III. Isolation, growth and analysis of ES clones

1. After electroporation, ES colonies will be ready to pick approximately 6-7 days after start of selection. By carefully monitoring the selected colonies every day, it will be possible to determine when the colonies are ready based on morphology and size.

One day before colonies are to be picked, prepare 96 and 24 well plates by coating with sterile 0.2% gelatin (leave the gelatin in the wells until just before the feeders are added). The 96 well plates will be used to prepare master plates, while the 24 well plates are used for DNA preparation. For the 96 well plates, the total growth area of a plate is 30.72 cm^2 (0.32 cm^2/well). Calculate the total area for all of the 96 well plates needed to determine how many vials of feeder cells to thaw. For example, for four 96 well plates, the total area is 122.88 cm^2, and five T25 vials of frozen, irradiated primary feeder fibroblasts would be sufficient. The feeders are plated using 150 &l/well. (For the example above, assume 100 wells at 0.15 ml/well, or 15 ml per plate. The thawed, pelleted fibroblasts would be resuspended in 60 ml ES media with G418 for preparation of four 96 well plates).

For preparation of DNA, most ES cells can be grown on gelatin-coated 24 well plates without feeders. Experience has shown, however, that R1 ES cells do not grow well at low density (which is the case after colony isolation) in the absence of feeder cells. Thus, the 24 well plates should have feeder cells (at lower than normal density). Although the feeder cells will contribute some DNA which may dilute the signal of the recombined allele, this is more than compensated for by the enhanced growth of the ES cells. Final characterization of positive clones will be performed with a large-scale preparation of ES cells grown in the absence of feeder cells.

To prepare the 24 well plates for DNA analysis, the plates are coated with gelatin, and fibroblasts are added to approximately 50% confluency. The growth area of a 24 well plate is 45.6 cm^2 (1.9 cm^2/well). Four 24 well plates will be needed for every 96 well master plate prepared. In the example given above for 4 master plates, 16 24 well plates will be needed. The total growth area of feeder cells is 16 plates x 45.6 cm^2/plate x 0.50 (50% confluency) = 365 cm^2. Thus, sixteen T25 vials will be sufficient for all sixteen 24 well plates. The 24 well plates will have 1 ml per well. (For the example above, each vial of thawed, pelleted fibroblasts would be added to 25 ml of ES media with G418 for each 24 well plate).

2. Each day that colonies will be picked, the media should be changed two hours before colony isolation. To isolate colonies, the plates are processed one at a time as follows. A plate is rinsed once with PBS, and a second change of PBS is then added and left on the cells. Colonies are isolated using a pipetteman with sterile pipette tips. It may be necessary to dislodge the colonies from the feeder layer by scraping around the colony with the pipette tip (pick only colonies which are rounded- do not pick flattened, differentiated colonies). Isolated colonies are added to 96 well v-bottom plates containing 20 µl of trypsin. Eight colonies are picked and processed together. After the eighth colony is picked, the cells are processed by pipetting up and down (20x) with a multi-channel pipette gun to dissociate cells. Add 75 µl of ES media to each well, and pipette up and down 5x to mix. Remove 35 µl to each well of a 96 well master plate which was prepared previously (the master plate has 150 µl of media before addition of the trypsinized cells from each colony). Remove remaining cell suspension to previously prepared 24 well plates.

Note: It works best if one person picks colonies, and a second person processes them.

3. The 96 well master and 24 well DNA plates are subsequently handled as described below:

a). 96 well master plates.

The media is changed on the master plates every day. The media is still supplemented with G418. (To change media, use a multi-channel pipette gun to remove old media, and add new media). Monitor the plates every day. They should be ready to freeze several days after addition of dissociated cells from individual colonies. It is usually quite obvious when they are ready to freeze, based upon colony size and density, as well as the color of the media. When they are ready to freeze, numerous wells will be starting to turn orange-yellow in color.

To freeze, change media, and wait two hours. Remove media, and rinse each well with 150 &l of PBS. Remove media, and add 30 µl of trypsin to each well. Return to 37°C incubator for two minutes, then add 170 µl of freezing media (10% DMSO) to each well and mix well by pipetting up and down 5x. (Freezing media - for each 96 well plate, mix 18.75 ml ES media with 2.5 ml DMSO). Wrap the plate edges with parafilm, seal in a plastic bag (such as a hybridization bag), and store flat in a -80°C freezer.

b). 24 well DNA plates.

The media is not changed on the 24 well plates (differentiation does not matter). Monitor the growth of the ES cells to be sure that everything is O.K., and to check for contamination (inevitably, there will be some colonies which develop bacterial or yeast infections). If there are only a few samples which become infected, they can be processed either by aspiration of media and treatment of the well with 70% ethanol to prevent spread of infection, or by careful rinsing with PBS, followed by addition of media supplemented with fungizone (for yeast infection) or another broad-scale antibiotic (ie., gentamycin) for bacterial infections.

When the media in a well becomes exhausted and turns yellow, the cells are processed for DNA. The media is aspirated, and 0.5 ml of DNA isolation buffer is added to each well.

DNA isolation buffer
100 mM Tris, pH 8.5
200 mM NaCl
0.2% SDS
5 mM EDTA
100 µg/ml proteinase K

After addition of DNA isolation buffer, the plates are returned to a CO_2 incubator at 37°C (there will still be clones which have not reached confluency). The samples being lysed are left for at least overnight at 37°C, although they can be left for several days before the processing is completed.

The lysed samples are removed to microcentrifuge tubes. The DNA is precipitated by the addition of 0.5 ml of isopropanol. The DNA is then pelleted by centrifugation for 3-5 minutes. The DNA pellet is then washed once with 100 µl of 70% ethanol, and pelleted by centrifugation

for 3-5 minutes. After removal of the ethanol supernatant, the pellet is resuspended in 50 µl TE. The trick to properly resuspending the samples is to leave them at 55°C overnight. Generally, 10 µl of each sample is sufficient for digestion and Southern analysis (use BSA in digests). 50 µl digests can be easily set up in 96 well v-bottom plates.

4. Once positive clones are identified by PCR or Southerns, the corresponding clones on the frozen master plates are thawed and expanded. The day before thawing a master plate, a 6 well plate is prepared by coating with gelatin, and the addition of irradiated feeder fibroblasts. The growth area of a 6 well plate is 57 cm^2 (9.5 cm^2/well). Thaw two T25 vials of feeders per 6 well plate.

To recover viable positive clones, a 96 well master plate is thawed by holding the plate on the surface in a 37°C water bath. This is best done before the plate is removed from the sealed bag, to minimize the chance of contamination from the water bath. Once the cells are thawed, remove the appropriate positive clones and add to 5 ml ES media. Pellet cells to remove DMSO, and resuspend in 5 ml ES media with G418. Aspirate media from 6 well plates, and add positive clones to feeder layers.

Change the media on the 6 well plates every day, and carefully monitor the growth of the cells by microscopic observation. When the colonies are a good size, and not overgrown, rinse with PBS and trypsinize with 5 ml trypsin. After dissociation, add to 5 ml ES media, pellet cells and resuspend in sufficient ES media (with G418) for a T75 flask (which has been prepared 1-2 days earlier by gelatin-coating and the addition of a feeder layer). After addition to the feeder layer in the T75 flask, the cells are grown to confluency (difficult to write down just what the cells look like at this stage, but they have proper morphology, and the colonies are not too large). At this point the cells are trypsinized and prepared for freezing. 9 aliquots are frozen down (each one representing one-tenth of the total number of cells from the T75), while the remaining one-tenth is added to a new T75 (gelatin-coated, but no feeders) for preparation of DNA.

The T75 flask for DNA is grown until the media is exhausted (turns yellow). DNA is then prepared as follows:

Large-scale purification of ES clone DNA

- Wash cells 2x in PBS.

- Add 10 ml of DNA isolation buffer (pre-heated to 37^{o}C).

DNA isolation buffer
20 mM Tris, pH 8.0
5 mM EDTA
0.1 M NaCl
0.5% SDS

- Add 200 µg/ml proteinase K, and incubate at 37^{o}C for 3 h.

- Extract 1x with phenol:chloroform (1:1).

- Chloroform extract 2x.

- Incubate with 40 µg/ml RNAse A (heat-inactivated) at 37^{O}C overnight.

- Extract 1x with phenol:chloroform (1:1).

- Chloroform extract 1x.

- Add NaCl to 0.2 M final (since isolation buffer contained 0.1 M NaCl, add additional NaCl to 0.1 M).

- Precipitate the DNA with the addition of 2.5 volumes of ethanol.

- Spool out the DNA, and resuspend in TE. Measure A_{260} and calculate concentration.

5. Once DNA has been prepared from the thawed clone, verify the genotype of this clone (by PCR or Southern) before using for blastocyst injection.