

## Ligation of cDNA into Vector (Subcloning)

- 1) To prepare the insert and vector for ligation, digest ~5ug of plasmid and cDNA vector with appropriate enzymes in 20ul volume and load on gel across 2 lanes.
- 2) **If you do not have cohesive ends, blunt-end your fragments.** Before loading on gel, heat inactivate the enzyme at 70°C for 15 minutes, add 1ul of each nucleotide from the High Prime kit (Roche) and 1ul of Klenow (Roche-red cap) to 10ul of each preparation before gel purification. (The Klenow can use the buffer that is already present.) Then proceed to gel purification. If you prefer to blunt without filling-in, Mung Bean Nuclease can be used.
- 3) After gel has completely separated the bands, gel purify the digested fragments using the Qiaquick gel extraction kit or, for larger plasmids, use the Qiaex II kit. See separate protocols for gel extraction.
- 4) Post gel-extraction, run an aliquot (1-2ul) diluted in 5ul of water and 1ul loading dye, on a gel with 5ul of DNA Ladder I for relative quantification.
- 5) Ligate as follows with the following controls: Roche and New England Biolabs T4 DNA Ligase both work well

When using different restriction sites on each end:

<p>1) Control for uncut vector in your vector prep</p> <p>Vector 50-100ng    1ul  10x Ligase buffer    1ul  H2O                    8ul</p>	<p>2) Control for complete digestion of vector</p> <p>Vector 50-100ng    1ul  10x Ligase buffer    1ul  H2O                    7ul  High Conc Ligase    1ul</p>
<p>3) Control of plasmid contamination in insert prep</p> <p>insert (5x molar)    5ul  10x Ligase buffer    1ul  H2O                    3ul  High conc Ligase    1ul</p>	<p>4) Actual Ligation 1:5 molar ratio</p> <p>Vector ( 50-100ng) 1ul  insert (5x molar)    5ul  10x Ligase buffer    1ul  H2O                    2ul  High conc Ligase    1ul</p>

6) If only using a single cutter, vector must be alkaline phosphatased to prevent self-ligation. Can use shrimp alkaline phosphatase from ROCHE. (Calf intestinal Phosphatase is also a good product). Keep some vector not treated for controls #1 and 2 above.

- a. Vector post gel extraction + water 8ul  
 10x SAP buffer 1ul  
 SAP 1ul  
 Incubate at 37°C for 15 minutes (for blunt-ends, incubate 1hr)  
 Incubate at 70°C for 15 minutes to inactivate

7) Set up reactions as follows when singly cut:

1a) Control for uncut vector in your vector prep	2a) Ligation control
Blunted Vector 50-100ng 1ul	Blunted Vector 50-100ng 1ul
10x Ligase buffer 1ul	10x Ligase buffer 1ul
H2O 8ul	H2O 7ul
	High Conc Ligase 1ul
3a) Control for uncut vector in your SAP'd prep	4a) Control for SAP
Vector SAP'd 50-100ng 1ul	Vector SAP'd 50-100ng 1ul
10x Ligase buffer 1ul	10x Ligase buffer 1ul
H2O 8ul	H2O 7ul
	High Conc Ligase 1ul
5a) Control of plasmid contamination in insert prep	6a) Actual Ligation 1:5 molar ratio
insert (5x molar) 5ul	Vector SAP'd ( 50-100ng) 1ul
10x Ligase buffer 1ul	insert (5x molar) 5ul
H2O 3ul	10x Ligase buffer 1ul
High conc Ligase 1ul	H2O 2ul
	High conc Ligase 1ul

NOTE: For all reactions, the amount of vector and insert should be kept constant so that you can compare the number of colonies per plate at the end.

- 8) Allow ligation reactions to incubate at 11-14°C overnight in the cold room water bath.
- 9) Using 5ul of the ligation reaction (freeze other 5ul) perform the transformation (see separate protocol). For large plasmids, over 10kb, use XL10 Gold cells (Stratagene) (don't forget to add 2ul of BME to 50ul cells). Otherwise, GC5 (PGC Scientific) cells, similar to DH5 alpha, are ok to use. Plate 150ul of transformants onto 10cm plate with appropriate antibiotics.
- 10) Incubate plates overnight at 37°C and assess growth.

#### Interpreting results:

Expected results for double cut plasmids:

Ligation #1) No colonies

Ligation #2) No colonies

Ligation #3) No colonies

Ligation #4) Lots of colonies

Expected results for singly cut SAP'd plasmids:

Ligation #1) No colonies

Ligation #2) Lots of colonies

Ligation #3) No colonies

Ligation #4) No colonies

Ligation #5) No colonies

Ligation #6) Lots of colonies

Notes:

- If you have colonies on plate #1 or 1a, then you have incomplete digestion. If this is the case, reisolate the vector with complete cutting.
- If you do not have colonies on 1a or 2a, then the ligase isn't working
- If you have colonies on 3 or 5a, then your insert preparation has uncut plasmid contamination. If this is the case, reisolate your insert with complete cutting.
- If 4a has more colonies than 3a, then the SAP did not work.
- If 1a has no colonies and 2a has colonies and 4a does not have colonies, then your phosphatase worked!
- If you have more colonies on 6a than either 3a, 4a or 5a, then your ligation probably worked and you should pick colonies.

If results are as expected, pick colonies and inoculate into 3-5mls of LB with antibiotics using a 14ml polypropylene tube with snap cap. Grow overnight and do miniprep (see separate protocol). Do a diagnostic digest to confirm insertion.

