96 well double-stranded template isolation

Basically colonies containing double-stranded plasmids are picked with sterile toothpicks into media and incubated at 37°C for 24 hours with shaking at 350 rpm. These cells are harvested by centrifugation and the pellets are manually resuspended by the addition of TE-RNase solution. An alkaline lysis solution is used to lyse the cells and the lysate is precipitated with KOAc. The lysate is cleared by filtration and further concentrated by ethanol precipitation. An aliquot from each DNA sample is subjected to agarose gel eletrophoresis to crudely assay concentration and purity. The yield of double stranded template is approximately 3 mg per sample.

Protocol:

Manual Double stranded isolation method

The following is a manual, 96 well, double stranded sequencing template isolation procedure that has been developed in our laboratory.

1. Pick individual shotgun clones off a plate with a sterile tooth pick and deposit each separately into 96 well block containing 1.5 ml of LB or SOC media per well. Keep toothpick in media for about 5 minutes to allow the cells to defuse into the media, remove the toothpicks after rotating several times between fingers, cover the 96 well block with the loose fitting lid (tin-foil), and allow the cells to grow for 24 hours in the 37°C shaker/incubator at 350 rpm.

2. Remove block from the shaker/incubator and collect the cells by centrifugation at 2500 rpm for 7 minutes. The cells can be stored frozen at -20°C in the block at this stage.

3. After thawing the cells, add 100ul TE-RNAse-A solution containing RNAse T1, mix by pipetting up and down 4-5 times to resuspend the cell pellet and then incubate in the 37degC incubator/shaker for 5 minutes at 350 rpm to mix more thoroughly.

4. Remove the block from the incubator/shaker and then add 100ul of alkaline lysis solution. Shake the block by hand to mix the reagents and then incubate at room temperature for 1 hour with intermittent swirling. 5. Then add 100ul of either 3M potassium or sodium acetate, pH 5, and place the block in the 37degC shaker/incubator for 5 minutes at 350 rpm to thoroughly mix and shear genomic DNA to reduce the viscosity of the solution. Place the block at -20°C for 30 minutes (this will result in a tighter forming pellet).

6. Centrifuge the block at 3000 rpm at 4°C for 30 minutes.

7. Transfer between 400 and 500ul of supernatant (containing your DNA) to a new deep well block. Add 1ml of 95% EtOH. Cover and store at -20°C for a minimum of 2 hours, but preferably O/N.

8. Centrifuge block at maximum rpm for 30 minutes. Decant, wash with 500ul 70% EtOH. Centrifuge for an additional 15 minutes. Decant again. Allow to air dry.

9. Resuspend in 100ul ddH_2O (warmed to 37°C), allow to go into solution (~15 minutes) and then transfer to a v-bottom microtiter plate.

Reagents:

Lysozyme solution: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 5 mg/ml lysozyme in sterile double distilled water.

5 ml	1 M Tris-HCl, pH 8.0
2 ml	0.5 M EDTA
0.5 g	lysozyme (Sigma L-6876)
sddH2O to 100 ml (make fresh)	

TE-RNase solution: 50:10 TE buffer containing 40 ug/ml RNase A and 40 U/ml RNase T1

1.2 ml	1 M Tris-HCl, pH 7.6
480 ul	0.5 M EDTA
50 ul	20 mg/ml RNase A
10 ul	100 U/ul RNase T1
22.3 ml	ddH2O
24.0 ml	

Alkaline lysis solution (NaOH/SDS): 0.2 N NaOH, 1% SDS in ddwater.

20 ml of 1 N NaOH (or 0.8 gms)
10 ml of 10% SDS (or 1.0 gms)
ddH2O to 100 ml (make fresh)