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IDENTIFICATION OF TARGETED CLONES BY MINI-SOUTHERN ANALYSIS

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When the majority of clones in the 96-well duplicate plates are fully confluent (6-7 days of culturing), the cells should be lysed and genomic DNA isolated for identification of targeted clones by hybridization analysis. The following procedure allows for the cell lysis, DNA precipitation, and enzymatic restriction to all be performed in the same 96-well dish in which the cells were grown. Both duplicate plates prepared from each Master plate should be taken through Step 1 of the protocol. However, at that point, only one replicate plate should be processed through the remaining steps, leaving the second plate in the freezer as a back-up for subsequent analysis, if necessary.

1. Aspirate the medium from the 96-well duplicate plates and wash each plate twice with 100 uL PBS. **It is essential to aspirate PBS completely or the DNA pellet is unlikely remain adhered to the plate after precipitation.** After aspirating the second wash, secure the lid to each plate using sealing tape (Wallac Catalog # 1450-461) and place the plates at -70°C for at least 3 hrs before moving to step 2. Freezing the dishes "cracks the cells, aiding the lysis and subsequent proteinase digestion, and assures complete digestion of the isolated DNA by the restriction endonuclease(s) used the analysis.

2. Remove only one copy of each duplicate plate from the freezer and allow it to warm to room temperature for 5 minutes. Using a multichannel pippettor, add 50 uL of mini-Southern Lysis Buffer (10 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 10 mM NaCl, 0.5% Sarcosyl or 0.5% SDS, and 1 mg/mL Proteinase K) per well. Seal the plates with Titer-Top™ (pre-cut adhesive film for sealing microplates, Diversified Biotech Catalog # T-TOPS-50). **DO NOT use parafilm to seal the plates, it melt at 55°C.**

3. Incubate the plate overnight at 55°C-65°C in a shaking incubator (100-150 rpm).

4. The next day, remove the plates from the incubator. In a low-speed tabletop centrifuge equipped with a swing-bucket rotor and microtiter plate carriers, briefly spin the plates to rid the lids of condensation.

5. Using the multichannel pipettor, add 100 uL of cold EtOH/NaCl (150 uL 5M NaCl in 10 mL cold 100% EtOH) solution per well. Allow the plates to rest on the bench at room temperature for 30-60 minutes or until the precipitated DNA is clearly visible. The DNA will adhere to the polystyrene dish (alternately, the plates can be parafilmed and plated at -20°C for 30 minutes). Spin in 96-well plate holder centrifuge, 2500 rpm, 5 minutes.

6. **Gently invert** the plates onto paper towels and allow the EtOH to drain from the wells (the DNA will remain adhered to the plate). Using the multichannel pipettor, add 100 uL 70% EtOH gently to wash each well.

7. Invert the plates onto paper towels to discard the EtOH wash and repeat the wash 2-3 times.

8. After the final wash, invert the plates to discard the EtOH wash, allow the plates to air-dry (for 10-30 minutes). **It is essential that all of the ethanol evaporate or the DNA will not digest**. To ensure adequate drying, leave the plate covered at 37°C until moisture just goes away. (Observe edges and center of well.)

9. Add to each well 30 uL sterile water or Resuspension Buffer (TE buffer) (in 5 mL sterile water add 50 uL 1M Tris-HCl, pH 7.5 and 10 uL 0.5M EDTA). **Pipet up and down several times. (Shaking briefly may be sufficient).** Seal and incubate the plate in shaking incubator at 37°C for at least 4 hours or O/N. If you are going to perform a primary PCR screen, you can resuspend the sample and take 1-2 ul for PCR and use the rest for digestion.

10. Next day, prepare the Restriction Digest Cocktail (1X restriction buffer specified for the enzyme being used, 1 mM spermidine, 100 ug/mL BSA, 50 ug/mL RNase, and 20 units of enzyme). The addition of RNase can be optional. Prepare enough for n samples (where n = number of clones) at 10 uL cocktail/sample. Select an enzyme for cutting that has one site outside of the targeting vector sequences.

11. Using the multichannel pipettor, add 10 uL of Restriction Digest Cocktail to each well and mix by pipetting up and down. Change pipette tips between one row and the next.

12. Once the cocktail has been added to all the wells, seal the plate with Titer-Tops, and incubate the plates O/N at 37°C (or the temperature specified for the restriction enzyme being used) in a shaking incubator, or in a humidified chamber.

13. The next day, prepare agarose gels (0.7-1.2% in 1X TAE for the electrophoresis of the samples. Use large gel trays with several rows of combs to accommodate the numerous samples plus molecular weight

markers, and cast gels thick enough to provide a loading capacity of 30-40 uL/well.

14. Remove the 96-well digest plates from the incubator and briefly spin to rid the lids of condensation. Add 7 uL 6X loading buffer to each sample and load the gel with 30-35 uL of each sample per well. Carry out the electrophoresis at 80 volts for approximately 4 hours or as long as needed.

15. Take picture of gel with ruler under UV light.

16. 10--15 minutes of depurination in HCl (19.2ml HCl/Liter of H20) might help the transfer of large fragments. If you depurinate then wash briefly with water afterwards and then proceed with denaturation. Soak the gel for 1 hour in Base solution with gently shaking (Base solution for 6 liters: 120 g NaOH and 526 g NaCl).

17. Replace Base solution with Neutralization solution (Neutralization solution for 6 liters: 405.6 g NaCl, 363 g Tris Base, pH 8.0) and gently shake for 30 minutes. Pour out Neutralization solution and replace with fresh Neutralization solution and shake for and additional 30 minutes.

18. Set up transfer DNA to filter as follows: Cut 2 filter paper strips at least as wide as the gel and long enough to extend into a reservoir of 10X SSC. We generally place a glass plate on top of a Tupperware container filled with 10X SSC. Soak the filter paper in 10X SSC and place it over the glass plate so each end extends into the 10X SSC (serves as a wick). Turn the gel upside down and gently place it on the filter paper. Around the edge of the gel place saran wrap up to the edge to prevent wicking around the gel. Cut a piece of Hybond N+ membrane to match the size of the gel and soak it in de-ionized water for 30 seconds. Place the membrane on the gel and carefully remove all air bubbles. Cut 2 more pieces of Whatman filter paper the size of the gel and soak briefly in 10X SSC. Put the 2 pieces of filter paper on top of the membrane. Finally, place paper towels on top of the filter paper at least 3inches in height and put a second plate of glass on top of the paper towels with a small weight (less than 500 g). Allow transfer to proceed overnight. (There are no hard and fast rules about the transfer. If you like using ZetaProbe GT Membrane or Hybond N+...go for it).

19. The next day, disassemble the stack and rinse membrane in 0.2M Tris-Cl pH8.0 1-2 times. This neutralizes the transfer buffer. Briefly rinse the membrane in 2X SSC 2-times. Then bake in 80^{o}C oven for 2 hours, or crosslink the DNA onto filter using Stratagene crosslinker.

20. Pre-wet membrane with 2X SSC just before adding prehyb solution.

21. Pre-hybridize membrane with Clontech Express Hybridization Solution (Catalog # 8015-1) for at least 1 hour at 65^{o}C, shaking water bath. (20-30

mL depending on the size of the membrane). Make sure Express Hyb is completely in solution before use. Heat in 37°C water bath and place on stir plate if necessary. You can add denatured salmon sperm DNA (100-200ug/ml final concentration) for both prehyb and hyb solutions. The DNA can increase signal to noise.

22. Prepare probe of choice and hybridize the membrane with a labeled probe comprising a target sequence external (5' or 3') to the targeting construct sequences. This will identify those recombination evens that occurred at the target locus versus random integration events. This is a particularly important screening strategy if only positive selection was employed in the ES cell transfection. Using an external probe, targeted recombinant clones are identified by hybridization to both the wild-type and mutant alleles of the targeted gene. Empty pre-hyb solution. Add probe to fresh Express Hyb Solution (20-30 mL) and add to bag. Hybridize for at least 2 hrs, 65oC in shaking water bath. Again, make sure Express Hyb is in solution. When labeling the probe, feel free to generate it in whatever manner you commonly use. The Hammer Lab uses a random primed DNA labeling kit from Roche and labels gel purified fragments. Many labs use PCR labeling which can give a stronger signal and a lower background. For PCR labeling 400 to 600 bp templates work very well. Optimally one should use a probe with 2-4 million cpms/ml, and a 60 to 90 % incorporation rate. One can use 6,000ci/mmol dATP, which makes for an extremely hot probe. If the probe is sufficiently hot and hyb conditions are optimal, you should be able to detect a nice signal within 12 hours or less.

23. After hybridization, remove membrane and wash at room temperature with 2X SSC/0.1% SDS solution for 5-10 minutes, gently shaking. Monitor the intensity of the blot frequently with a Geiger counter. Replace wash solution with 0.1X/0.1% SDS and wash in 45-50°C water bath for 5 to10 minutes. Check blot intensity and background with Geiger counter. Wash longer if needed. In general washing is an art not a science. General rule of thumb: Check the intensity of the blot every 5 minutes or so to get a sense of the loss of signal...if the signal is depleting rapidly...stop the washing.

24. Briefly blot membrane dry between 2 pieces of 3M Whatman paper. Wrap membrane in Saran wrap and expose to film -80°C generally overnight or longer.

Note:

1. This procedure produces a crude DNA preparation and not all restriction enzymes work well under these conditions. Therefore you should check digestion efficiency on DNA prepared from normal ES cells before starting your gene targeting experiment. The following enzymes work well with this procedure: Eco RI, Eco RV and Hind III.

2. An alternative method to produce cleaner DNA is to spool the DNA out of the 70% EtOH from the 96-well plate into an eppendorf tube, rinse twice in 70% EtOH and resuspend in 40 uL of TE. Restriction enzyme digestion is then carried out in the tubes.

Restriction Digest Cocktail for 96-well Plate ES Cell And Digestion

Construct: Name
Plate: Number

After DNA precipitation, 70% EtOH wash and air drying of the plate, restriction digest cocktail is prepared for digestion of DNA before Southern blotting

Make (10 ul/well) x (number of wells)= X Volume
(Number of wells should be 10 wells more than what you really need)

Reagents	Stock Conc.	Final Conc.	Amount/ well
1- Spermidine	100 mM	1 mM	0.4 uL
2- BSA	10 mg/mL	0.1mg/mL	0.4 uL
3- RNase	0.5 mg/mL	0.05 mg/mL	4 uL
4- Enzyme	40 unit/uL	20 unit/well	0.5 uL
5- 10X buffer	10X	1X	4 uL
6- Sterile water	------	-------	up to 10 uL

Make fresh mix for each use. Add 10 uL of restriction digest cocktail to each well of the 96-well plate. Incubate the plate at 37^{o}C in a shaking incubator (or humidified chamber) O/N after sealing the plate to avoid evaporation.