Ligation of DNA for cloning
by Howard Judelson 11.06

1. Determine concentration of vector and insert. The easiest way to do this is to run a standard agarose gel containing ethidium bromide, along with standards of known concentration. For the standards, prepare a 10 µg/ml stock solution of linear DNA (such as lambda or a digested plasmid). Load 10, 30, and 100 ng as standards on the gel (you may wish to add water or gel buffer to the 10 and 30 ng samples to make loading the gel easier). In nearby lanes, run a minimal amount of your DNA, maybe about 30 ng. If you have purified your DNA using a kit from a prior reaction step (such as using the GeneClean or Wizard kits, you can assuming a 50% yield). Run the gel briefly (let the bromophenol blue dye run about 1 cm). View the gel.

By comparing the fluorescence of your samples and the standards, you can judge the DNA concentration with reasonable accuracy. This also confirms that your DNA is not degraded.

2. Calculate the amount of insert and vector needed. Aim for a DNA concentration about 10-20 ng/µl. Typically, I try to get to 20 ng/µl in a ligation of 10-20 µl. For sticky end ligations, aim for an average insert to vector molar ratio of about 2:1, or 1:1 if the vector DNA has been treated with alkaline phosphatase. A 5:1 ratio is suggested for blunt-end ligations. Please note that this is a molar ratio: you need to consider the size of the DNA, not just its concentration.

Some theory: Optimal conditions for ligation depend on the length of the molecule, the size of the DNA, whether the ends are cohesive or blunt, and whether both ends of the vector have the same restriction site. This involves calculations related to both the DNA concentration of DNA ends (not the total DNA concentration), the flexibility of DNA, etc.

A common error is to add too much insert, or have too high an overall DNA concentration. In such cases, there will be a tendency to ligate 2 or more insert fragments into the vector, or a to form dimers, trimers, etc. of vector or insert that are non-productive for cloning. In other words, the optimum results occur when the absolute concentration of termini is high enough to favor intermolecular ligation but not so high as to cause the formation of extensive oligomers.

Therefore, insert:vector molar ratios between 2 and 6 are best. Ratios below 2:1 result in lower ligation efficiency. Ratios above 6:1 promote multiple inserts or the formation of long chains. For critical cloning experiments, such as making a library, it is generally useful to test several ratios of insert to vector. Nevertheless, here are some guidelines:

In general, for ligations of fragments with cohesive ends, one can achieve the greatest number of recombinants when the molar concentration of insert is 2x that of the vector. For a 3 kb vector, the optimum concentration of vector (not total DNA) is 10-20 ng/µl; for a 10 kb vector, the optimum is 5-10 ng/µl (a lower concentration of DNA is required for larger vectors as the likelihood that the ends of the same molecule will interact is less than for smaller vectors).

3. Set up the ligation. I normally aim for a minimum volume of 10 µl. Anything greater than 25 µl is usually just a waste of reagents. Also, Step 2 will help indicate the best volume. The following is a typical recipe:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X ligase reaction buffer (containing ATP, BSA)</td>
<td>1 µl</td>
</tr>
<tr>
<td>vector, insert, and water</td>
<td>8.5 µl</td>
</tr>
<tr>
<td>T4 DNA ligase (NEB, low conc.)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>total volume</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
Mix gently by flicking with your finger, and spin for 2 seconds in a microcentrifuge to get the liquid down to the bottom of the tube.

Incubate at 4°C to 20°C (room temperature) for 4 to 24 hours.

**Considerations in picking the best temperature:** The optimal incubation temperature for T4 DNA ligase is 16°C; when very high efficiency ligation is desired (e.g. making libraries) this is recommended. However, ligase is active over a broad range. For routine purposes such as subcloning, convenience often dictates incubation time and temperature; ligations performed at 4°C overnight, or at room temperature for 30 min to a couple of hours also can work well. Nevertheless, for sticky end ligations, between 4°C to 12°C often works best since this helps the DNA termini to anneal. It follows that for blunt ends, there is no harm doing ligations at room temperature.

After ligation has proceeded (don't go longer than 24 hour, even at 4°C, freeze the ligation just in case DNAses are present.

4. **Other tidbits:** Not all manufacturer's sell T4 ligase at the same concentration, or use the same unit definition. Be careful!

T4 DNA ligase is impaired at high salt concentrations. That is one reason why it is generally good to purify DNA from restriction digests, which are often in 50-100 mM salt. Ligations can be performed in low-salt restriction buffers (such as NEB 1 and 4), or other low-salt buffers such as most Klenow buffers, as long as ATP is added to 1 mM. Ligations work moderately in NEB buffer 2.

ATP is provided with most ligation buffers (for example, ligase from NEB). Note, however, that ATP breaks down over time so buffers should be discarded after ~2 years. For the same reason, thaw the buffers in your hand or at room temperature, not at 37°C.

ATP concentrations above 0.5 mM inhibit ligation of blunt ends.

Rapid ligation can be achieved by adding PEG6000 to the ligation (5% w/v). This is normally what is added to many of the "fast ligation" kits on the market. However, at high DNA concentrations one may get too many oligomers, rather than simple circles.

The DNA concentrations described above are for vector-insert ligations, not other applications such as linker addition.

Make sure that at least one of your molecules contains a 5’ phosphate! Oligos, for example, lack phosphate at their 5’ ends.

There are ligases other than T4 DNA ligase! Make sure that you use the correct one!