ES CELL CULTURE

Gelatin Plates:

1. Make up a stock of 0.1% gelatin in water and autoclave.
2. Mix up solution, then put 3 ml on 10 cm plate
3. Let sit in hood for 20 min.
4. Aspirate, let plates dry 20 min.
5. Store in saran wrap or sleeve at 4°C. Stable for 1 mo.

Medium

1. For 1liter:
   - 780 ml DMEM
     - 7 ul BME TC grade
   - 200 ml FCS Lot#
     - 10 ml 100x glutamine
     - 10 ml 100x EAA
     - 10 ml LIF conditioned medium
   - Filter sterilize through 0.2 um filter
2. G418:
   - 150 ug/ml (active) final conc.
   - 15 mg/ml 100x stock in DMEM, pH with 1 N NaOH till color is right, then filter sterilize.
3. HygroB:
   - 200 ug/ml (active) final conc.
   - 20 mg/ml 100x stock in DMEM, as above.
   - Add drugs to ES medium prior to filtering, then refilter.

Growing ES Cells

1. Plate at $5x10^6$ cells/10 cm dish. Usually ready to pass in 3 days. Cells will grow in clumped colonies. Density at 3 days should be $5x10^7$ cells.
2. Feed cells 2 hrs prior to passing.
3. Wash plate with 10 ml sterile PBS.
4. Add 3 ml trypsin/EDTA, then remove 1 ml
5. Place in incubator for 4 min.
6. Take 2 ml pipette and break up cells.
7. Place back in incubator for 4 min.
8. Add 8 ml medium and breakup cells futher, 3–4x.
9. Split 1:10 into new plates.
10. Feed cells every day.

Electroporation

1. Need $2x10^7$ cells/cuvette i.e., 1 10 cm dish = 2 cuvettes.
2. Harvest the cells as usual, count and resuspend in electroporation medium at $2x10^7$ cells in 0.8 ml.
3. Transfer 0.8 ml cells to sterile cuvettes.
4. Add linearized DNA, 20–25 ug, to cells in cuvette.
   - a. Cut DNA with restriction enzyme as usual
   - b. Phenol/chloroform, chloroform and etoh ppt.
   - c. Wash with sterile 70% etoh in hood.
   - d. Resuspend in sterile TE at 2 mg/ml (10 ul).

5. Electroporate:
    a. Disconnect pulse controller, by plugging the cuvette holder directly into the electroporater.
    b. 0.45 Kv
    c. 25 uF
    d. Time = .4 msec
6. Mix cells and plate
    a. 5 ul cells into 1 ml ES medium and then plate 5 ul of that onto 6 cm plate on gelatin, about 500–600 cells. This will be to test for viability.
    b. Plate 2 dilutions of cells, aim for 500–600 cells, on 10 cm plates in duplicate in presence of HygroB.
    c. Electroporation samples:
        i. Control cells, no DNA, no electroporation. This gives indication of cell death in presence of selection.
        ii. Control cells, pGKneo/Hygro, vector positive control plated in selection medium allows scoring of efficiency of selection
        iii. Recombinant cells, targetting vector selects the recombinants of interest.
    d. Cells need to be fed every day.
    e. By day 10 most control cells (i) will be dead, and recombinant colonies should be forming.
    f. By day 14 should see nice isolated colonies.

Screeining of Colonies

1. Under scope mark colonies to be collected.
2. Wash plates 1x with 10 ml PBS.
3. Put 10 ml more PBS in plate.
4. With tip on p200, set at 100 ul, tap top of colony and suck up into tip.
5. Transfer to a well in 96 well plates containing 100 ul trypsin/EDTA.
6. Trypsinize for 9 min 37°C.
7. Put 100 ul (1/2) into 1 ml PBS in microfuge tube.
8. Put 100 ul into 500 ul 90%FCS/10%DMSO and freeze –80°.
9. Spin microfuge tube 3.5 K for 5 min in microfuge.
10. Aspirate all but 5-10 ul liquid with thin tip.
11. Add 10 ul 1x lysis buffer and 5 ul 20 mg/ml proteinase K.
12. Tap to mix, incubate 30 min to O/N at 55°C.
13. Boil 5 min, put on ice, spin. Use 5 ul/PCR reaction.

2x Lysis Buffer

8.6 ml water
1.0 ml 1M KCl
30 ul 1M MgCl
200 ul 1M Tris, pH8.3
90 ul NP40
90 ul Tween20
Must be sterile for PCR.

Electroporation Medium

DMEM
Hepes
glucose
BME