

## **Barber Lab Cloning with Invitrogen TOPO TA Cloning kit**

This procedure will insert your PCR fragment into a suitable vector in this case (pCR2.1-TOPO). This vector has antibiotic resistance (Ampicillin and Kanamycin) as well as having a gene for LacZ production. The successful insertion of a fragment into the ligation site will interrupt the LacZ gene and when grown on X-gal plates will result in white colonies. Blue colonies result from cells with a vector, but not PCR fragment. Cells without a vector will not grow on antibiotic plates.

### **Major Steps (Each step represents a logical stopping point if necessary)**

1. Creation of template DNA via PCR and gel purification (3.5 hours)
2. Ligation of vector and template (setup 15 min, incubation: 1 hour - overnight)
3. Transformation of cells (setup 1.5 hours, incubation: overnight)
4. Analysis of transformants via PCR (3 hours)

### **Creation of template DNA via PCR and gel purification (3.5 hours)**

1. This is for PCR fragments that may be resulting in multiple bands etc.
2. Run 50uL PCR reactions as per normal protocols
3. Pour agarose gel slightly thicker using extra wide comb
4. Prepare buffer but only fill until just level with top of gel but NOT covering gel
5. Add 3uL of loading dye to total PCR reaction and load total volume into gel
6. Run gel at low Voltage (45) for 20-30 minutes or until template is completely into gel.
7. Stop gel and top buffer to appropriate level. Run gel at normal voltage for 2x normal length to ensure good band separation.
8. Stain gel as per normal protocol and take a picture for reference.
9. At this point you want to minimize exposure to UV. Using light box and a face shield cut out bands of interest. Minimize the size of the band (<.2g) and place each band in individual gel purification tube in filter basket.
10. Add 3 volumes of GelBind to the gel slice (0.1g gel = 300uL GelBind)
11. Incubate for 2 minutes at 65C, invert to mix, then incubate another minute to melt gel. Continue the inversion process if gel is not melted right away (this can take up to 5 minutes).
12. Centrifuge spin filter tube for 10s at 10K g
13. Remove the filter, vortex the flow through, then pipette liquid back onto filter and replace filter into tube.
14. Centrifuge spin filter tube for 10s at 10K g then Discard flow through
15. Add 300uL GelWash buffer to filter basket
16. Centrifuge spin filter tube for 10s at 10K g then Discard flow through
17. Centrifuge spin filter tube for 30s at 10K g
18. Transfer filter basket to new collection tube.
19. Add 50uL of Elution buffer (or dH2O) directly onto center of white spin filter membrane.
20. Centrifuge spin filter tube for 30s at 10K g
21. Discard filter basket and refrigerate template DNA for later use.

### Vector Ligation (pGEM-T)

1. Make up a master mix containing (multiply by # of samples)
  - 2.5 uL 2X Rapid Ligation Buffer
  - 0.5 uL T4 DNA ligase
  - 0.5 ul pGEM-T vector
  - 1ul dH2O
2. Aliquot 4.5uL of master mix and add 0.5 uL of template and incubate at room temp for 60mins (Maximum transformants will result from an incubation at 4 C overnight)
3. Set reactions on ice (or freeze at -20 for long term storage)

### Transformation

1. Set heat block to 42 C.
2. Thaw (on ice) one tube of cells for each ligation (or half if you are doing 25 uL reactions).
3. Pipette 2uL of vector ligation into each cell tube and incubate on ice for 5min.
4. Heat shock cells at 42 for 30 seconds then transfer back to ice for 2 minutes
5. Add 250 uL of SOC to each tube (125 uL for half reactions).
6. Shake at 200 rpm horizontally at 37 C for 1 hour
7. Spread 2 dilutions (25uL and 100uL) onto appropriate plates and let soak in for 5-20 minutes in incubator before flipping. Spreading 25uL is plenty for good reactions, 50 uL for weaker bands.
8. Incubate at 37 overnight (18 hrs) then transfer to fridge for color resolution and longer storage. Seal with parafilm for very long term storage.

### Analysis of Transformants

1. Set up 10 PCR reactions (25uL) for each plate of successful transformants. These do not need to be hot start and use the T7 and M13R primers.
2. Using a sterile toothpick, pick colony from plate then swirl in PCR reaction. You may want to re-streak colonies on a new plate that is properly labeled.
3. Run PCR products out on Agarose gel and then analyze as you would any PCR reaction.