1. **<u>What growth medium and culture reagents are used to culture mesothelial cell lines?</u>**
   Medium is 15% FBS in M199:MCDB110 (1:1) with 10 ng/ml EGF plus 400 ng/ml hydrocortisone. If MCDB 110 is unavailable, Ham's F12 may be substituted.
   a) MCDB 110 (Sigma #M6520-10L or equivalent)
   b) Ham's Nutrient Mixture F12 (HF12; Invitrogen # 11765-062 or equivalent)
   c) Medium 199 (M199; Invitrogen # 11150-059 or equivalent)
   d) Fetal Bovine Serum (FBS; Sigma-Aldrich # F2442 or equivalent)
   e) Epidermal Growth Factor (EGF; Peprotech #100-15 or equivalent)
   f) Hydrocortisone (Sigma #H4001 or #H0888 or equivalent) see #2
   g) Gelatin (Sigma #G2650 or equivalent); Gelatin solution, 1% in 0.9% NaCl, sterilized by autoclaving
   h) Versene 1:5000 (0.2 g/L EDTA•4Na in PBS, Invitrogen #15040-066 or equivalent)
   i) 0.05% trypsin-0.02% EDTA (0.05% Trypsin – 0.53 mM EDTA•4Na in Ca^{2+}- Mg^{2+}- Free Dulbecco's PBS or CMF-Hank's Balanced Salt Solution; Invitrogen #25300-054 or equivalent)
   j) Bovine serum albumin (BSA; Sigma #A4503 or equivalent)

2. **<u>How should the hydrocortisone stock be preparred?</u>**
   1. Dissolve 40 mg hydrocortisone in 10 ml 95% ethanol (*non*-denatured) and filter sterilize using 0.2 µm pore *nylon* syringe filter. Store at -70°C or below.
   2. To make 1000X working stock, dilute 1:10 in sterile calcium and magnesium-free D-PBS.
   3. Dispense 0.5 ml aliquots and store at at -70°C or below.

3. **<u>How should the EGF stock be preparred?</u>**
   1. Dissolve 100 µg recombinant human EGF (Peprotech rhEGF #100-15 or equivalent) in 10ml 0.2% BSA in D-PBS + 10mM acetic acid.
   2. Filter sterilize using 0.2 µm pore syringe filter.
   3. Dispense 0.5 ml aliquots and store at -70°C or below.

4. **<u>How is a mesothelial cell line subcultured?</u>**
   Volumes are for 25-cm^2 flask or 60-mm dish.
   1. Coat a flask with gelatin by adding 3-5 ml sterile gelatin solution to a T25 and incubate for 30 minutes. Flasks may be stored at 4°C. Aspirate gelatin solution immediately before use.
   2. Aspirate the medium from the stock culture and rinse with 3 ml Versene.
   3. Add 1ml 0.05% trypsin-0.02% EDTA.
   4. Incubate the flask at 37°C until cells round up (1-5 minutes).
   5. Add 5ml of growth medium to the flask and dislodge any undetached cells by gently pipetting the medium against the bottom surface of the flask.
   6. Triturate gently to make a single-cell suspension and transfer suspension to a 15ml centrifuge tube.
   7. Remove a 0.5 ml aliquot to count the cells.
   8. Centrifuge at 100 x g for 5 – 10 minutes.

9. Aspirate supernatant, flick tube to break up pellet, and re-suspend cells in growth medium.
10. For stocks, generally plate 25cm^2 or 75 cm^2 flasks with 1e4 – 1e5 cells/cm^2.

5. <u>What is the freezing medium used to cryopreserve mesothelial cell lines?</u>

Growth medium + 15% FBS + 5% DMSO

6. <u>How should mesothelial cell lines be cryopreserved and stored?</u>

1. Place cells into a single-cell suspension, count and pellet as indicated in the subculture protocol above.
2. Resuspend the cells in freezing medium to a seeding density of 5.0e5 viable cells per ml
3. Aliquot 1 ml into each cryovial or ampule.
4. Cells resuspended in freezing medium should be immediately placed in a controlled rate freeze machine that reduces temperature at a controlled rate of -1°C/min. Alternatively, cryovials can be placed in an ethanol bath at -80°C overnight before being placed in liquid nitrogen vapor.
5. Frozen cell stocks are stored in liquid nitrogen tanks. Glass ampules are submerged in liquid, plastic cryovials are stored in vapor phase.

*Suppliers of reagents are listed for the convenience of culture recipients only. Such lists are not intended to be either selective or exhaustive, and Coriell Institute does not recommend specific products or suppliers. Other media and reagents may be satisfactory, but have not been tested.