Picking ES Cell Colonies and Analysis of ES Cell Clones via Mini-Southern

Initial Cloning

1. Aspirate selective media off the plates containing colonies of interest. Wash the plates twice with PBS. After aspirating the second wash, add enough PBS to just cover the surface of the dish.

2. Prepare the 96-well U-bottom cloning dish by pipetting 25 μl of trypsin into each well using a multichannel pipettor. Up to two full plates of clones can be picked at one sitting without problems of the trypsin drying.

3. Pick colonies from the washed plates and transfer them into the trypsin solution, one colony per well. Proceed until colonies have been cloned into each of the 96 wells. After you have completed one plate, place the plate into the incubator for 10 minutes to ensure complete trypsinization of the colonies. Proceed with step 4 -in other words, do one plate at a time.

4. Using the multichannel pipettor, add 25 μl of M15 media per well. Pipette up-and-down multiple times to disaggregate the cells.

5. Transfer the clones to an appropriately labelled 96-well feeder plate, having first removed the STO media from the 96-well plates and replacing it with 100 μl M15 media. Allow the colonies to grow under selection. The cells will take about 3-5 days to grow to such a confluence as to turn the media yellow at one-day intervals.

Duplicate Preparation

1. Once the clones reach near-confluence, trypsinize, duplicate, and freeze the cells [refer to the protocol "Freezing Embryonic Stem (ES) Cell Clones in 96-Well Plates"].

2. Allow the cells to grow on the gelatinized 96-well duplicate plates. Refeed the plates with fresh M15 media as the old media turns yellow until the cells are ready for use.

Mini-Southern Analysis

1. When the cells are ready, wash the plates twice with PBS and aspirate. Using the multichannel pipettor, add 50 μl of Lysis Buffer [10 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 10 mM NaCl, 0.5% Sarcosyl, and 1 mg/ml Proteinase K (added fresh)] per well. *Note: From this point on, all manipulations are to be performed **outside** the tissue culture facilities.*

2. Incubate the plates overnight @ 60° C in a humidified chamber (such as a plastic container with a wet sponge on the inside, or a seal-a-meal bag with a damp paper towel).

3. Next day, prepare a fresh solution of 75 mM NaCl in ethanol (add 150 μl of 5 M NaCl per 10 ml of cold absolute ethanol and mix well; the salt will precipitate, but this is of little consequence).

4. Using the multichannel pipettor, add 100 μl of the NaCl/ethanol solution per well. Allow the plate to rest on the bench @ room temperature for 15-30 minutes, or until the precipitated DNA is clearly visible under low-power magnification. The DNA adheres to the plastic, so look at the perimeter of each well to see the precipitated DNA.

5. Invert the plate to discard the solution (the DNA will remain adhered to the plate). Using the multichannel pipettor, add 70% ethanol to wash each well. Alternatively, a squirt bottle may be used, but a strong stream could detach the DNA from the plate. Invert the plate to discard the 70% ethanol and repeat the wash 2 times.

6. After the final wash, invert the plate, discard the 70% ethanol, and allow the plate to air-dry a few minutes. At this point, the dried plates may be frozen at -80 º C. Allow the plates to thaw and resuspend the DNA in the Restriction Enzyme Cocktail (see below).

7. While the plate is drying, prepare the Restriction Enzyme Cocktail (1X Restriction Buffer specified for the enzyme being used, 1 mM Spermidine, 100 μg/ml Bovine Serum Albumin, and 10-15 units of enzyme).

8. Using the multichannel pipettor, add 30 μl of Restriction Enzyme Cocktail to each well and mix by pipetting up-and-down (especially if the DNA had been frozen down). **Change tips between one row and the next.**

9. Once the cocktail has been added to all the wells, incubate the plates overnight @ **37º C** (assuming that this is the temperature favored by the restriction endonuclease) in a humidified chamber [place a wet paper towel along with the taped plate in a seal-a-meal bag].

10. Next day, prepare the agarose gel(s) for electrophoresis. Prepare a large gel tray (15 cm x 24 cm) with three 33-teeth combs (add 3 extra teeth per comb, secured with tape) evenly distributed along the length of the tray. Pour 300 ml of molten agarose into the tray; remove any bubbles with a needle. Allow the gel to solidify for about 30 minutes. This size of gel (3 lanes with 99 wells total) will accommodate one 96-well mini-Southern digest plate at 32 samples per lane plus one well per lane for molecular weight markers.

11. Remove the 96-well mini-Southern digest plate from the incubator and add 4-5 μl of loading buffer to each well. Load the gel (30-35 μl per well) and run @ 80 V for approximately 4-6 hours. When deciding how far to allow the samples to migrate, take into account the size of the fragment(s) being distinguished. The gel can be run further as long as the bands from one lane of samples being detected with a probe do not overlap the bands from the next lane of samples.

12. After the electrophoresis is complete, transfer the DNA to GeneScreen *Plus* membranes according to the usual protocol [refer to the protocol "Genomic DNA: Restriction Enzyme Digests, Agarose Gel Electrophoresis, and Southern Transfer (Blotting)"].

NOTES: *This protocol may not work efficiently for all restriction enzymes. Therefore, make a pilot experiment to test the desired enzyme(s) before proceeding to the large experiment.*