VIII. APPENDICES

1. Acid-fast stain

1.1 preparation of acid fast stain

1. Fuchsin : Dissolve 0.3 g basic fuchsin in 10 ml 95% ethanol.
2. Phenol : Weigh 5.0 g phenol crystal ; melt with gentle heat ; add 100 ml water.
3. Mix solution (1) with 90 ml of solution (2) ; this is now called carbol fuchsin.
4. Acid alcohol : Carefully add 3.0 ml concentrated hydrochloric acid (HCl) to 97 ml 95 % ethanol and mix.
5. Methylene blue : Dissolve 0.3 g methylene blue chloride in 100 ml distilled water.

2. Nonspecific esterase stain

2.1 Buffered formaldehyde-acetone fixative

Dissolve 20 mg Na_2HPO_4 and 100 mg KH_2PO_4 in 30 ml distilled water. Add 45 ml acetone and 25 ml formaldehyde, mix well, adjust pH of the solution to 6.6 and store in a refrigerator.

2.2 0.15 M phosphate buffer pH 7.4

A : Prepare 0.15 M Na_2HPO_4 (9.47 g Na_2HPO_4/liter H_2O)

B : Prepare 0.15 M KH_2PO_4 (20.4 g KH_2PO_4 /liter H_2O)

Mix 250 ml of A with 750 ml of B (or other appropiate volume at the same ratio 1:3). Adjust pH of the solution to 7.4.

2.3 Hexazotized pararosaniline

Dissolve pararosaniline (Sigma) 0.1 g in 2.0 ml of distilled water and 0.5 ml concentrated hydrochloric acid (HCl), mix well, and this solution is filtered

through Whatman No.1. The pararosaniline HCl can be used effeciently for one month. Store in a refrigerator.

2.4 α-naphthyl acetate

Dissolve 0.05 g of α-naphthyl acetate in 1.0 ml of ethylene glycol monoethyl ether (EGME) (Fisher) immediately before use. Mix 8.9 ml of 0.15 M phosphate buffer, pH 7.4, 50µl hexazotized pararosaniline, and 500µl α-naphthyl acetate. Filter the cloudy white solution before use.

3. Preparation of Middlebrook 7H-9 broth from commercial base

1. Suspend 4.7 g of Middlebrook 7H-9 basal medium in 900 ml distilled water containing 2.0 ml glycerol, or 0.5 g tween 80. Do not use tween 80 and glycerol together.
2. Autoclave at 121 °C for 15 min.
3. Remove from autoclave and cool to 45°C
4. As soon as cool to 45°C, aseptically add 100 ml of albumin-dextrose-catalase (ADC) enrichment

4. Preparation of Middlebrook 7H-10 agar from commercial base

1. Suspend 3.6 g of Middlebrook 7H-10 basal medium in 180 ml distilled water and add 1.0 ml of glycerol.
2. Swirl base into suspension and sterilize by autoclaving at 121 °C for 15 min
3. Remove medium from autoclave and place in a water bath at 50 °C
4. As soon as cool to 50 °C, add 20 ml of oleic acid-albumin-dextrose-catalase (OADC) enrichment.
5. Allow to solidify at room temperature without exposure to daylight.

5. Phosphate buffer saline (PBS) pH 7.2

Dissolve 0.23 g of NaH_2PO_4 (anhydrous) and 1.15 g of Na_2HPO_4 (anhydrous) in 900 ml distilled water. Adjust pH of the solution to 7.2 with 1.0 M HCl. Add distilled water to 1000 ml. Autoclave at 121 °C for 15 min.

6. 1.0 M Hydrochloric acid (HCl)

Mix in the following order:

913.8 ml distilled water and 86.2 ml concentrated HCl (Add acid to water).

7. RPMI 1640 medium

1. Dissolve RPMI 1640 powder (GIBCO) 10.4 g in steriled deionized water 700 ml.
2. Add 26.6 ml of steriled 7.5% $NaHCO_3$, mix.
3. Add 10.0 ml of steriled HEPES buffer (1.0 M, pH 7.3), mix.
4. Adjust pH of the medium to 7.2 with 1.0 M HCl.
5. Add steriled deionized water to 900ml.
6. Sterilize by filter through filter membrane (pore size 0.2 μm).

8. Complete medium (RPMI-10)

Add 20 ml of heat-inactivated fetal bovine serum (FBS) (GIBCO), 70 μl of 2-mercaptoethanol (2-ME) (freshly dilute1:100 with steriled distilled water), 2.0 ml of L-glutamine (200mM) (Biochrom AG), 2.0 ml of non-essential amino acid (100x) (Biochrom AG), and 2.0 ml of penicillin/streptomycin (10000 U/10000μg/ml) (Biochrom AG) to 200 ml of RPMI 1640 medium.

The percentage serum used in a protocol step is indicated by a numeral hyphenated to the base medium name . Thus, " complete RPMI-10" indicates that 10% FBS is used. The absence of a numeral indicates that no serum is used.

9. HEPES buffer (1.0 M, pH 7.3)

1. Dissolve 23.83 g of HEPES (MW 238.3) in 90 steriled distilled water.
2. Adjust pH of the solution to 7.3 with 1.0 N NaOH.
3. Add steriled distilled water to 100 ml.
4. Sterilize by filter through Acrodisc® Syringe Filter 0.2 μm Supor® membrane (Gelman Laboratory).

10. 7.5% $NaHCO_3$

$NaHCO_3$	7.5	g
distilled water	100	ml

Autoclaved 121 °C for 15 min. Storage at room temperature.

11. 0.4% Trypan blue

Trypan blue dye	0.4	g
Phosphate buffer saline (PBS) to	100	ml

Store in the dark bottle and filter after prolong storage.

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