomly selected (1set) and incubated at 37°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.

MIDDLEBROOK’S 7H 9 LIQUID MEDIUM

Salt solution:

Disodium anhydrous hydrogen phosphate (Na₃HPO₄) : 2.5g
Potassium dihydrogen orthophosphate (KH₂PO₄) : 1.0g
Ammonium sulphate (NH₄SO₄) : 0.5g
L-sodium glutamate : 0.5g
Trisodium citrate (2H₂O) : 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 (MgSO₄.7H₂O) : 1.0 ml of 5% aq. soln.
Calcium chloride (CaCl₂.2H₂O) : 1.0 ml of 0.05% aq. soln.
Zinc sulphate (ZnSO₄.7H₂O) : 1.0 ml of 0.1% aq. soln.
Cupric sulphate (CuSO₄.5H₂O) : 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°C and store in the cold.

**OADC 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 : 100 ml.

Mix well and sterilize by Seitz filtration or membrane filtration.
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$_2$SO$_4$
• 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$_2$SO$_4$ 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)

<table>
<thead>
<tr>
<th>ZN staining grading (RNTCP)</th>
<th>Reporting /Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;10 AFB/field after examination of 20 fields</td>
<td>Positive, 3+</td>
</tr>
<tr>
<td>1-10 AFB/field after examination of 50 fields</td>
<td>Positive, 2+</td>
</tr>
<tr>
<td>10-99 AFB/100 field</td>
<td>Positive, 1+</td>
</tr>
<tr>
<td>1-9 AFB/100 field</td>
<td>Positive, Scanty</td>
</tr>
<tr>
<td>No AFB per 100 fields</td>
<td>Negative</td>
</tr>
</tbody>
</table>