THE NORTHWESTERN UNIVERSITY
TRANSGENIC AND TARGETED MUTAGENSIS LABORATORY

Preparation of BAC DNA by Double Acetate Precipitation and CsCl Gradient

(by Shiaoching Gong, 14 October 2004)

General Considerations:

a) Never vortex cells or DNA suspensions
b) We recommend using wide bore pipette tips to avoid damaging DNA during solution transfer
c) Use caution when handling ethidium bromide; a potent mutagen

1. Pick a single colony of transformed bacteria from a freshly streaked chloramphenicol (20μg/ml) and ampicillin (50μg/ml) agar plate; inoculate 3 ml of Luria Broth medium containing chloramphenicol and ampicillin (same conc as agar). Incubate at 37°C for 8 hours.
2. Transfer 0.4 - 1.0 ml of inoculated broth (depends on the cell density) into 500 ml of Luria Broth containing chloramphenicol and ampicillin (conc as above); incubate at 30°C for 14 – 16 hours
3. Spin down the bacteria at 4000 rpm for 30 mins at 4°C (J6-MI Beckman-Coulter centrifuge, JS-4.2 rotor). Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.
4. Resuspend cells in 40 ml of 10 mM EDTA, pH 8.0 by pipetting and transfer to a 250 ml bottle.
5. Add 80 ml of alkaline lysis solution (0.2N NaOH in 1% SDS: 2 ml of 10N NaOH, 10 ml of 10% SDS into 88 ml dH_2O). Mix by *very* gently swirling and incubate for 5 min at RT.
6. Add 60 ml of cold 2M KOAc (50 ml of 7.5M KOAc, 23 ml of glacial acetic acid and 127 ml of dH_2O, stored at 4°C). Mix by *very* gently swirling and incubate on ice for 5 min. Spin at 11650 rpm for 30 mins at 4°C (J-25I Beckman Avanti centrifuge, JLA-16.250 rotor).
7. Transfer supernatant into a 250 ml bottle, add 180 ml of isopropanol. Mix by gently swirling. Spin at 4000 rpm for 30 mins at 4°C (J6-MI centrifuge, JS-4.2 rotor). Decant the supernatant.
8. Dissolve the DNA pellet in 18 ml of 10:50 TE (1ml of 1M Tris, 10 ml of 0.5M EDTA into 89 ml dH_2O). Add 9 ml of 7.5M KOAc and mix and incubate at -70°C for 30 min.
9. Thaw solution and centrifuge at 6000 rpm for 10 mins at 4°C (J-25I Beckman Avanti centrifuge, JA-25.50 rotor).
10. Transfer supernatant to a new tube and add 2.5 volume of ethanol. Spin at 11650 rpm for 30 mins at 4°C to precipitate the DNA (J-25i Beckman Avanti centrifuge, JLA-16.250 rotor).
11. Decant supernatant and gently resuspend pellet (while still moist) in 4.4 ml of TE. Dissolve, as best possible, 10.2 g of CsCl in another 4.4ml of TE. *Gently* mix CsCl solution with 4.4 ml of DNA until the CsCl has dissolved. Add 0.2 ml ethidium bromide solution (10 mg/ ml dH_20) and mix immediately. Spin at 4000 rpm for 10 mins at 4°C to remove debris (J6-MI centrifuge, JS-4.2 rotor).
12. Remove the supernatant and load into a Beckman Quick-Seal tube (16 x 76 mm, #342413) using a syringe and 18-gauge needle. Seal tubes *carefully* and place in a NVT65 rotor. (It is very important to equilibrate the tubes to be centrifuged in opposing positions: weigh them very carefully to make sure they do not differ by more than 0.05g). Spin at 65,000 rpm overnight (>8 hours) at 18°C.
13. Remove tubes from rotor carefully, taking care not to disturb the gradient. Use a 23-gauge needle to poke a hole in the top of the tube. Utilizing a UV light, carefully remove the band (choose bottom band if there are two) with an 18-gauge needle with the needle bevel up. Take the band and no more (usually about 200μl). Transfer it to a 15 ml tube and bring it up to 2 ml with TE. Extract 4-5 times with NaCl-saturated butanol (20ml of 3M NaCl in 100ml of butanol) until there is no more

orange color. (To extract add equal volume of NaCl-saturated butanol to TE solution, mix gently, let mixture sit 30 sec to allow for separation, remove and discard top layer.)

14. Add 1 ml of H_2O to DNA solution and then 2.5 - 3.0 volumes of EtOH and mix. Place at -20^oC for 30 mins. Spin solution at 11650 rpm for 30 mins at 4^oC to precipitate the DNA (J-25i Beckman Avanti centrifuge, JA-25.50 rotor). Resuspend DNA in 0.5 ml of 0.3M NaOAc. Transfer DNA to 1.5 ml Eppendorf tube and add 1 ml EtOH. Spin down the DNA at 14000 rpm for 30 mins at 4^oC (Eppendorf microcentrifuge model 5417R). Discard the supernatant, fill the tube with 70% EtOH and allow the tube sit at room temperature for 5 mins. Spin the DNA again as in previous step but shorten time to 10 mins. Dry the pellet at RT for 1 min; use paper towel to get rid of the trace amount of ethanol. Resuspend DNA gently in 20-40μl (or more if the pellet is large) of TE. Place DNA in 37^oC incubator for 20-30 min. You are likely to get 5-20 ug of BAC DNA. You should store BAC DNA at 4^oC. (Do not store it at -20^oC!!!)
15. Determine the concentration with UV and check the DNA on pulse field gel. For PFG, linearize 2μl BAC DNA (ie, digest with PI-SceI) and compare with commercially available linear DNA standard markers.
16. Digest the BAC DNA with PI-SceI (New England Biolabs, Cat # R0696S): Add together 5 – 10μl (about 100 ng) of BAC DNA, 2μl of PI-SceI enzyme, 5μl of 10x buffer and dH_2O to produce a final volume of 50μl. Incubate in a 37^oC incubator for 3 to 4 hours.
17. To dialyze the DNA, start by placing 20 ml of injection buffer (recipe below) into a sterile Petri dish and float a 25 mm, 0.025 um filter (Millipore, Cat. # VSWP02500) on top with the shiny side up. Load the 50μl of digested DNA on the top of the filter and cover the Petri dish with lid. Allow set-up to sit at RT for 4-6 hours. Transfer the DNA-containing solution on top of the filter to a microcentrifuge tube and add enough injection buffer to return solution to original volume of 50μl.
18. Check the DNA on pulse field gel again as describe in step 15 to confirm the concentration.

Injection buffer for BAC (use high-quality distilled water and filter):

10 mM Tris, pH 7.5
0.1 mM EDTA
100 mM NaCl