SUE'S LARGE SCALE CESIUM CHLORIDE PLASMID PREP

Materials:

500 ml centrifuge bottles
Sorvall centrifuge
Sorvall GSA rotor
Sorvall rotor SA-600
50ml oakridge tubes
Beckman ultracentrifuge
Beckman Vti50 tube (cat#343665)
Beckman Vti50 rotor
Beckman Ti70.1 tube cat.#342413
Beckman Ti70.1 rotor
18-guage needles
20 & 10 cc syringes
30 & 15 ml Corex tubes

Reagents:

5ml LB culture
LB (1 liter in a 2-liter flask)
chloramphenicol (34 mg/ml)
25% sucrose/50 mM Tris pH 8.0
lysozyme (10mg/ml)
0.25 M Tris pH 8.0
0.5 M EDTA (cold).
Triton Lysis solution
cesium chloride
ethidium bromide (10 mg/ml)
TE (pH8.0)
sec-Butanol:1 M NaCl solution
phenol:choroform:*iso*-amyl alcohol (25:24:1)
100% Ethanol
3 M NaOAc (pH5.2).

LB Media

Tryptone	10 g
Yeast	5 g
NaCl	10 g
dH_2O	1 liter

For plates, add (per liter):
Agar 20 g

Autoclave media 30 min.

Antibiotic	stock conc.	Dilute in	working conc.
Ampicillin	100mg/ml	dH_2O	100 µg/ml
Kanamycin	10 mg/ml	dH_2O	30µg/ml
Tetracycline	5 mg/ml	EtOH	10µg/ml

25% Sucrose/50 mM Tris pH 8.0
500 ml:
125 g Sucrose
25 ml 1M Tris pH 8.0
dH_2O up to 500 ml
filter

sec-Butanol/1 M NaCl
500 ml:
250 ml *sec*-Butanol
250 ml 1M NaCl
mix well and let phases separate

10 mg/ml Lysozyme
10ml:
100 mg lysozyme (Sigma# L-6876)
0.25M Tris (pH 8.0) up to 10ml
store at –20°C

TE pH 8.0
10mM Tris-Cl (pH 8.0), 1mM EDTA (pH8.0)
500 ml:

1M Tris pH 8.0	5 ml
0.5 M EDTA	1 ml
dH_2O	494 ml

autoclave and store at room temp.

0.25 M Tris pH 8.0
500 ml:
125 ml 1M Tris pH 8.0
375 ml dH_2O

Phenol:Choroform:*iso*-amyl alcohol (25:24:1)
100ml:

phenol	50ml
chloroform	48ml
iso-amyl alcohol	2 ml

store in a light-tight bottle at 4°C for 6 months

Triton Lysis Solution
Final Concentration:
0.4% Triton
62.5 mM EDTA
50.0 mM Tris pH 8.0

400 ml:	10% Triton X-100	4 ml
	0.5 M EDTA	50 ml
	1 M Tris pH 8.0	80 ml
	dH_2O	216 ml

Procedure:

Day 1:

1. Prepare a streak plate from a stab or transformed cells on a drug-resistant agar plate and place in 37°C incubator until single colonies appear (15-18 hours).

Day 2:

2. Inoculate a 5ml LB culture (with proper antibiotic) with a single bacterial colony. Place in 37°C shaker 15-18 hours.

Day 3:

3. Flame the top of the culture tube and pipet 4 mls of the culture into a 1 liter of LB in a 2-liter flask.
4. Approximately 4 hours later, take a 1 ml sample of the culture. Take an OD reading at 595□. When the sample reads 0.7-1.0, add 5 ml of chloramphenicol (34 mg/ml in EtOH) to each flask. Chloramphenicol affects the degree of amplification of plasmids, thus higher yields will be obtained. Keep in 37°C shaker approximately 15-18 hours.

Day 4:

5. Collect cells. Pour culture from flasks into 500 ml centrifuge bottles and fill to approximately three-fourths full (to prevent leakage). Spin in Sorvall centrifuge (rotor

#GS-3/mdeol SLA-3000) at 5Krpm for 10 minutes. Pour off LB and refill bottle with remaining culture. Repeat procedure until entire liter is spun down.

6. Resuspend cells in 6 ml of 25% sucrose/50 mM Tris pH 8.0 solution (cold). Keep cells on ice.
7. Add 1.5 ml of 10 mg/ml lysozyme (in 0.25 M Tris pH 8.0). Mix well and ice 5 minutes.
8. Add 1.5 ml 0.5 M EDTA (cold). Mix and ice 5 minutes.
9. Add 11.5 ml of Triton Lysis solution. Mix and ice 15-30 min.
10. Transfer contents to 50 ml oakridge tube (for Sorvall rotor SA-600). Spin tubes in Sorvall centrifuge at 10K rpm for 30 min at 4°C.
11. Pour supernatant into a 50 ml conical tube. Record volume in mls (typical volume will be approximately 20 mls) and add an equivalent amount of solid cesium chloride in grams (1 g: 1 ml). Dissolve CsCl completely. Prepare a blank solution of CsCl in TE (1g: 1ml)--approximately 40 ml for 8 samples.
12. Add 50µl 10 mg/ml EtBr (ethidium bromide) to each tube. **EtBr is a known mutagen and very toxic**. Wear gloves at all times when handling this solution and dispose of all liquid/solid waste into their proper containers. Also, avoid leaving the tubes exposed to light as long term exposure in combination with EtBr could nick the DNA.
13. Pour solution into a Beckman VTi50 heat-sealable tube (Beckman cat#343665; 25x64 mm/27 ml). To do this, attach an 18-guage needle to a 20cc syringe with the suction end removed. Set the needle into the top of the heat-sealable tube and use the barrel of the syringe as a funnel to pipet the solution into. It is important to fill the tube completely (to the base of the neck) with the blank CsCl solution. Balance the tubes exactly (by weighing them in pairs) and heat seal them.
14. Spin tubes in a VTi50 rotor at 45 Krpm, 15°C for 16 hours.

Day 5:

15. Carefully remove the tubes from the VTi50 rotor (gently and as level as possible as to not disturb the gradient).
16. In a dark room, prepare an area with bench paper. Set a ringstand with a clamp-holder on top of this area and place a waste tray on the base of the stand. Carefully clamp the tube onto the ringstand. Using a hand-held long-wavelength UV light, check for the appearance of 2 distinct bands (the upper band being *chromosomal* DNA and the lower band being *plasmid* DNA). The upper chromosomal DNA band is usually much smaller if visible at all. Carefully puncture a hole into the top of the tube using a needle (this hole is for airation). Remove the lower band using a 10cc syringe with an 18guage needle. The trick here is to gently twist or screw the needle-with-syringe into an area ~2-3mms below the lower band. Keep steady pressure on the needle and be careful not to let the needle to come out or the contents will quickly leak out and the band will be lost. Using the syringe, transfer the band to a Beckman Ti70.1 heat-sealable tube (Beckman cat.#342413; 16x76 mm/13.5 ml). Again, fill tube completely to the top (to the base of the neck of the tube) using blank solution. If possible, be careful to avoid getting any of the chromosomal DNA (the upper band) during extraction.
17. Balance the tubes exactly and heat seal them. Spin tubes in the Ti70.1 at: 45Krpm, 20°C, for 40 hours –or--55Krpm, 20°C, for 24 hours.

Day 6:

18. Carefully remove the tubes from the Ti70.1 rotor (again, as gently and as level as possible as to not disturb the gradient). In a dark room, carefully clamp the tube onto a ringstand. Using a hand-held long-wavelength UV light, you should see one very distinct band of plasmid DNA (any appearance of an upper band indicates the presence of chromosomal DNA). Carefully puncture a hole into the top of the tube using a needle. Remove the lower band using a 10cc syringe with an 18 g needle and transfer the contents to a 15 ml Falcon tube. This time avoid all contact with the upper band--even if this results in compromising a small amount of plasmid DNA.
19. Record the volume of each tube. Add an equivalent volume of *sec*-Butanol:1 M NaCl solution to each tube and mix well. Allow the phases to separate and remove the upper (purple/pink) phase. Repeat this process until the upper phase is clear (approximately 2-3 times). Dispose the *sec*-Butanol/EtBr waste into EtBr liquid waste.
20. Record the volume of each tube and transfer the contents to a 30ml Corex tube.
21. Add 2x the volume of dH_20 and 4x the volume of -20°C EtOH. Parafilm and mix well. Place tubes in -20°C for 2 hours (or overnight).

22. Spin tubes in the Sorvall centrifuge (rotor SA-600) at 8K rpm for 30 min at 4°C.
23. Carefully pour off EtOH and let pellets dry completely. It's convenient to lean the tube upside-down against a test-tube rack on top of a paper towel. The pellets usually are held firm to the bottom of the tube but be careful as occasionally one may slide down.
24. Resuspend pellets in 2 ml TE (pH8.0).
25. To denature and remove proteins, add 2 ml phenol:choroform:*iso*-amyl alcohol (25:24:1). Parafilm and gently vortex ~5 seconds on a medium speed. Spin tubes in Sorvall centrifuge (rotor SA-600) at 7K rpm for 5 min to separate the phases. Normally, the aqueous phase frms the upper phase. If the aqueous phase is dense because of salt or sucrose, it will form the lower phase. In this case, double the volume of the aqueous phase with dH_2O and add phenol:choroform:*iso*-amyl alcohol to 1:1.
26. Transfer upper phase to a 15ml Corex tube (be careful to avoid any contact with the lower/phenol phase).
27. To each tube, add 4 ml -20°C 100%EtOH and 200µl 3 M NaOAc (pH5.2). Parafilm and mix well. Place tubes in -20°C for 2 hours (or overnight).
28. Spin tubes in the Sorvall centrifuge (rotor SA-600) at 12K rpm for 30 min at 4°C. Pour off EtOH and allow pellets to dry completely using the same method as previously done.
29. Resuspend pellets in TE (pH8.0). Suggested volumes/pellet size: 800µl/size of a nickel; 500µl/size of a dime.
30. To calculate the concentration, make a 1:100 dilution with dH_2O (add 10µl DNA/TE to 990µl dH_2O). Take an OD reading at 260nm and 280nm on the UV spectrum (program #10 on our Hitachi Spec). The final concentration in µg/ml will be 5000 multiplied by the OD reading at 260.

    5000 because:
    Spectrophotometric conversion: 1 A_{260}dsDNA = 50µg/ml
    Dilution: 1:100

The OD_{260}/OD_{280} ratio provide an estimate of the purity/quality of the DNA. Pure preparations of DNA have an OD_{260}/OD_{280} value of 1.8. If there is contamination with protein or phenol the OD_{260}/OD_{280} will be significantly less than 1.8 and accurate quantitation is not possible.

Reference: Maniatis, T. Molecular Cloning A Laboratory Manual (1989) p1.33-1.43.