UChicago IGSB ENCODE K562 Transfection Protocol
March 5, 2013

BAC Preparation

1. Prepare four inoculation tubes for each BAC with 10 ml LB Agar and 10 ul Kanamycin solution (Sigmal Aldrich, K0254) for a final concentration of Kanamycin of 50 ug/ml.

2. With a P1000 tip scratch off a small amount of frozen BAC and mix for each inoculation tube.

3. Incubate tubes overnight at 37C with shaking.

4. Store tubes on ice for 5 minutes then centrifuge them at 3,000 RPM, 7 min, 4C. Remove the supernatant and dab the tubes on a paper towel to fully remove the supernatant.

5. Purify the BAC's using Macherey-Nagel's Clontech's NucleoBond 20 PC 20 - Plasmid miniprep kit according to manufacturer's protocol with the following modifications (Macherey-Nagel, 740571.100). Two spins are used to clarify the precipitated protein and cell debris prior to loading the columns. In the precipitation step, 2 ul of glycogen is added in addition to the isopropanol. Cold 70% ethanol is used to wash the BAC DNA precipitate instead of room temperature ethanol.

6. Run a 0.5% Agarose, 0.002% EtBR gel with a 24 Kb ladder (Fisher Scientific, BP2580100) to confirm the presence of each purified BAC. Use 5 ul sample + 1 ul 6x gel loading dye blue (New England BioLabs, B7021S) and run the gel for 40 minutes at 80V. The gel should have two distinct bands per well. Each band should be higher than the 24,000 bp ladder band. The top band corresponds to the purified BAC DNA and the lower band is super-coiled *E. Coli* DNA. If the two bands smear together, or the BAC DNA band is jagged and non-distinct, then the BAC purification most likely failed and it may not be worth transfecting.

7. Measure concentrations of each BAC with a Nanodrop 1000 (Thermo Scientfic). We transfect using 1 ug of BAC DNA.

Transfection

1. Seed 500,000 of freshly growing pure K562 cells (should be less than p6) in each well of a **12 well plate** in 2 ml of RPMI 1640 media (Invitrogen, 22400-105) supplemented with 10% HI-FBS (Invitrogen, 10438-026) and 2% Antibiotic-Antimycotic (Invitrogen, 15240-062) per well. (EX: If you are transfecting 5 lines and one negative control, you would need 6X500,000 = 3 million cells total in 6X2 ml media = 12 ml of total media and seed 2 ml of this into each of your wells).

2. Label 1.5 ml eppendorf tubes for each transfection and add 100 ul of Opti-MEM media (Invitrogen, 31985-062).

3. Add 1000 ng of BAC DNA to each corresponding tube containing the Opti-MEM, flick gently to mix well.

4. Mix Plus reagent (Invitrogen, 15338-100) gently, add 1 ul Plus:1 ug BAC (in our case 1 ul PLUS) to each tube and incubate at room temp for 5 min.

5. Add 5ul Lipofectamine LTX (Invitrogen, 15338-100) to each tube and mix thoroughly by flicking tube. Incubate at room temp for 30 min.

6. Add entire entire volume of DNA-Lipid complex DROPWISE to each corresponding transfection well, shaking **12 well plate** entire time.

7. One day after transfection (D1), spin down cells and change media to 3 ml of complete media (including negative) and replate them in the same **12 well plate.**

8. Two days after transfection (D2). spin down cells and place in a **6 WELL PLATE** containing 4 ml of complete media per well. Add Geneticin G418[1] (Invitrogen, 10131-027) to a concentration of 50ug/ml to each well to begin light selection.

9. Five days after transfection (D5), spin down cells and replace media with full selection (G418 concentration of 500 ug/ml) and replate them in the same **6 well plate**.

10. Refresh media every two days (if transfection was started on a Wednesday, then change media every Monday, Wednesday and Friday) with full selection media (RPMI 1640, 10% HI-FBS, 2% Anti-Anti, 500ug/ml G418) and replate them in the same **6 well plate**.

11. After two weeks of full selection (D19), expand cells to a T-25 flask.

12. Once negative is completely dead and transfected cells are growing rapidly (about 2.5-3 weeks, D22-D26), expand cells to a T-75 flask.

13. Once cells have reached a log growth phase (doubling approximately every day), collect 7 million cells and spin down the cells.

14. Resuspend pellet of 7 million cells in 7 ml of Recovery Cell Culture Freezing Medium (Invitrogen, 12648-010). Aliquot 1 ml of resuspended cells (1 million) into a cryogenic freezing vial, repeat six times for a total of 7 vials with 1 million cells.

15. Place cells in a room temperature freezing box (with isopropanol) and store in -80 C overnight. After this, the cells should be transferred to liquid nitrogen storage.

[1] There appears to be significant lot-to-lot variation in effective concentration of G418, so it is important when ordering a new lot of Geneticin to calibrate its effective concentration by making a kill curve. Our kill curve was set up so that our negative control (no BAC - no geneticin resistance) will die after two weeks of full selection (500 ug/ml of G418).