SOP: SP016

Ligation of DNA fragments with blunt ends (vector/insert ligation)

Materials and Reagents:

1. Quantitated vector and insert DNA (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. 16-25°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 insert/vector ratio, 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 16-25°C for minimum of 4 hours to overnight. (note 9)

10. _____ Incubate in bath or thermal cycler.
    Tetrad cycler, STD menu, program LIG16 (doesn't include head deactivation).
    Hybaid cycler, program A-09 (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 not been dephosphorylated and has no 5' or 3' 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 elecrophoresis (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 microcentrifuge vials.
4. Blunt-end ligations can be done successfully over a range of DNA concentrations (20-50 ng/µl per ligation) 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×
ie, 1 mole insert requires 3.75 moles vector for equal number of molecules

a. <u>For a 1:1 insert:vector ratio</u>:
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. 60 ng insert contains same number molecules as 225 ng vector, etc.

2. Determine ligation volume for 1:1 insert and vector at 20 ng/µl
using 60 ng insert and 225 ng vector
(60 ng insert + 225 ng vector)/20 ng/µl = 14.3 ul ligation

See example ligation note 7, below.

b. <u>For a 3:1 insert:vector ratio</u>:
If 1 ng insert has same number molecules as 3.75 ng vector for 1:1 ratio then
<u>3 ng</u> 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:
90 ng insert is required for 112.5 ng vector

2. Determine ligation volume for 3:1 insert and vector at 20 ng/µl
(90 ng insert + 112.5 ng vector)/20 ng/µl = 10.0 µl ligation

7. Example blunt ligation reactions at *1:1 vector:insert molar ratio* described in note 6
20 ng/µl, 14 µl final vol,

Assume quantitated vector is 50 ng/µl, with 225 ng to be used in ligation
Assume quantitated insert is 15 ng/µl with 60 ng to be used in ligation

	V+I	V alone
Vector 225 ng (V)	4.5 µl	4.5 µl
Insert 60 ng (I)	4.0 µl	–
H_2O	3.1 µl	7.1 µl
10X ligation buffer	1.4 µl	1.4 µl
T4 DNA ligase stock	1.0 µl	1.0 µl
	14.0 µl	14.0 µl

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 recommends 16-25 °C incubation for T4 ligase. Blunt end joining requires longer incubation than sticky end ligation. Most users incubate reactions overnight at 16−25 °C, however conditions are flexible. Consider removing part of the ligation mixture after 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