Cultivation of *Leptospira kirschneri* serovar grippotyphosa, strain RM52 And *L. interrogans* serovar copenhageni, strain L1-130

Important Warning: Leptospiral cultures are a well-documented laboratory hazard and must be handled in a laminar flow biological safety cabinet using standard microbiological safety practices, including a laboratory coat, gloves, mask, eye protection, and appropriate disposal of contaminated material. Ingestion, accidental parenteral inoculation, and direct and indirect contact of skin or mucous membranes are the primary routes of exposures.

1) Preparation of Supplement

1 Add 100 g Bovine Albumin Fraction V slowly to 500 ml of distilled water. Stir slowly to avoid the formation of foam. Alternatively, add the BSA to water and hold at 4°C overnight. Stir slowly while solution is warming to room temperature.

[Notes: Leptospiral growth and morphology are affected by the quality of Bovine Serum Albumin, we are currently using Intergen Cat #81-003-3 (lot 612) and Millipore Cat #84-065-3 ("Probumin") to prepare media for strains RM52 and L1-130, respectively. BSA should be stored in a dessicator at 4° after opening the bottle. Triple glass distilled water or GIBCO Distilled Water (cat# 15230) can be used. In either case, the water must be autoclaved prior to use.]

2 Prepare the following stock solutions:

Zinc sulphate ($ZnSO_4.7H_2O$)	0.4 gm/100 ml d water
Calcium chloride ($CaCl_2.2H_2O$)	1.0 gm/100 ml d water
Magnesium chloride ($MgCl_2.6H_2O$)	1.0 gm/100 ml d water
Thiamine chloride	0.5 gm/100 ml d water
Vitamin B_{12}	0.02 gm/100 ml d water
Manganous sulphate ($MnSO_4.4H_2O$)	0.3 gm/100 ml d water

Filter each of these stock solutions and store at 4°C until required. Best if prepared freshly, but may be stored for up to one month.

3 Prepare and filter the following solutions the day supplement is prepared:

Ferrous sulphate ($FeSO_4.7H_2O$)	0.5 gm/100 ml d water
Tween 80	20 ml/180 ml d water
Tween 40	20 ml/180 ml d water

4 When the BSA is fully dissolved, add stock solutions slowly, in order, while stirring. Continue to stir for one hour.

Thiamine stock	10 ml
$CaCl_2$	10 ml
$MgCl_2$	10 ml
$ZnSO_4$	10 ml
$MnSO_4$	1 ml
$FeSO_4$	100 ml
Vitamin B_{12}	10 ml
Tween 80 (for EMJH)	125 ml

5 Adjust pH to 7.4 with 10% NaOH (filtered).

6 Adjust volume to one liter.

7 Filter (.22 μm) and dispense aseptically in 100 ml aliquots. Store at -20^{o} C

2) Preparation of Basal Medium (we autoclave the basal media after it has been pH'ed)

8 In a 2L Beaker with a stir bar, add:

Distilled water	998 ml
Disodium phosphate (Na_2HPO_4)	1.0 gm
Monopotassium phosphate (KH_2PO_4)	0.3 gm
Sodium Chloride (NaCl)	1.0 gm

9 Add the following filtered stock solutions:

Glycerol (10% v/v)	1.0 ml
Ammonium Chloride (25% solution)	1.0 ml

10 Stir ingredients until fully dissolved.

11 Adjust pH to 7.4 using 10% NaOH (filtered).

3) Preparation of Complete Medium

Prior to preparation of the complete medium, rabbit serum must be collected from a MAT negative rabbit. The blood should be collected in a separation tube, placed at 37°C for 30 minutes and centrifuged @ >3000 x g for 30 minutes. Heat inactivate the serum at 56°C for 30 minutes and filter (.22 μm). If possible, serum should be collected the same day the medium is prepared. If not possible, serum can be stored at -20°C and used at a later date.

12 To 100 ml of supplement, add:

Lactalbumin hydrolysate	1 g
Superoxide dismutase	0.001 g (add 100 μl of a 10 mg/ ml solution in PBS)
Sodium pyruvate	0.04 g

13 Add normal rabbit serum and 5-fluorouracil as desired (see chart below). Filter (.22 μm) this mixture into previously sterilized basal medium.

14 Dispense aseptically in 7 ml aliquots.

Media	Basal	Supplement	Rabbit serum	5-FU
EMJH+	890 ml	100 ml T80	10 ml	0
EMJH++	880 ml	100 ml T80	10 ml	10 ml

5-Fluorouracil Stock Solution

Dissolve 1 gm of 5-Fluorouracil in 90 ml of filtered basal media (without agar) by swirling in an Erlenmeyer flask at 37°C. Adjust pH to 7.4 using 2N NaOH (filtered) and add sterile Basal Medium to a final volume of 100 ml. Filter (.22 μm) and store at 4°C.

Semisolid or Solid Leptospiral Media

Add 2.0 gram (12.0 grams for plates) of Bacto agar per liter when preparing Basal Medium and autoclave to dissolve and sterilize agar. When the agar-containing Basal Medium has cooled to 55°C, add other media components (warmed to room temperature) and mix well. Dispense 10 ml into 15 ml blue-cap conical Falcon tubes and store at 4°C.

Storage in Liquid Nitrogen is performed in essentially the same way as for tissue culture cell lines. Add 1 volume of 4% sterile glycerol mixed in EMJH to a liquid culture in cryovials. Cool slowly to -80°C then place in liquid nitrogen.