

PROTOCOL- BAC DNA Preparation for Microinjection

Allow 5 days for preparation of BAC DNA for microinjection

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| Reagents: | P1 | 50mM Tris-HCl 10mM EDTA, pH 8.0 Store at 4°C |
| | P2 | 200 mM NaOH 1% SDS Store at room temperature |
| | P3 | 2.8M KOAc pH 5.5 Store at 4°C |
| | 7.5M KOAc | 184 g KOAc 250 mL sterile diH ² O Filter or autoclave |
| | BAC-MIB | 10 mM Tris-HCl pH 7.5 10 M EDTA 30 uM spermine 70 uM spermidine 100mM NaCl |

Method:

1. Inoculate 800 mL (2 x 400 mL) selective LB with 0.5 mL of overnight culture. Start from a single colony on a streak plate – This Is A Very Important Point. Shake overnight at 37°C (no longer than 18 hours).
2. Pellet the cells at 2,000xg for 15 minutes, discard the supernatant.
3. Resuspend the pellet in 30 mL Buffer P1 without RNase.
4. Add 100,000U Ready Lyse (Epicentre) to each 30 mL prep and incubate for 15 minutes at room temperature. DON'T MIX OR VORTEX PAST THIS POINT.
5. Add 30 mL of Buffer P2 and incubate for 15 minutes at room temperature to lyse the cells. It's OK to roll the tubes gently on the countertop but don't mix, invert, shake or vortex.
6. Add 30 mL of chilled Buffer P3 and incubate for 15 minutes on ice then spin at 15,000xg for 30 minutes at 4°C.
7. Pour supernatant into a clean bottle and spin again. (15 minutes is ok this time).
8. Pour the supernatant through a filter paper funnel and collect in a clean bottle. Add 45 mL (1/2 volume) of P1+P2+P3, mix gently but thoroughly.
9. Spin at 6,000xg for 15 minutes at 4°C, decant and drain the pellet well.
10. Resuspend the pellet in 9 mL TE and place in a 30- or 50-mL Oak Ridge tube.
11. Add 4.5 mL of 7.5M sterile KOAc, mix gently and chill at -80°C for 30 minutes.

12. Thaw at room temperature then spin at 5000xg for 12 minutes.
13. Place supernatant in a clean Oak ridge tube and repeat the spin.
14. Add 27 mL of absolute ethanol and spin at 5000xg for 12 minutes.
15. Resuspend the pellet in 1 mL of Buffer P1 containing RNase and incubate at 37°C for 30 minutes.
16. Add 100 uL of 10% SDS, mix gently and add proteinase K to a final concentration of 0.2 mg/mL, incubate at 50-60°C for at least 3 hours up to overnight.
17. Remove protein with buffered phenol (pH 8) and extract the phenol with chloroform (3X).
18. Add 7.5M NH₄OAc to a final concentration of 2.5M (1/2 of original volume) and precipitate the DNA with 0.5 volume room temperature isopropanol.
19. Spin in 1.5 mL eppendorf tubes at 12,000 rpm for 5 minutes.
20. Wash the pellets with 1 mL 70% ethanol and 300 uL TE (to stabilize DNA). Incubate at 37°C for at least 30 minutes up to overnight.
21. Add 12 mL Clonetech Buffer N2 and run through an equilibrated Clonetech column following the manufacturer's instructions. Resuspend the DNA pellet in TE, don't let the DNA pellet dry too much before resuspension. Incubate at 37°C for 30 minutes then at 4°C overnight.
22. Linearize the BAC with an appropriate enzyme (or use terminase) for at least 3 hours up to overnight.
23. Extract with phenol:chloroform.
24. Dry the pellet 15 minutes then dissolve the DNA in 20 uL of fresh BAC MIB, incubate for 30 minutes at 37°C.
25. the A₂₆₀:A₂₈₀ ratio must be between 1.7 and 2.0; if higher or lower phenol:chloroform again until it is between 1.7 and 2.0.
26. Store at 4°C until used.
27. On injection day thaw the DNA, check the A₂₆₀ and dilute in BAC MIB to 1 ng/uL.

NOTE: Check the integrity of the BAC DNA prior to injection. This should be done with 0.8% agarose using pulse field electrophoresis with 1) 1 uL of linearized BAC DNA (this should give a sharp band of the expected size without any gDNA contamination or smearing) and 2) BamHI digested DNA (should give clean fragments in a ladder pattern without any smearing).

