MEDIA PREPARATION
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RECEIPIES FOR INVIVO STUDIES IN MICE

1. PREPARATION OF STOCK GIEMSA SOLUTION :-

Giemsa powder (Koch – Light Laboratories, London)  800 mg
Glycerol (Qualigens , India , A. A. Grade)    50 ml
Methanol (Qualigens , India A. R. Grade)    50 ml

i) Dissolve Giemsa in glycerol at 60°C with regular shaking.
ii) Cool to room temperature and add methanol.
iii) Mix thoroughly for 5 – 10 minutes.
iv) Allow to stand overnight.
v) Filter next day and store in dark coloured bottles at 0 – 4°C.

Working Stain :- (5 % Giemsa) :- Take 5 ml filtered stock Giemsa and adjust final volume to 100 ml help of PO₄ buffer.

2. Preparation of stock May – Grunwald stain:-

May Grunwald powder (Koch – Light Laboratories Ltd., London)  250 mg
Methanol (Qualigens, India, A. R. Grade)  100 ml

i) Dissolve May – Grünwald powder in methanol.
ii) Mix well for 5 minutes.
iii) Filter with Whatman filter paper 541 and store in bottle at 0 – 4°C.

(May – Grünwald stain may be prepared freshly just before use). Filter both the stains before use. (pH 6.8) (Do not reuse the stains).

Working Stain:-

i) Undiluted May Grunwald stain:- Stock is used as undiluted stain after filtration.

ii) Diluted May Grunwald stain :- (1:1) Take 50 ml of the filtered stock May – Grunwald stain and make the final volume up to 100 with help of distilled water and used as working stain (fresh preparations should be
iii) **Phosphate buffer :- (pH 6.8 ) Stock Solution**

a) 0.2 M solution of Na$_2$HPO$_4$, 2H$_2$O (BDH, India)
   35.61 g in 1 lit. D. H$_2$O

b) 0.2 M solution of NaH$_2$PO$_4$, 2H$_2$O
   31.21 g in 1 lit. of D H$_2$O

Mix the stock solutions and dilute to 200 ml with water. For pH 6.8 add 49.0 ml of 0.2 M Na$_2$HPO$_4$, 2H$_2$O into 51.0 ml of 0.2 M NaH$_2$PO$_4$. H$_2$O (for 0.1 M again equal vol. of D.H$_2$O) Use fresh preparation only.

**STOCKS**

a) **1 % tri – sodium citrate** (Loba Chemic, India, AR Grade)

Weigh 1 g of tri – sodium citrate on one pan balance (Sico, SB4, India) and dissolved in distilled water. Adjust the final volume up to 100 ml in volumetric flask (Use fresh preparation).

b) **Fixative :-** Methanol : Glacial acetic acid

   Methanol : (Qualigens, India) AR Grade : 3 parts
   Glacial acetic acid : (Loba Chemic, India, AR Grade) : 1 part

Take 75 ml of methanol and add to it 25 ml of glacial acetic acid. Store it in freeze. Use freshly prepared chilled fixative.

c) **Colchicine :** (Loba Chemic, India M. W. 399.45)

Dose required 4 mg / kg body weight (EHC 51, 1985) 0.096 mg / 24 g of animal (24 g is average body weight of the animal).

Weigh accurately 5 mg of colchicine on one pan balance (Sico, SB4, India) and add to it 12 ml distilled water. From the above stock give 288µl/animal, intraperitoneally with the help of tuberculin syringe and 26 G needles (Use fresh preparation only).
d) Positive Control :-
Methyl methane Sulphonate (MMS) : ( Sigma U. S. A.)
MMS original concentration : 1.39 ml
Required concentration : 40 mg/kg body wt.
Take 10 ml stock and add to it 1990 µl of distilled water. Feed 0.148 ml/24g animal body weight by oral gavage.
(Use fresh preparation only).

e) Phosphate buffer :- pH 6.8 (0.1 M)
Stock Solutions :-

A) 0.2 M solutions of Na$_2$HPO$_4$·H$_2$O (BDH, India)
35.61 g in one lit. of distilled water (Prepare 100 ml)
B) 0.2 M solution of NaH$_2$PO$_4$·H$_2$O (Qualigens, India)
31.21 g in one lit. of distilled water (Prepare 100 ml)
For phosphate buffer (0.1 M) with pH 6.8
Add solution (A) 49 ml and (B) 51 ml. Mix (A) & (B) well and dilute to 200 ml with distilled water.

f) Giemsa powder :- (Koch – Light Laboratories Ltd. London ):-
Stock :- Giemsa powder : 800 mg
Glycerine (Qualigens, India, AR Grade) : 50 ml
Methanol (Qualigens, India, AR Grade) : 50 ml
800 mg of Giemsa powder weighed accurately on single pan balance (Sico, SB4, India) and dissolved in 10 ml of glycerin by using pestle and mortar and final volume adjusted to 50 ml. In the same mixture 50 ml of methanol is added and stirred. Labelled as stock and preserved at 4°C.
5% working stain :-Take 5 ml of the filtered stock and make the final volume 100 ml. Do not reuse. Filter before use.
A. **NURTIENTS :-**

1. **Nutrient broth :-**
   
   Nutrient broth (Hi Media, India) 8 g
   
   Distilled water 500 ml
   
   The nutrient broth was dissolved in boiling water and autoclaved at 15 lbs pressure for 15 minutes.

2. **Minimal glucose agar :-**
   
   a) **Difco bacto agar (Difco, U. S. A.)** 15 g
      
      Distilled water 930 ml
      
      This is autoclaved at 15 lbs for 15 minutes.

   b) **Vogel – Bonner (VB) medium (50x)**
      
      Warm distilled water (45°C) 670 ml
      
      Magnesium sulfate (MgSO₄.7H₂O) (Loba, India) 10 g
      
      Citric acid monohydrate (C₆H₈O₇.H₂O) (BDH, India) 100 g
      
      Potassium Phosphate dibasic (K₂HPO₄) (Qualigens, India) 500 g
      
      Sodium ammonium Phosphate [NaHNH₄ (PO₄, 4H₂O)]
      
      (Qualigens, India) 175 g
      
      The salts were added to warm water in a two litter flask placed on magnetic stirrer. The medium was autoclaved at 15 lbs pressure for 20 minutes.

   c) **Glucose (40%)**
      
      Glucose (C₆H₁₂O₆) (Qualigens, India) 40g
      
      Distilled water 100 ml
      
      This was autoclaved at 15 lbs for 20 minutes.

**Minimal glucose agar**

a. Difco bacto agar 930 ml
b. VB medium E (50 x) 20 ml
c. Glucose (40 %) 50 ml
A teflon coated magnetic stir – bar was placed in a flask used for compounding the above ingredients before autoclaving it and the mixture was later stirred thoroughly. About 30 ml of this glucose agar was poured in each petridish. Petridishes were allowed to cool at room temperature. Salts and glucose are autoclaved separately.

3. **TOP AGAR :**

   Difco bacto agar (Difco, U. S. A.)  6 g  
   Sodium Cholride (NaCl) (Qualigens, India)  5 g  
   Distilled water  1000 ml  

This was autoclaved at 15 lbs pressure for 15 minutes.

4. **L– Histidine + D – Biotin (0.5 mm)**

   D –Biotin (Romali , India )  12.2 mg  
   L – Histidine – HCl (Ronali, India)  24.0 mg  
   Distilled water  250.0 ml  

The ingredients were dissolved by heating. The solution was autoclaved at 15 lbs pressure for 15 min. stored in glass bottle at 4°C.

5. **NUTRIENT AGAR :**

   Nutrient Broth (Hi Media, India)  8 g  
   Sodium chloride (NaCl) (Qualigens, India)  5 g  
   Difco bacto agar (Difco, U. S. A)  15 g  
   Distilled water  1000 ml  

The ingredients were mixed in distilled water and autoclaved at 15 lbs pressure for 15 minutes. This mixture was used for testing viability of bacteria.

A. **LIVER HOMOGENATE (S9 Fraction):**

1) **SALT SOLUTION :**

   Potassium Chloride (KCl) (Sarabhai M. chemi, India)  61.5 g  
   Magnesium Chloride (MgCl2 .6H2O) (Loba, India)  40.79 g
Distilled water 500 ml

The ingredients were mixed in distilled water and the solution was autoclaved at 15 lbs pressure for 2 minutes. Store it in glass bottles in refrigerator.

2) **0.2 M SODIUM PHOPHATE BUFFER: (pH 7.4)**
   a) 0.2 M sodium dihydrogen phosphate (Lobia, India)
      \[
      \text{(NaH}_2\text{PO}_4\text{.H}_2\text{O)} \quad 2.76 \text{ g} \\
      \text{Distilled Water} \quad 100 \text{ ml}
      \]
   b) 0.2 M Disodium hydrogen phosphate (Loba, India)
      \[
      \text{(Na}_2\text{HPO}_4) \quad 2.84 \text{ g} \\
      \text{Distilled Water} \quad 100 \text{ ml}
      \]

60 ml of solution (a) and 40 ml of solution (b) were compounded. The pH was adjusted to 7.4 and the mixture was autoclaved at 15 lbs pressure for 20 minutes.

3) **0.1 M NADP SOLUTION (Nicotine adenine dinucleotide phosphate)**
   NADP (Sigma, U. S. A.) 383 mg

Sterile distilled water was added to pre weighed NADP. This solution was stored at –20°C.

4) **1M GLUCOSE - 6 – PHOSPHATE ( G - 6 – P ) :-**
   G - 6 – P (Sigma, U. S. A.) 2.82 g

   Sterile distilled water 10 ml

This solution was sterilized by passing through 0.22 µm millipore filter and was stored at –20°C.

5) **S9 mix :-**

Using above ingredients the S9 mix was prepared as follows:-
   Rat liver S9 5 ml
   MgCl₂ KCl salt 1 ml
   1M G-6-P 0.25 ml
0.1 M NADP  
0.2 M sodium phosphate buffer  
Sterile distilled water 

The above solution was prepared fresh prior to each experiment and kept on ice. All ingredients were chilled. The S9 mix filtered through 0.45 µm millipore filter. Any leftover of S9 mix was discarded after the experiment.

B. **CHECKING OF THE STRAINS** :-

i. **Test for ampicillia resistance** –
   Master plates for R- factor strain
   Ampicillin solution (8 mg / ml)
   Ampicillin trihydrate  
   Sodium hydroxide (0.02 N) (NaOH) 

   Store in glass bottle at 4°C.

ii. **Test for crystal violet sensitivity** :-
   To confirm rfa mutation
   Crystal violet solution (0.1 %)
   Crystal violet  
   Distilled water 

   Store at 4°C. Wrap the bottle with aluminium foil to protect against light.

iii. **Test for histidine requirment** :-
   Master plates for non – R factor strains
   Histidine / Biotin plates.
   Agar  
   Distilled water 
   50 X VB salt 
   Glucose (40%)  
   Sterile histidine – HCl H₂O (2 g/ 400ml H₂O) 
   Sterile biotin (0.5 mm) 

   15 g  
   914 ml  
   20 ml  
   50 ml  
   10 ml  
   6 ml
In autoclaved agar + water, sterile 40% glucose + 50X VB salts and histidine is added to hot agar solution. After slight cooling, sterile biotin is added to above mixture and plates were prepared.

iv. Test for ampicillin/tetracycline resistance:-
Mater plates for strains carrying the plasmid pKM 101 and pKM 101+PAQ1.

Ampicillin plates and tetracycline plates:-

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>910 ml</td>
</tr>
<tr>
<td>50X VB salt</td>
<td>20 ml</td>
</tr>
<tr>
<td>Glucose (40%)</td>
<td>50 ml</td>
</tr>
<tr>
<td>Sterile histidine, HCl, H₂O (2 g / 400 ml of DH₂O)</td>
<td>10 ml</td>
</tr>
<tr>
<td>Sterile biotine (0.5 mm)</td>
<td>6 ml</td>
</tr>
<tr>
<td>Sterile ampicillin solution (8 mg/ml of 0.02 N NaOH)</td>
<td>3.15 ml</td>
</tr>
<tr>
<td>Sterile tetracycline solution (8 mg/ml 0.02 N HCl)</td>
<td>0.25 ml</td>
</tr>
</tbody>
</table>

In the autoclaved agar + water + glucose + 50X VB salts + histidine is added. After mixing cool to 50°C & add sterile biotin and ampicillin aseptically. Tetracycline was added only for use with TA102 which is tetracycline resistant.