

SOP: SP015**Ligation of DNA fragments with sticky ends (vector/insert ligation)****Materials and Reagents:**

1. Quantitated vector and insert DNAs (notes 1,2)
2. Sterile 0.2 ml PCR vials or 0.65 ml microcentrifuge vials (note 3)
3. Ice
4. T4 DNA ligase (recommended: New England BioLabs T4 DNA ligase cat#M0202S)
5. 10X T4 DNA ligase buffer containing ATP
6. Sterile MilliQ water
7. Sterile 10 or 20 µl pipet tips
8. Pipetman p10 or p20
9. Microcentrifuge
10. 16°C bath or thermal cycler
11. 65°C bath or thermal cycler

Protocol:

1. ____ On ice, add sterile water to 0.2 ml vial, volume based on ligation design (notes 4,7)
2. ____ Add insert and vector DNAs in 1:1 and/or 3:1 molar ratio (notes 5 – 7)
3. ____ For each vector/insert variation, assemble an identical vector-alone control ligation (note 7).
4. ____ Thaw 10X T4 DNA ligase buffer (note 8).
5. ____ Add 10X T4 DNA ligase buffer to DNA solution at 0.1X final reaction volume
eg. 1.5 µl to 15.0 ligation. (note 7)
6. ____ Add 1.0 µl of T4 DNA ligase per 10-20 µl ligation. To avoid glycerol toxicity, dilute enzyme at least 10×.
7. ____ Mix by tapping vial or by gentle pipet mixing.
8. ____ Spin tube briefly at 5-10,000 rpm to ensure all material is at bottom of vial.
9. ____ Incubate ligations at 16°C for minimum of 4 hours. (note 9)
10. ____ Incubate in bath or thermal cycler.
Tetrad cycler, STD menu, program LIG16 (doesn't include heat deactivation).
Hybaid cycler, program A-09 (Ligation, 9 hr at 16°C, 15 min at 65°C). (note 10)
11. ____ Deactivate ligase by incubating at 65°C for 10 minutes in bath or thermal cycler (note 10).
12. ____ Ligation is ready for transformation into competent cells or other downstream application.

Notes:

1. This procedure is based on vector DNA that has been dephosphorylated or contains non-compatible sticky ends (restriction endonuclease generated overhangs).
2. Vector and insert DNAs must be quantitated either by spectrophotometric evaluation (SOP014) or by comparison of an aliquot with DNA quantitation standards by agarose gel electrophoresis (SOP018).
Recommended standards: Invitrogen High Mass DNA Ladder cat no. 10496-016
Invitrogen Low Mass DNA Ladder cat no. 10068-013
Follow manufacturer's instructions for load volumes of standards.

3. Assemble ligation to be incubated in thermal cyclers in 0.2 ml vials. Ligations incubated in baths can be assembled in 0.2 ml or 0.65 ml microvials.

4. Ligations can be done successfully over a range of DNA concentrations (1-20 ng/μl per ligation reaction) in a range of volumes necessary to achieve these concentrations. Typical ligation volumes are 5-20 μl.

5. Insert to vector molar ratios of 3:1, 1:1 and 1:3 are common. Because quantitation of vector and insert is usually done with small quantities of DNA, concentrations are estimates within a range of ~2-3× the true value. As a consequence, selecting a ratio that will give the most efficient match of vector and insert is difficult. Efficiency of ligation is also dependent on completeness of digestion of component ends. Combining components at 1:1 and/or 3:1 insert:vector will usually generate recombinants. If either component is present in large imbalance (eg. greater than 5×), concatamer artifacts can skew the outcome.

6. *Calculation of insert and vector quantities and ligation volumes for 1:1 and 3:1 ratios*

NOTE: Choice of ligation volume and concentration varies. They depend on quantities of vector and insert available.

Example: Vector is pET23b, 3666 bp

Insert is 800 bp

Divide bp vector by bp insert: $3666/800 = 3.75\times$

ie, 1 mole insert requires 3.75 moles vector for equal number of molecules

a. For a 1:1 insert:vector ratio:

1.
 - Insert requires 3.75× quantity of vector for a 1:1 molar ratio
 - So, 1.0 ng insert contains same number molecules as 3.75 ng vector
 - Expanding to realistic quantities:
 - a. 10 ng insert contains same number molecules as 37.5 ng vector
 - b. 20 ng insert contains same number molecules as 75 ng vector, and so on
2. Determine ligation volume for 1:1 insert and vector at 5 ng/μl using 20 ng insert and 75 ng vector
 $(20 \text{ ng insert} + 75 \text{ ng vector})/5 \text{ ng}/\mu\text{l} = 19.0 \text{ ul ligation}$

See example ligation note 7, below.

b. For a 3:1 insert:vector ratio:

If 1 ng insert has same number molecules as 3.75 ng vector for 1:1 ratio then 3 ng insert is required for 3:1 ratio

1. 3 ng insert is required for 3.75 ng vector (3:1 ratio)
Expanding to a realistic quantity:
30 ng insert is required for 37.5 ng vector
2. Determine ligation volume for 3:1 insert and vector at 5 ng/μl
 $(30 \text{ ng insert} + 37.5 \text{ ng vector})/5 \text{ ng}/\mu\text{l} = 13.5 \mu\text{l ligation}$

7. Example ligation reactions at 1:1 vector:insert molar ratio described in note 6
5 ng/μl, 19 μl final vol,

Assume quantitated vector is 50 ng/μl, with 75 ng to be used in ligation

Assume quantitated insert is 15 ng/μl with 20 ng to be used in ligation

	<u>V+I</u>	<u>V alone</u>
Vector 75 ng (V)	1.5 μl	1.5 μl
Insert 20 ng (I)	1.3 μl	-

H ₂ O	13.3 μ l	14.6 μ l
10X ligation buffer	1.9 μ l	1.9 μ l
T4 DNA ligase stock	<u>1.0 μl</u>	<u>1.0 μl</u>
	19.0 μ l	19.0 μ l

8. Minimize time 10X Lig buffer (containing ATP) and T4 DNA ligase are kept above -20°C . ATP activity depletes quickly. Hold both on ice.

9. New England BioLabs T4 DNA ligase (cat#M0202S) instruction lists 16°C incubation with no time defined. Alternatively, NEBL recommends room temperature ($20\text{-}25^{\circ}\text{C}$) ligation for 10 minutes. Most users incubate reactions overnight at 16°C , however conditions are flexible. Consider removing part of the ligation mixture after 2-4 hr for transformation into competent bacteria, incubating the remaining volume overnight.

10. Deactivation of ligase before bacterial transformation is not absolutely necessary. However, deactivation contributes to higher transformation efficiency and is easily done at the end of a thermal cycler incubation.

Reference:

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual (2nd Edition). pp 1.53-1.71