CLONING IN M13

Materials:

2% x-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside):
20 mg/ml stock in N-N'-dimethyl formamide, stored at -20°C.
0.1 M IPTG (isopropyl-ß-D-thio-galactopyranoside): 0.5 M stock made in H_2O, stored at -20°C.; dilute to 0.1 M with H_2O as needed.
YT medium: 8 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 5 g/L NaCl. For plates, 15 g/L agar. For top agar: 8 g/L agar.

Transfection of JM103 with M13 mp8/mp9 DNA subclones

1. Prepare competent JM103 cells as described for E. coli transformation ($CaCl_2$-treatment). Best if used immediately after $CaCl_2$ treatment.
2. Use DNA amounts from ligation mixes, etc. as described for E. coli transformation.
3. Place 0.2 ml of competent JM103 cells in sterile tube on ice and add 25 µl of diluted DNA.
4. Let DNA and competent cells sit on ice for 30 minutes.
5. Place at 42°C for 3 minutes then back on ice.
6. Place 50 µl -100 µl of this mixture into a tube containing 0.2 ml of freshly grown (non-competent) JM103 (middle-late log phase cells); These cells will form the bacterial lawn.
7. Make a 1:1 solution of 2% x-gal (20 mg/ml stock in N-N' - dimethyl-formamide, stored at -20°C) and 0.1 M IPTG (0.5 M stock made in H_2O and stored at -20°C).
8. Add 80 µl of 1:1 IPTG:x-gal solution to each tube, then plate onto YT plates by mixing with 3 mls YT top agar.
9. Place plates at 37°C.
10. Plaques will form after 12-16 hours, recombinant plaques are colorless, non-recombinant plaques are blue.

Growth of M13 mp8/mp9 phage

1. Pick a single colorless plaque with tip of Pasteur pipet and place into 2 mls of YT medium.
2. Place at 37°C with shaking for 12-18 hours.
3. Transfer cells to Eppendorf tubes and spin out cells in microfuge.
4. Transfer supernatant to test tubes, avoiding cells.
5. This phage stock is stored at 4°C.
6. To check size of recombinant phage, place 30 µl of phage supernatant into Eppendorf tube and add 5µl of gel loading buffer containing SDS (50% glycerol, 130 mM Tris-Cl pH7.5, 10 mM EDTA, 2.5% SDS, 0.1% x.c., 0.1% B.P.B.)
7. Run on agarose gel and visualize bands by staining with EtBr.

Titer determination/plaque purification of M13 mp8/mp9 phage supernatants

1. Serially dilute phage supernatant to achieve 10^8 and 10^{10} dilutions.
2. Mix 0.1 ml of 10^8 dilution or 0.1 ml of 10^{10} dilution with 0.2 ml of fresh JM103 cells in sterile tubes.
3. Add 80 µl of a 1:1 solution of 2% x-gal and 100 mM IPTG.
4. Add 3 mls molten YT top agar and plate onto a YT plate.
5. Place at 37°C for 12-16 hrs.
6. Titer is generally 10^{11} - 10^{12} plaque forming units (pfu)/ml.

M13 mp8/mp9 single-stranded and replicative form DNA isolation

This procedure yields very clean single-stranded M13 DNA for dideoxy sequencing. Following this procedure greatly increases the amount of sequencing information gained from a given cloned segment. Also, double-stranded RF DNA can be isolated from the same preparation.

1. Add 2 mls of dense overnight culture of JM103 cells to 100 mls YT.
2. Place at 37°C with shaking until $O.D._{595nm}$ reaches approximately 0.1 - 0.2.
3. Add phage supernatant to 10^9 pfu/ml (i.e., 1 ml of 10^{11} pfu/ml phage stock into 100 mls).
4. Incubate at 37°C for 16-18 hours with shaking.
5. Spin down cells in 250 ml bottles for 5 minutes at 5,000 rpm at 4°C.
6. Pour supernatant into fresh 250 ml bottle.
7. For replicative form DNA preparation, treat pellet exactly as outlined for plasmid DNA isolation.
8. Spin supernatant again, 5 minutes at 5,000 rpm to remove any cells.
9. Place supernatant into fresh 250 ml bottle.
10. Add 3 g polyethylene glycol (PEG) 8000 and 3 g NaCl; dissolve completely.
11. Let stand at room temperature for 30 minutes.
12. Spin down at 10,000 rpm for 20 minutes at 4°C.
13. Discard supernatant, spin precipitated phage again at 10,000 rpm for 3-5 minutes and pipet off remaining supernatant. Phage pellet should be clearly visible.
14. Wipe out bottle carefully with tissue to remove all traces of PEG, this step is critical for achieving clean DNA sequencing information.
15. Add 6 mls TE to each pellet and let resuspend slowly at room temperature. Do not vortex or shake to resuspend; swirl gently.
16. Add TE until weight of sample is 10.0 g.
17. Add 4.2 g CsCl to each; dissolve completely.
18. Fill Oakridge screw cap Ti50 tubes.
19. Centrifuge at 40,000 rpm in Ti50 rotor for 24 hours.
20. Collect blue phage band with a pasteur pipet, be careful to avoid PEG and cellular debris.
21. Dialyze vs. TE overnight, changing buffer three times.
22. Phenol extract dialyzed phage two times or until clear; phenol: chloroform extract once.
23. Add sodium chloride or sodium acetate to 0.3 M final concentration.
24. Ethanol precipitate with two volumes -20°C ethanol and place at -20°C overnight.
25. Spin down ethanol precipitates in microfuge for 30 minutes at 4°C.

26. Resuspend pellets in 100-500 µl 10mM Tris pH 8, 0.1 mM EDTA, depending on yield as judged by intensity of phage band in gradient; desired concentration is 0.5 - 1.0 mg/ml.
27. Read optical density at 260 nm to determine concentration; 1 $O.D._{260nm}$ unit = 40 µg/ml for single-stranded DNA.

Procedure adopted from Joan Kobori in the Hood laboratory at California Institute of Technology, Pasadena, CA.

Reference:

For a complete and detailed description of many techniques in M13 cloning and sequencing, the Amersham handbook M13 cloning and sequencing handbook is an excellent source.