

# Oligonucleotide-directed mutagenesis using pBluescript

Courey Lab

## A. Making CJ236 cells competent for transformation and Transformation.

1. Grow CJ236 in 5 ml 2XYT medium + 30  $\mu\text{g/ml}$  chloramphenicol o/n.
2. Dilute the overnight culture 100-fold in 50 ml 2XYT and grow until  $\text{OD}_{600} = 0.6$ .
3. Harvest and resuspend in 1/10 volumes (5 ml) of freshly made 100 mM  $\text{CaCl}_2$  and incubate on ice for 30 minutes.
4. Harvest cells and resuspend in 1 ml of 100 mM  $\text{CaCl}_2$  and incubate on ice for several hours.
5. Transform template into these cells (usual procedure) and plate on amp + chlor. It is ESSENTIAL to plate on amp AND chlor, as the F1 plasmid or whatever it is that allow the phage to properly uptake the DNA is chlor resistant. Grow up and miniprep 1/2 of each colony (usual procedure); use other half for step 6.

## B. Isolation of uracil-containing ssDNA of the plasmid.

6. Grow the transformed bacteria in 5 ml 2XYT + amp (50  $\mu\text{g/ml}$ ) o/n.
7. Dilute the overnight culture 100-fold in 100 ml 2XYT medium with uridine (5.0  $\mu\text{g/ml}$ ) and ampicillin (50  $\mu\text{g/ml}$ ) in 500-ml flask to get good aeration.
8. Grow until  $\text{OD}_{600} = 0.3$  ( $3.0 \times 10^8$  cells/ml). Add 5 moi of helper phage. Shake @  $37^\circ\text{C}$  overnight.
4. Remove the cells by centrifugation at 13K (17,000 g) (or 11K in SA-600 rotor) for 15 minutes. Use the SUPERNATANT for the next step!
5. Add 1/4 volume of 20% PEG in 2.5M NaCl to the supernatant, mix, and let stand on ice for 1 hour.
6. Collect by centrifugation (11,000 g for 20 minutes at  $4^\circ\text{C}$ ). Respin for 1 minute to remove residual supernatant. Use the PELLETT for the next step!
7. Resuspend the pellet in 1 ml TE (pH 8.0). After resuspension transfer to eppendorf and spin to remove debris. Save 10  $\mu\text{l}$  to test the phage (see below). Store at  $4^\circ\text{C}$ .
8. Extract with phenol, then phenol:chloroform until no interface is visible.
9. Precipitate the ssDNA by adding 1/2 vol 7.5M  $\text{NH}_4\text{OAc}$  or 0.375 vol 10M  $\text{NH}_4\text{OAc}$  and 2 vol 100% EtOH. Incubate on ice for 15 min and spin for 20 min at  $4^\circ\text{C}$ . Resuspend in TE and examine product by agarose gel electrophoresis.

# Oligonucleotide-directed mutagenesis using pBluescript

Courey Lab

## C. Testing the phage.

Add 5  $\mu$ l of the phage suspension to 3 ml of 2XYT medium that contains 0.3 ml of overnight culture (DH5 or CJ236). Make  $10^{-2}$  and  $10^{-4}$  dilutions and use 0.1 ml to plate on amp plates.

## D. Primer extension and product analysis:

### 1. Mix the following in a microfuge tube:

2  $\mu$ l 10X T4 forward kinase buffer  
2  $\mu$ l 10 mM ATP  
oligonucleotide (15-50 nt long) at a molar ratio of 10:1 (oligo:template)  
water to 20  $\mu$ l

Add 2 units of T4 kinase and incubate at 37°C for 60'. Terminate reaction by adding 1  $\mu$ l of 0.5 M EDTA and heat to 70°C for 10'.

2. To the phosphorylated oligonucleotide (20  $\mu$ l), add the ssDNA template (typically 1  $\mu$ g) and 1.25  $\mu$ l 20X SSC. The final volume should be 25  $\mu$ l. Mix thoroughly, spin 5", and put into a beaker containing 70°C water and allow to cool to RT. Spin briefly.

### 3. Meanwhile, make 5X polymerase mix:

100 mM TrisHCl, pH 8.0  
10 mM DTT  
50 mM MgCl<sub>2</sub>  
2.5 mM each dNTP  
5 mM ATP

To the hybridization mix (volume 25  $\mu$ l) add:

20  $\mu$ l 5X polymerase mix  
2.5 units DNA polymerase (T4 or Klenow)  
2 units T4 DNA ligase  
water to 100  $\mu$ l

4. Mix and incubate for 5 minutes at 0°C (i.e. ice water), 5 minutes at RT, and one hour at 37°C. Spin 5", add 2 more units each enzyme, and incubate for 1 more hour at 37°C.

5. Run 20  $\mu$ l out on a gel. Run some ssDNA and dsDNA out as well for controls.

6. Transform remaining 80  $\mu$ l into bacteria.