II. Electroporation and selection of ES cells

1. Prepare one T25 and two T75 flasks of irradiated primary embryo fibroblasts (γ-EF), 1-2 days before needed. Thaw the cells onto gelatinized plates (coat plates with autoclaved 0.2% gelatin for 5 minutes immediately prior to the addition of cells).

2. On a Friday, thaw one vial of frozen ES cells and add to the T-25 flask with γ-EF cells. The cells are thawed rapidly at 37°C, added to 9 ml of ES media, and pelleted at 1,000 rpm for 5 minutes. After aspirating the supernatant, the cell pellet is resuspended in an appropriate volume of ES media for the T25 flask.

ES media:

DMEM, high glucose
+ 0.1 mM non-essential amino acids (100x stock from GIBCO)
+ 1 mM sodium pyruvate (100x stock from GIBCO)
+ 10 μM ß-mercaptoethanol (100x stock stored at -20°C)
+ 15% heat-inactivated fetal calf serum (HyClone)
+ penicillin/streptomycin (final concentration of 50 μg/ml of each)
+ 1000 U LIF/ml (LIF is from GIBCO)
+ 4 mM L-glutamine

***Once ES cells are plated, the media should be changed every day!!**

3. Two days later, the cells should be ready to split into the two T75 flasks with γ-EF cells. Trypsinize the T25 flask using 5 ml of trypsin/EDTA (GIBCO). After the cells detach from the flask, pipette to dissociate clumps, and then add 5 ml of media (with serum). Pellet the cells at 1,000 rpm for 5 minutes, and resuspend the cell pellet in an appropriate volume of ES media for the two T75 flasks.

4. The day before electroporation, plate γ-EF cells into ten 100 mm tissue culture plates which have been coated with gelatin (coat plates with autoclaved 0.2% gelatin for 5 minutes immediately prior to the addition of cells). Use two T25 vials of frozen cells per 100 mm dish - 20 vials total (surface area of 100 mm dish is 55 cm^2). These plates will be used for the ES cells after electroporation.

5. After approximately another 2 days (should be around Tuesday or Wednesday), the cells will be ready for electroporation. Wash one T75 flask of ES cells with Dulbecco's PBS (GIBCO). Add 10 ml of trypsin/EDTA and leave for 2-3 minutes. Pipette well to disperse cells, then add 10 ml of ES media. Pellet cells at 1,000 rpm for 5 minutes. Resuspend cell pellet in 10 ml ES media. Make a 1:10 dilution (0.2 ml cell suspension + 1.8 ml ES media), place a drop in a hemacytometer, and count ES (small) cells in the center area. Multiply this number by 10^6 to obtain the total number of cells in the 10 ml cell suspension. For example, if there are 50 cells in the center area, then there are
5×10^7 cells/10 ml, or 5×10^6 cells/ml. Pellet cells again and resuspend to 2×10^7 cells/800 μl of electroporation buffer (see recipe for electroporation buffer below). Combine 800 μl of this cell suspension with 50 μg of DNA (1 μg/μl - linearized for replacement vectors) in a sterile tube.

Mix, then transfer to a sterile electroporation cuvette (eg., Bio-Rad gene pulser cuvette, cat # 165-2088, 0.4 cm electrode gap). Electroporate at 400 V, 25 µF (time constant 0.4). Let cells sit for 10 minutes, then add to 100 ml ES media, and distribute 10 ml of this cell suspension to each 100 mm dish.

The remaining ES cells from the trypsinized plate, as well as the ES cells on the T75 which wasn't used, can be frozen (prepare 16 freezing vials from the equivalent of one T75 flask of ES cells).

6. 24 h after electroporation, change the media to ES media supplemented with 350 µg/ml G418 (and any other antibiotics/compounds used for positive/negative selections).

7. Change media every day, continuing with the same selection process.

8. Colonies should be visible and ready to start isolating at around 6-7 days after electroporation.

Electroporation buffer:

20	mM HEPES (pH 7.0)
137	mM NaCl
5	mM Kcl
0.7	mM Na_2HPO_4
6	mM glucose
0.1	mM ß-mercaptoethanol

This buffer can be prepared from the following six 10x stocks:

1. 200 mM HEPES, pH 7.0 (10x)
   FW = 238.3
   0.2 M (200 mM) = 47.66 g/L

   For 50 ml, use 2.383 g HEPES - dissolve solid in 40 ml dH_2O, adjust pH to 7.0 with 10N NaOH and then adjust volume to 50 ml with dH_2O.

2. 1370 mM NaCl (10x)
   MW = 58.44
   1370 mM = 1.37 M = 80.06 g/L

   For 50 ml, dissolve 4.00 g NaCl in 50 ml dH_2O.

3. 50 mM KCl (10x)
   MW = 74.55
   50 mM = 0.05 M = 3.7275 g/L

   For 50 ml, dissolve 0.1864 g KCl in 50 ml dH_2O.

4. 7 mM Na_2HPO_4 (10x)
   FW = 141.96

0.007M = 0.99372 g/L

To avoid weighing errors, prepare 1 L.

5. 60 mM glucose (10x)
   MW = 180.2
   0.06M = 10.812 g/L

   For 50 ml, dissolve 0.5406 g glucose into 50 ml dH_2O. This solution should be filter sterilized to prevent bacterial growth.

6. 1 mM ß-mercaptoethanol
   MW = 78.13
   density = 1.1143 g/ml
   1114.3 g/L = 14.3 M stock

   For 50 ml, add 3.5 µl of stock ß-mercaptoethanol to 50 ml dH_2O.

To prepare electroporation buffer from stocks, add 5 ml of each 10x stock to 20 ml dH_2O. Filter in tissue culture hood, and store in sterile 50 ml tube.