Linker Ligation

This is a protocol for ligating a linker (prepared from two annealed oligonucleotides) in to a plasmid. Useful for modifying vectors (ie, changing the multiple cloning site, adding poly-his tails, etc)

Special Materials

Linker buffer (50mM Tris-HCl pH 8.0, 100 mM NaCl, 1mM EDTA)
PNK (polynucleotide kinase, plus the buffer included with this enzyme)
ATP (1 mM solution)
T4 Ligase (and included buffers)
Qiaquick Gel Extraction Kit

1. Order top and bottom oligonucleotides in 100 nmole scale, HPLC-purified.

2. Dilute both oligonucleotides to 100 µM in **Linker Buffer** (see above)

3. Mix 10 µL of each oligonucleotide together in a PCR tube to net 20 µL of a 50 µM linker solution.

4. Place linker tube in a PCR thermocycler and anneal the linker using the following cycle:
   - 95^0C for 2 min.
   - 52^0C for 10 min.
   - Hold at 4^0C

5. Meanwhile, cut the target plasmid with the appropriate restriction enzyme(s) and gel-purify it on a 0.8 % agarose gel using the Qiagen Qiaquick Gel Extraction Kit (follow kit instructions). Elute the mixture from the Qiaquick column in **30 µL EB** (instead of the prescribed 50 µL).

6. After annealing the linker, prepare the following 20 µL phosphorylation reaction mixture (use PCR tubes):

2 µL	Annealed linker (from step 4)
1 µL	PNK (polynucleotide kinase, 10 units)
2 µL	10X PNK Buffer A (using NEB PNK system)
1 µL	1 mM ATP solution (1 nmol)
14 µL	ddH_2O (sterile)

7. Place the phosphorylation reaction in the thermocycler and process as follows:
   - 37^0C for 30 minutes
   - 65^0C for 15 minutes (heat inactivation)
   - Hold at 4^0C

8. Set up the following Ligation Mixture (also set up a negative control that uses 5 µL of plasmid, omits the linker, and uses ddH_2O to make up the difference). *NOTE that for troubleshooting purposes, you can vary the plasmid:linker ratio to optimize the ligation, ie try 5 µL plasmid and 1 µL linker*:

0.5 µL	phosphorylated linker (from step 7)
15.5 µL	digested, purified plasmid (from step 5)
2 µL	10x T4 ligase buffer (NEB system, well-thawed and rediluted)
2 µL	T4 ligase (5 Weiss units)

9. Incubate ligation reaction overnight at 16^0C in the PCR machine.

10. Heat-inactivate the ligation reaction (65^0C for 15 minutes), then transform entire ligation reaction in to E. coli using the standard method. Plate on appropriate selection.

11. Perform minipreps and restriction analysis, as well as sequencing, to confirm the presence and reading frame of your linker. A good strategy is to cut the plasmid with a restriction enzyme that should only cut within the linker, and not the original plasmid. Pair this cut with one that will cut both wild-type and linker-ligated versions of the vector and then look for a miniprep that produces two bands on a gel, representing a plasmid that has incorporated you linker.