7.13 Experimental Microbial Genetics

Fall 2008

<h3 style="text-align:center">Dephosphorylating with CIP and Ligation Reactions</h3>

1. Preventing Vector Recircularization using Calf Intestinal Alkaline Phosphatase:
Alkaline phosphatase catalyzes the removal of 5′ phosphate groups from DNA, RNA, ribo- and deoxyribonucleoside triphosphates. Since CIP-treated fragments lack the 5′phosphoryl termini required by ligases, they cannot self-ligate (1). This property can be used to decrease the vector background in cloning strategies.

DNA ligase requires that one of the two pieces of DNA to be joined have a 5'-phosphate (donor). Vector DNA cut with a single enzyme will have two 5'-phosphate ends and two 3' hydroxyl ends. Since they are on the same molecule, there is nothing to stop these from rejoing during a ligation. In fact, this reaction will proceed quite well, since it is essentially unimolecular. In order to minimize this reaction and favor ligation of the insert DNA, one can treat the digested vector DNA with Alkaline Phosphatase prior to purification. This enzyme will remove the 5'-phosphate groups. Since the insert will still have 5'-phosphates, vector-insert ligation will occur but vector-vector ligation will not. This procedure is also suggested when double-digesting a vector when the two cuts are extremely close. When the two sites are close, analysis by gel electrohoresis will not enable one to assess the extent that both enzymes have worked. If the DNA consists of a pool of single and double cut DNA, the single cut DNA can easily religate if 5'-phosphate is present and a high background of vector religation may result, even if the vector is gel purified.

Applications
- Removing of 5′ and 3′ phosphoryl groups from nucleic acids
- Preparing of templates for 5′ end labeling
- Preventing fragments from self ligating
- Dephosphorylation of proteins

Reaction Buffer
10X NEBuffer 3:[100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 @ 25°C)]. On thawing, DTT may precipitate, vortex to dissolve.

Dephosphorylating with CIP:
1. Suspend DNA in 1X NEBuffer (0.5 µg/10 µl).
2. Add 0.5 unit/µg vector DNA.
3. Incubate 60 minutes at 37°C.
4. Purify DNA by gel purification immediately after step 3.

Note
1) Slightly lower activity may be observed in any reaction buffer containing a total salt concentration of less than 50mM, under these conditions a 2-fold excess of CIP is recommended.
2) Since CIP treatment of vectors is the final vector preparation step before ligation it is better to gel purify the vector than use the PCR/enzyme clean up kit after CIP treatment. This will ensure that the cut vector is separated away from any uncut vector as well as

removing the CIP. Transformation with uncut vector occurs at a significantly higher efficiency than ligation reactions, and therefore must be removed before setting up ligations.

2. Ligation of DNA:

T4 DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA. This enzyme will join blunt end and cohesive end termini as well as repair single stranded nicks in duplex DNA, RNA or DNA/RNA hybrids.

Applications:
- Cloning of restriction fragments
- Joining linkers and adapters to blunt-ended DNA

Reaction Buffer:
T4 DNA Ligase Reaction Buffer (10X)

Setting up a Ligation Reaction:
In general, use a 1:1, 1:3 or 3:1 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector. However, if you have plenty of cut vector and insert set up four ligation reactions- vector:insert 1:1, 1:3, 3:1 and a control containing vector but no insert (**always** set up this control). These ratios will vary with other types of vectors, for example, cDNA and genomic cloning vectors. The following example illustrates the conversion of molar ratios to mass ratios for a 3.0kb plasmid and a 0.5kb insert DNA fragment.

$$\frac{\text{ng of vector } \times \text{ kb size of insert}}{\text{kb size of vector}} \quad \times \quad \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Example: How much 0.5kb insert DNA should be added to a ligation in which 100ng of 3kb vector will be used? The desired vector:insert ratio will be 1:3.

$$\frac{100\text{ng vector} \times 0.5\text{kb insert}}{3\text{kb vector}} \quad \times \quad \frac{3}{1} = 50\text{ng insert}$$

The following ligation reaction of a 3kb vector and a 0.5kb insert DNA uses a 1:1 vector:insert ratio. Typical ligation reactions use 100–200ng of vector DNA.

1. Assemble the following reaction in a sterile microcentrifuge tube:

vector DNA	100ng
insert DNA	17ng
Ligase 10X Buffer	1µl
T4 DNA Ligase	0.5-1µl
Nuclease-Free Water to final volume of	10µl

2. Incubate the reaction at: 16°C overnight.