Protocol 8: Ligating DNA fragments together

**Background:**

DNA fragments are “glued” together by enzymes referred to as ligases. Ligases catalyze the formation of a phosphodiester bond between directly adjacent 3’-hydroxyl and 5’-phosphoryl termini of nucleic acid molecules. To effectively suppress re-circularization of vector DNA, an enzymatic dephosphorylation reaction is often performed to remove the 5’-phosphate. Ligases may join either DNA or RNA substrates and often require cofactors such as ATP or NAD+. DNA ligases are used as tools to create novel combinations of nucleic acid molecules and to attach them to vectors for molecular cloning. We will be using a bacteriophage ligase cloned from the T4 bacteriophage. This enzyme has a $K_m$ of $6 \times 10^{-7}$ M for cohesive ends, $5 \times 10^{-5}$ M for blunt ends, and $\sim 5 \times 10^{-5}$ M for ATP. It is capable of taking DNA-DNA, DNA-RNA, and RNA-RNA substrates. For less efficient ligations, agents such as polyethylene glycol are often used in T4 DNA ligation reactions to increase macromolecular crowding and thereby increase the rate of ligation by up to three orders of magnitude. In addition, this enzyme has the ability to discriminate between perfectly and imperfectly paired ends.


**Materials:**

<table>
<thead>
<tr>
<th>DNA vector</th>
<th>DNA insert</th>
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<tbody>
<tr>
<td>T4 DNA ligase</td>
<td>10X T4 ligase buffer</td>
</tr>
<tr>
<td>assorted pipetmen</td>
<td>assorted pipet tips</td>
</tr>
<tr>
<td>1.5 ml microfuge tube</td>
<td>bench top microfuge</td>
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</tbody>
</table>

**Method:**

1. Place the ligase buffer, DNA vector, and DNA insert to be ligated together on ice. Label a 1.5 ml microfuge tube accordingly.
2. Add the following components to the microfuge tube in the order listed with a L20 pipetman:

\[
\begin{align*}
\text{nanopure water} & \quad (8.5 - x - y) \text{ µl} \\
\text{DNA vector} & \quad x \text{ µl} \\
\text{DNA insert} & \quad y \text{ µl} \\
\text{ligase buffer} & \quad 1.0 \text{ µl} \\
\text{T4 DNA ligase} & \quad 0.5 \text{ µl} \\
\text{total volume} & \quad 10.0 \text{ µl}
\end{align*}
\]

3. Mix the contents of the tube by pipetting the solution up and down with the L20 pipetman.
4. Leave the tube on your bench top at room temperature for 15 - 20 minutes. For cohesive (sticky) ends, the ligation reaction can proceed in only 20 minutes; however, for a blunt-ended ligation, the ligation reaction should be allowed to proceed for at least 2 hours prior to transformation. For larger inserts, ligations can be carried out at 16°C overnight to allow for proper circularization of DNA.
5. Proceed to transformation protocol to transform the ligated plasmid DNA. Alternatively, the ligation reaction may be stored in the -20°C freezer until ready for use.

The volumes of the DNA added to the ligation reaction depend on the final concentration of your DNA samples. Ligation reactions are set up with varying insert:vector molar ratios, where ratios of 5:1, 10:1, or 20:1 are typically used. The ratio that yields the highest ligation efficiency depends on the relative sizes of the insert and vector DNA, the purity of the DNA, ligation time, and laboratory technique. Ratios are typically optimized by trial-and-error for each researcher in the laboratory. In typical ligation reactions, approximately 25-50 ng of vector DNA is added. The corresponding amount of insert DNA is then determined based on the selected molar ratio and concentration of the insert sample. As an example, for a ligation reaction between a 4,500 bp vector and a 700 bp insert at respective concentrations of 100 ng/µl and 120 ng/µl the required amounts of each sample are determined as follows:

- Volume vector (µl) = (desired amount) / (concentration) = 50 ng / (100 ng/µl) = 0.5 µl
- Volume insert (µl) = (desired amount) * (molar ratio) *[(size insert) / (size vector)] / (concentration)
  = 50 ng * 10 * (700 bp / 4500 bp) / (120 ng/µl) = 0.65 µl

More information about ligation troubleshooting can be found at NEB website for T4 DNA ligase: http://www.neb.com/nebecomm/products/faqproductm0202.asp