Standard Operation Procedure (S.O.P.) for Ligase Reaction

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This S.O.P. will direct you in the ligation reaction procedure so as to subclone cDNA in plasmids designed for protein expression in different heterologous expression systems including *Xenopus laevis* oocytes (through *in vitro* cDNA transcription and cRNA microinjection into oocytes), e. coli (*Escherichia coli*), yeast (*Pichia pastoris*), and baculovirus systems in insect (*Spodoptera frugiperda*, *Sf9* and *Sf21*, and *Trichoplusia ni*, *High Five*) and mammalian (human embryonic kidney, HEK293) cells. The reaction is mediated by the enzyme T4 DNA Ligase (New England BioLabs, Cat. No. M0202S), which catalyze the formation of a phosphodiester bond between two double-stranded DNA (dsDNA) strands by joining the 5'-phosphate and the 3'-hydroxyl groups of adjacent nucleotides. This ligase can be used for joining blunt-ended and cohesive ended DNA segments. Furthermore, the T4 ligase can join dsRNA to either dsDNA or dsRNA but will not join single-stranded nucleic acids. Ligase enzyme concentration is given in different units per mL, depending on the company. A Cohesive Unit (not Weiss Units) is usually defined as the amount of T4 Ligase enzyme that is required to catalyze the ligation of greater than 50% of the *HindIII* fragments of 1 μg of lambda DNA (linear DNA obtained from the bacteriophage lambda) in 30 minutes at 16 °C in a reaction volume of 20 μL.

Different factors could affect the T4 Ligase reaction, including:

- Component storage temperature: T4 Ligase enzymes and its corresponding buffer must be kept at –20 °C. The buffer contains adenosine triphosphate (ATP); therefore, the buffer must be aliquoted to minimize degradation of the ATP and Dithiothreitol (DTT). The DTT may precipitate upon cooling.
- Conditions such as temperature, buffer composition, pH, ionic strength, enzyme co-factors, and vector-to-insert molar ratio affect T4 Ligase activity. Of particular interest are Mg^{2+} concentration (10 mM), ATP, and DTT.
- The balance between the optimal temperature of theT4 Ligase (25 °C) and the temperature necessary for ensure annealing of DNA fragment ends. The annealing temperature vary with the length and base composition of the overhangs. Overhangs shorter than 16 bases require smaller temperatures. Ligation reactions at lower temperatures require longer incubation times. Blunt-end ligations generally are efficient at temperatures between 15–20 °C for 4–18 hours. Sticky ends are efficiently ligated at 22 °C for 3 hours. Both ligations can also be performed at 4–8 °C overnight.
- The size of the DNA fragment to be subcloned. The smaller the size the easier it is to clone. As the fragment size increases over 5 kb the cloning efficiency drops. Fragment as large as 10 kb has been subcloned. DNA fragments larger than 15 kb are more easily cloned in lambda vectors and still larger fragments, up to 50 kb, can be cloned into cosmid vectors. Very large DNA fragments can be cloned into P1, BAC, and YAC vectors.
- DNA Purity. Relative pure DNA is required for high ligation efficiency. The DNA fragment to be subcloned and the vector must be isolated and purified from agarose gels.
- EDTA. DNA storage buffer, such as Tris–EDTA (TE), usually contains low concentrations of EDTA (1 mM) to prevent DNA degradation by nucleases. EDTA interferences the ligation reaction by chelating Mg^{2+} ions.
- The vector-to-insert molar ratio. The ideal vector-to-insert molar ratio is typically 1:1 or 1:2, but usually this must be empirically determined. Vector-to-insert ratio could range from 10:1 to 1:1 to 1:10. The molar ratios 3:1, 1:1, and 1:3 are commonly used.
- The overall concentration of vector + insert should be between 0.001–0.01 μg/μL. A typical

ligation reaction uses 25–100 ng of vector and the DNA fragment mass calculated based on the vector-to-insert molar ratio. The total reaction volume is usually 20 μL

- Pretreated of the vector DNA with alkaline phosphatase. This treatment prevents vector reconstitution when the experimental design is based on one restriction enzyme. Be sure that the phosphatase was completely inactivated or purify the vector
- Addition of polyethylene glycol 8000 (PEG 8000). PEG to a final concentration of 5% (w/v) can promote ligation of blunt-ended fragments by "molecular crowing". However, PEG is not recommended due to undesirable concatemerization. Only use PEG as last resort.

Important:

- **If the incubators and baths have not been recently used, check that the temperature is correctly set by using a calibrated thermometer.**
- **Enzyme should be the last component added to the reaction and should be taken from the freezer at the right moment of adding it to the reaction. Keep the enzyme in ice all the time.**
- **Thaw and resuspend the buffer at room temperature. Assemble the reaction at room temperature to avoid precipitation of the DTT.**
- **If your DNA sample was storage in EDTA-containing buffers or you do not know the buffer of your sample, purify your DNA first and resuspend it in nuclease-free water.**
- **The T4 Ligase could be heat inactivated at 65 °C. This procedure should be used to stop the ligation reaction.**
- **If the volume that is needed for any of these reagents is less than 1 μL, a stock dilution should be prepared to add 1 μL of the reagent to the reaction tube.**

Materials:

- 70% Alcohol
- Micropipettes and Tips for 2, 10, and 20 μL
- 0.5 μL Microtubes
- T4 DNA Ligase Enzyme (stored at −20 °C)
- T4 DNA Ligase Reaction Buffer (10X; stored at −20 °C)
- Linearized DNA To Be Ligated (vector + fragment; stored at 4 or −20 °C)
- Nuclease-free Water (stored at room temperature)
- Ice Bucket
- Water Bath
- Incubator for Cold Temperatures (optional)
- Dry Bath at 65 °C (optional, for T4 DNA Ligase inactivation)
- Microcentrifuge for 0.5 μL Microtubes
- Vortex

Procedures:

This procedure is designed to set-up a ligation reaction using 100 ng of **Vector** at a 1:1 vector-to-insert molar ratio in a total reaction volume of 20 μL. The incubation temperature is 22 °C. *In case this condition does not work, the table in the Appendix section describes, in sequential order, the conditions that must be screened for optimizing the ligation reaction*. The table includes vector-to-insert molar ratio and incubation temperature and time

a) Calculate the volume of **Water** and **DNA Fragment** to be ligated based on the mass of **Vector** and **DNA Fragment,** molecular weight or size of the **Vector** and **DNA Fragment**, and the vector-to-insert molar ratio.

If you know the molecular weight of the **Vector** and **DNA Fragment**, the **DNA Fragment** mass is

$$Vector\ mass\ (ng) \times \frac{1\ g\ Vector}{10^9\ ng\ Vector} \times \frac{1}{M.W.\ Vector} \left(\frac{mol}{g}\right) \times$$

$$Molar\ Ratio\ \left(\frac{mol\ DNA\ Fragment}{mol\ Vector}\right) \times M.W.\ DNA\ Fragment\ \left(\frac{g}{mol}\right) \times \frac{10^9\ ng\ DNA\ Fragment}{1\ g\ DNA\ Fragment}$$

Simplified,

$$DNA\ Fragment\ mass\ (ng)\ = \frac{100 \times 1\ mol\ DNA\ Fragment \times M.W.\ DNA\ Fragment}{M.W.\ Vector \times 1\ mol\ Vector}$$

If you do not know the molecular weight of the **Vector** and **DNA Fragment**, the **DNA Fragment** is

$$\frac{Vector\ mass\ (ng) \times size\ DNA\ Fragment\ (kb)}{size\ Vector\ (kb)} \times Molar\ Ratio\ \left(\frac{mol\ DNA\ Fragment}{mol\ Vector}\right)$$

Simplified,

$$DNA\ Fragment\ mass\ (ng) = \frac{100\ \times size\ DNA\ Fragment\ (kb) \times 1 mol\ DNA\ Fragment}{size\ Vector\ (kb) \times 1\ mol\ Vector}$$

Reagent	Volume (Total Volume 20 μL)
T4 DNA Ligase Reaction Buffer (10X)	*2 μL*
Vector	$\frac{100\ ng}{Vector\ Concentration\ (ng/\mu L)}$
DNA Fragment	$\frac{DNA\ Fragment\ mass\ (ng)}{DNA\ Fragment\ Concentration\ (ng/\mu L)}$
T4 DNA Ligase Enzyme (400 U/μL)	*1 μL*
Nuclease-free Water	(*20 − 2 − 1 − Vol. Vector − Vol. DNA Fragment*)

b) Equilibrate the water bath and the dry bath at 22 °C and 65 °C, respectively, for at least 30 minutes before placing the ligation reaction mixture.

c) The working area should be sanitized using 70% ethanol prior to placing any materials on the bench.

d) Make sure that the **T4 DNA Ligase Reaction Buffer** is properly thawed and resuspended at room temperature before use. ***The reaction must be assembled at room temperature to avoid DTT precipitation***.

e) Add the required volume of **Water** to a 0.5 μL microtube.

f) Add the 2 μL of **T4 DNA Ligase Reaction Buffer** (10X) and mix by pipetting up and down.

g) Add the required volume of **Vector** and mix by pipetting up and down.

h) Add the required volume of **DNA Fragment** and mix by pipetting up and down.

i) Briefly centrifuge the ligation reaction mixture and add 1 μL of **T4 DNA Ligase Enzyme** (400 U/μL) to a final concentration of 20 U/μL.

j) Spin-down the reaction in a microcentrifuge and vortex to ensure proper mixing.

k) Incubate for 3 hours in the water bath at 22 °C.

l) *Optional*: Incubate for 10 minutes in the dry bath at 65 °C.

m) Place the microtube on ice and transform 5 μL of the ligation reaction mixture in 50 μL of chemically-competent bacterial cells using the *Standard Operation Procedure for Bacterial Transformation of an Expression Plasmid*.

n) The rest of the ligation reaction can be stored at −20 °C for a further transformation, in case the appropriate transformation control does not work.

o) Decontaminate the work area and materials with 70% ethanol and be sure all equipment are shut down: water bath and dry bath. The pipettes must be returned to their maximal volume.

Appendix:

Possible Conditions for Screening

Order	Vector-to-Insert Molar Ratio	Temperature (°C)	Time (Hours)
1	1:3 and 3:1	22	3
2	1:1	16	6
3	1:3 and 3:1	16	6
4	1:1	4	Overnight
5	1:3 and 3:1	4	Overnight