Bi 1X, Spring 2012

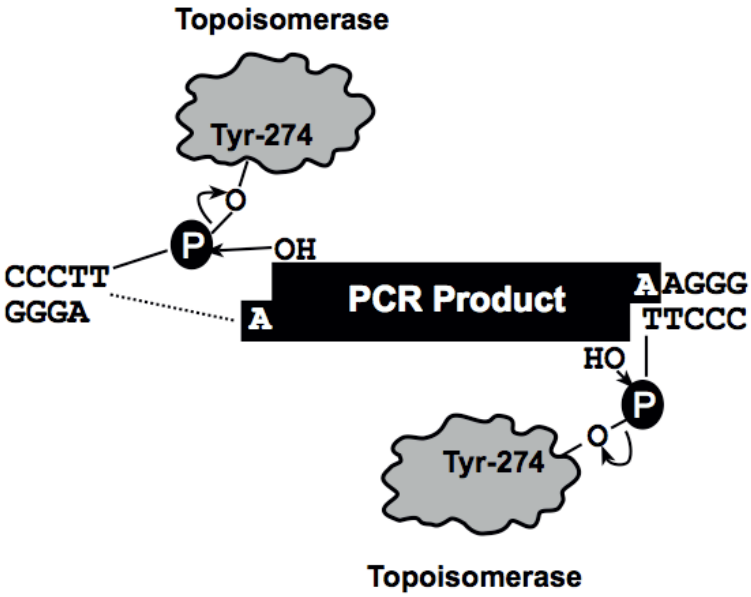
Week 6

- **TOPO TA Cloning**

Background:

TOPO mediated DNA cloning

When sequences are amplified with PCR, the Taq polymerase enzyme adds an extra (untemplated) deoxyadenosine ("A") on the 3'-end of the DNA fragments. These "A-overhangs" are complementary to the deoxythymidine "T" overhangs on the cloning site of the vector. The vector is supplied in the linear form and when you add a PCR product the complementary ends base pair to form a circular plasmid containing both vector and insert. The TOPO name in the kit refers to the fact that the vector contains topoisomerase I that is covalently bound to the vector at the insertion site. The topoisomerase enzyme has a ligase activity that ligates the phosphodiester backbone of the insert to the vector.[1]



The vector

In addition to 5'- "T-overhangs", the plasmid vector pCR4-TOPO contains the genes encoding for ampicillin and kanamycin resistance. Bacteria transformed with the plasmid can then be selected with media that contains either antibiotic (in this case, we will use kanamycin because it exhibits greater stability). The pCR4-TOPO vector contains a lethal gene, ccdB, which is fused to the LacZa gene. Upon disruption with a ligated PCR product, it permits only the growth of positive recombinants--i.e. cells that take up a vector without an insert will destroy themselves.

[1] Portions of this background section are from the Woods Hole microbial diversity course lab manual.

The cells

We will be using *E.coli* TOP10 competent cells ("One-Shot Cells") supplied with the kit. The term "competent" refers to the fact that these cells have been chemically treated to allow rapid uptake of DNA such as the pCR4-TOPO vector. The cells will be grown on agar plates containing Luria-Bertani broth (LB) and kanamycin.

Protocol:

1. **PCR Purification:**

   For more efficient cloning, you will first purify your PCR products from all sources of contamination using the Qiagen kit.

   a. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.**
      In your case, add 100 µL of Buffer PB to 20 µL PCR sample.
   b. **Place a QIAquick spin column (purple) in a provided 2 mL collection tube.**
   c. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 60s.**
   d. **Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.**
   e. **To wash, add 0.75 mL Buffer PE to the QIAquick column and centrifuge for 60s.**
   f. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the *empty* column for an additional 1 min.**
      IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
   g. **Place QIAquick column in a clean 1.5 mL microcentrifuge tube.**
   h. **To elute DNA, add 30 µL sterile water to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**
      IMPORTANT: Ensure that the sterile water is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. **Be careful not to poke the membrane with your pipet tip!**

2. **NanoDrop on purified PCR product:**

   Follow the protocol in Week 2.

3. **Cloning reaction:**

   a. Set up the following reaction on ice:

      4 µL PCR product
      1 µL salt solution
      1 µL TOPO vector*
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      6 µL Total volume

      *TOPO vector is extremely expensive. A TA will add the TOPO vector to your reaction when you are ready.

b. Mix gently with a pipettor. Spin it down briefly. Then incubate at **room temperature** for 10 minutes. After the reaction is complete, place the tube back on ice.

During this brief incubation time, the topoisomerase enzyme will ligate the ends of the pCR4-TOPO vector to your PCR products.

4. **Transformation reaction & plating:**

a. Place a tube of the TOP-10 competent cells on ice to thaw. Five minutes after the tube is thawed, proceed to the next step.

b. Add 2 µL of the TOPO cloning reaction and mix gently into the cells by flicking the tube lightly with your finger several times. **Do not mix by pipetting up and down, as the cells are very delicate.**

c. Incubate the cells on ice for 5 minutes.

d. **Heat shock** the cells by placing the tube in a 42ºC water bath for **exactly** 30 seconds **without** shaking.

Heat shocking causes these cells to uptake DNA by a mechanism that is not entirely understood. One theory is that the heat shock lowers the membrane potential to allow DNA to enter the cell more easily.[2]

e. Immediately transfer the tube to ice and add 250 µL of room temperature SOC medium.

SOC is a very rich medium that will help the cells recover after their "shocking" experience.

f. Ensure that the tube is screwed on **tightly**. **Securely** tape the tube horizontally to the bottom of the 37ºC incubator. Incubate at 37ºC, 200 rpm shaking for 1½ hours to allow the cells to recover.

g. Spread 75 µL and 150 µL of cells on two LB+Kan plates respectively. *See the demo performed by your TAs.* Incubate overnight at 37ºC.

[2] Panja S, *et al*. "**How does plasmid DNA penetrate cell membranes in artificial transformation process of Escherichia coli?**" Mol Membr Biol. 2008 Aug;25(5):411-22.