Bi 1x, Spring 2009

Week 5

- **Session 1**
  - Agarose gel electrophoresis of PCR products

- **Session 2**
  - TOPO cloning reaction
  - Bacterial transformation and plating

Session 1 – Agarose gel preparation and electrophoresis

In week 4, you loaded and ran agarose gels that your TAs had prepared for you. Today, you will run your pond sample PCR products on a gel that you prepare yourself.

Note: Each small gel box can accommodate two individual's samples. The large gel box holds up to eight individual's samples.

1. Set up your gel box for casting:

   i. For the small boxes, rotate the gel tray inside the gel box so that the open, rubber gasketed sides are blocked by the sides of the box. Place a purple comb in, 1.5 mm side down.

   ii. For the large gel box, place the tray into the casting apparatus. Insert both combs, one at the top of the tray and one at the midway point.

   Ask a TA if you are unsure about the set up!

2. Prepare 50 ml (if you are using a small gel box) or 100 ml (if you are using the large gel box) solution of 2% agarose in TAE (tris acetate EDTA) buffer.

   For the 50 ml solution: Mix 1 gram of agarose with 50 ml TAE.
   For the 100 ml solution: Mix 2 grams of agarose with 100 ml TAE.

   TAE stands for Tris-acetate plus EDTA. Tris-acetate is a buffer that maintains proper pH control during gel electrophoresis. EDTA is a chelator of divalent ions. EDTA is often used in experiments involving DNA, since DNA-degrading enzymes (DNases) require the divalent ion Mg^{2+} for activity.

3. To melt the agarose, heat the suspension in a microwave until it begins to boil. This may take less than a minute. Gently swirl the flask from time to time. **Be extra cautious, since super heated solutions of agarose have a tendency to boil explosively. Wear protective mittens when you handle the hot flask and keep it away from your face!**

Continue heating and swirling the agarose solution until it is **completely** clear and free of translucent agarose particles. [1]

4. Let the flask stand on your bench for about 1-2 minutes. You want to give the solution enough time to cool such that it does not damage the plastic casting tray, but not so much time that it begins to solidify. Pour the solution into your tray.

   If bubbles are present in your gel after pouring, use a clean pipette tip to pop them or move them to the end of the gel, where they will be out of the way of your samples.

5. **While the gel is hardening**, prepare your samples:

   To 10 µl of each of your 3 PCR reactions (no primer control, no template control, and full reaction), add 2 µl of 6X DNA loading dye.

6. **After the gel hardens**, set your gel box up for running:

   i. For the small gel boxes, remove the comb (by pulling upwards slowly), pull out the gel tray, rotate it 90º and place it back in the gel box, with the wells at the end that corresponds to the negative (black) electrode. Add TAE buffer to the box such that the gel is barely covered by buffer.

   ii. For the large gel box, remove the combs (by pulling upwards slowly), remove the gel tray from the casting device and place it in the gel box. Add TAE buffer to the box such that the gel is barely covered by buffer.

7. Load your gel:

   i. First, pipette in 5 µl of 100 bp ladder solution.
   ii. Next, pipette in each of your three dye/sample mixtures.

8. Plug your gel into a power supply and run it at 120 V (constant amperage) for 45 minutes to an hour.

9. After your gel is complete, stain it:

   i. Place your gel into the staining solution for 10 minutes.

      The staining solution contains ethidium bromide (EtBr). EtBr is a planar molecule that intercalates between the stacked base pairs of a DNA double helix. Upon irradiation with UV light, the EtBr fluoresces an orange-pinkish color, making it easy to locate the DNA fragments within the agarose gel matrix.

   ii. Place your gel into the destaining solution for 10-15 minutes.

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1. [1] Bertani, L. E. "Cell Biology Laboratory Manual, 2003/04" Caltech.

The destaining solution contains buffer without EtBr. Excess, non-DNA bound EtBr will seep out of your gel during the destaining step, reducing background.

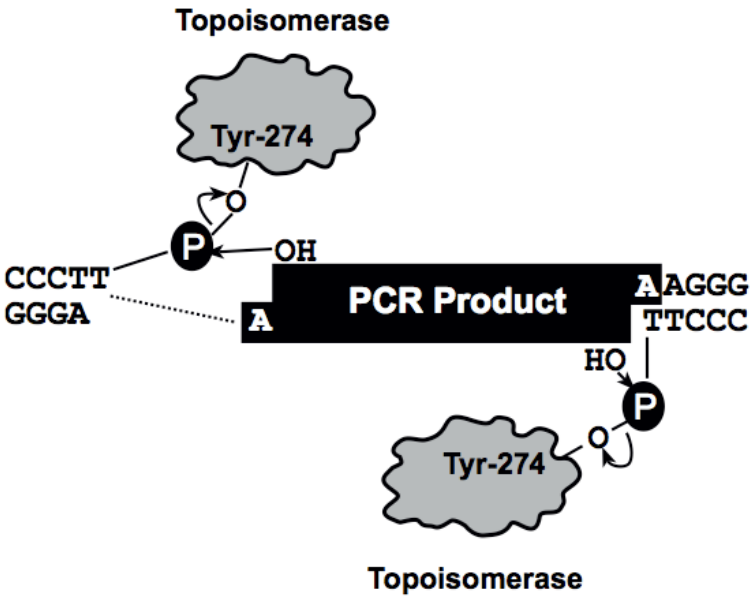
10. Image your gel on the AlphaImager (a TA will help you with this).

Session 2 – TOPO cloning of 16S PCR products

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.[2]



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

[2] Portions of this background section are from the Wood's Hole microbial diversity course lab manual.

exhibits greater stability). The pCR4-TOPO vector contains a lethal gene, ccdB, which is fused 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.

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.

Cloning reaction

1. 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.

2. Mix gently with a pipettor and incubate at room temperature for 5 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.

Transformation reaction & plating

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

2. Incubate the cells on ice for 7 minutes.

3. **Heat shock** the cells by placing the tube in a 42ºC water bath for **exactly** 30 seconds.

   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.[3]

[3] 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.

4. 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.

5. Ensure that the tube is screwed on tightly. Tape the tube horizontally to the bottom of the 37ºC incubator. Incubate at 37ºC, 200 rpm shaking for 1 hour to allow the cells to recover.

6. Spread 75 µl of cells on one plate and 150 µl of cells on another plate. *See the demo performed by your TAs.* Incubate overnight at 37ºC.