Cloning of DNA fragments in plasmid vectors

Please note that today’s work will be under S1 condition. Make sure that you follow the S1 rules at all times.

In today’s lab we will be cloning a gene amplified by Polymerase Chain Reaction (PCR) into a commercially available cloning vector called pCR2.1-TOPO. You will begin with PCR products from a reaction that used genomic DNA as a template, gene-specific primers, and Taq polymerase. One of the characteristics of Taq polymerase is that it adds a single deoxyadenosine (A) to the 3’ ends of PCR products. The resulting PCR products therefore have 3’ A-overhangs. The plasmid vector we will be cloning into has been linearized (it is no longer circular) and has 3’ deoxythymidine (T) overhangs. The “sticky ends” of the PCR product and the linearized vector are compatible and can form hydrogen bonds. This type of cloning, called TA cloning, is quicker and easier than traditional subcloning because it avoids the use of restriction enzymes.

Typically an enzyme called DNA ligase is used to join pieces of DNA together. Ligase repairs nicked DNA by creating a new phosphodiester bond between nucleotides. However, another, more efficient way to join the vector and insert together is to use an enzyme called topoisomerase. The advantage of using this method, called TOPO cloning (or more specifically in this case, TOPO TA cloning), is that the “ligation” step requires only 5 to 20 minutes at room temperature versus the longer incubation times required for ligase, which are most often performed at 16°C overnight for single base overhangs.

With TOPO cloning, the linearized ends of the vector are bound to a topoisomerase enzyme, which cleaves the phosphodiester backbone of DNA and covalently binds to the 3’ phosphate of the cleaved strand. Topoisomerases are normally used by cells to unwind and repair coiled DNA. This enzyme has been adapted by a biotech company (Invitrogen) to ligate the PCR product and the vector DNA together. Upon formation of the phosphodiester bond between the PCR fragment and the vector, the topoismerase is released from the vector.

After the ligation is complete, we will have a mixture of many circular molecules of DNA in the solution. Some of these molecules may be re-ligated vector without the PCR product, but we hope that many will be the plasmid containing our target PCR fragment. Please study the plasmid map and note that the amplified gene will be inserted into the LacZ gene (which encodes β-galactosidase) and disrupt the LacZ open reading frame (ORF). This means that E. coli cells that are transformed with plasmids that have the cloned gene will be white. Those bacteria that are transformed with plasmids that do not have the cloned gene inserted into them will be blue. This is because the LacZ gene is not disrupted and encodes a functioning β-galactosidase enzyme.

This vector also contains genes for resistance to ampicillin and kanamycin, so either of these antibiotics can be used to select for E. coli that have been successfully transformed with the plasmid. E. coli that do not have the plasmid will not grow on plates containing ampicillin or kanamycin.

For more background information on today’s protocol, please refer to the PDF file on the course website. This is the instruction manual for this particular cloning vector and will be useful when you are preparing your lab reports.
Once ligation is complete, we will use the heat-shock method to transform *E. coli* competent cells with the plasmid. After heat-shocking the bacteria, we will grow the bacteria for one hour at 37 °C to allow them to recover and begin synthesizing protein. Then we will plate out the bacteria on plates containing antibiotic and X-gal (substrate for β-galactosidase).

The bacteria will be allowed to grow overnight on the plates. We will then select white colonies and use them to start overnight liquid cultures. In the next lab, we will purify plasmid DNA from the liquid cultures to determine if it contains the target PCR fragment.

**Protocol:**

The ligation reaction:

- H₂O: 3 µl
- PCR product: 1.5 µl
- Vector: 0.5 µl

1) Let the ligation reaction sit at room temperature for 20 minutes.

2) Add 2 µl of ligation reaction to the *E. coli* competent cells and incubate on ice for 20 minutes.

3) Give the cells a “heat-shock” by incubating at 42 °C for exactly 30 seconds.

4) Put the cells back on ice for 2 minutes.

5) Add 250 µl of SOC media to the cells and incubate for 1 hour at 37 °C.

6) Prepare two plates: label one plate with your group number and ‘75’, label the other plate with your group number and ‘200’, and plate out 30 µl of X-gal on each plate. Incubate the plates at room temperature for 10 minutes.

7) Plate out 75 µl of transformed bacteria on the plate labelled with ‘75’.

8) Plate out the remainder of the transformed cells on the plate labelled with ‘200’.

9) Incubate for 12-16 hours at 37 °C. Afterwards, store plates at 4 °C.
The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. The complete sequence of pCR®2.1-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 24).

Comments for pCR®2.1-TOPO®

3931 nucleotides

LacZu fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809