T4 DNA Ligase

(T4 bacteriophage)

Cat. No.	size
E1060-01	20 000 units (~300 Weiss units)
E1060-02	100 000 units (~1500 Weiss units)

T4 DNA Ligase which catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA.

Description:

- Enzyme of choice for almost all ligation assays (2).
- Catalyzes the joining of duplex DNA molecules at blunt ends (1).
- Covalently joins DNA fragments with complementary cohesive ends.
- Seals single-stranded nicks in duplex DNA, RNA or DNA/RNA hybrids.
- Ultrapure recombinant enzyme.
- Suitable for cloning of restriction fragments and joining linkers or adapters to blunt-ended DNA (2).

Unit Definition:

One unit is defined as the amount of enzyme required to give 50% ligation of HindIII fragments of Lambda DNA (5' DNA termini concentration of 0.12 µM [300 µg/ml]) in 20 µl of 1 x T4 DNA Ligase Reaction Buffer in 30 minutes at 16°C.

Note: 67 cohesive end ligation units are the equivalent of one *Weiss* unit.

Storage Conditions:

Store at -20°C.

References:

1. *Weiss, B., Jacquemin-Sablan, A., Live, T.R., Fareed, G.C. and Richardson, C.C. (1968) J. Biol. Chem. 243, 4543-4555.*
2. *Sambrook, J. et al. (1989) Molecular cloning: A laboratory Manual, second edition, pp. 1.53-1.73, Cold Spring Harbor, New York.*
3. *Dugaiczyk A. et.al. (1975) J mol Biol 96: 171–184.*
4. *Damak S., Bullock D.W. (1993) Biotechniques 15:448–452.*
5. *Cranenburgh R.M. (2004) Appl Microbiol Biotechnol. 65: 200–202.*

Assay Conditions:

66 mM Tris (pH 7.6 at 22°C), 6.7 mM $MgCl_2$, 67 mM ATP, 10 mM dithiothreitol, 3.3 µM [α-^{32}P]$Na_4P_2O_7$. Reaction volume is 30 µl.

1 x Reaction Buffer:

50 mM Tris-HCl (pH 7.5 at 25°C), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ml BSA.

Avoid multiple cycles of freezing/thawing of the stock reaction buffer. Thawing should be performed at temperatures not exceeding 10°C. Recommended procedure is to divide the provided reaction buffer into smaller portions.

Recommended Reaction Condition:

Even though much less units of the ligase are required for ligation to take place, due to the ligation kinetics for the best cloning results we recommend using 0.3-1 µl of the enzyme per 10-20 µl reaction at 16°C for overnight.

It is known that phenol-chloroform extraction, followed by ethanol precipitation of ligated DNA, improves yield of transformation.

Storage Buffer:

10 mM Tris-HCl (pH 7.5 at 22°C), 1 mM dithiothreitol, 50 mM KCl and 50% (v/v) glycerol.

Quality Control:

All preparations are tested for contaminating endonuclease, exonuclease and non-specific single- and double-stranded DNase activities. The preparation is approximately 95% pure as judged by SDS polyacrylamide gel electrophoresis.

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Preparation of Ligation Reactions:

Cohesive and Blunt Termini Ligation Protocol (2)

1. Digest plasmid and insert DNA with appropriate restriction enzymes.
2. Purify DNA by Phenol-Chloroform extraction or by spin column purification (e.g. EURx PCR/DNA Clean-Up Kit, Cat. No. E3520).
3. Transfer 20-100 ng vector DNA and 1-3 x molar amounts of insert DNA (see formula below) to a plastic reaction tube. Total volume: 8 to 8.8 µl (dependent on amount of T4 DNA Ligase).
4. Heat to 45°C for 5 min to melt any reannealed cohesive termini.
5. Chill briefly on ice.
6. Mix:

Component:	Amount:
10 x T4 DNA Ligase Buffer	1 µl
T4 DNA Ligase	50-400 U
Vector and Insert Mix	8 to 8.8 µl
Nuclease-free Water	to 10 µl

7. Incubate for 1–4 h (min. 10 min) at 16°C or 25°C (RT) (for cohesive ends) or for 1-16 h at 16°C (for blunt ends).
8. Use 1-2 µl of each ligation reaction for transformation.

Note: T4 DNA Ligase Buffer contains ATP

Note: Ligation of blunt-ended DNA is less efficient as compared to sticky ends ligation. For blunt end ligation, these additional prerequisites have to be met (2):

- Low ATP concentrations (<0.5 mM).
- Complete absence of Polyamines (e.g. spermidine).
- High Ligase concentrations.
- High substrate concentration (blunt ended termini).

Note: Standard Restriction Endonuclease Buffers, T4 Polynucleotide Kinase Buffer or certain other low salt buffers can serve as reaction buffer as well, provided 1 mM ATP is added. High salt concentrations (>200 mM) strongly inhibit T4 DNA Ligase.

Two-step ligation protocol for Blunt-End Termini (4)

This procedure is only recommended for blunt-end ligation if extremely high efficiency is required, e.g. for cloning libraries. It takes into account that the first step in ligation, intermolecular ligation events (vector to insert ends) preferentially occurs at high DNA concentrations, whether low DNA concentrations favor the second ligation step, joining of both DNA strand ends for circularization.

1. Digest vector and insert DNA with appropriate restriction enzymes.
2. Ensure high vector and insert DNA concentrations (>50 ng μl^{-1}).
3. Mix 0.1 µg vector DNA and an equimolar amount of insert DNA. Total volume 10 µl. **Note:** Under these conditions, vector to insert ligation is favored.
4. Incubate for 1 h at room temperature.
5. Dilute reaction 20 times with 1 x T4 Ligase Buffer and T4 DNA Ligase. **Note:** Under these conditions, self-ligation is favored and ligated molecules are circularized.
6. Incubate overnight at room temperature.

Electroporation

Use 1-2 µl (max. 5 µl) for transformation of 50 µl electro- or chemical competent cells. Transformation efficiency is enhanced by prolonged reaction time (1 h or longer), by heat inactivation (10 min, 65°C; not applicable with PEG) or by DNA ethanol precipitation.

Calculation of Required Vector and Insert DNA Solution Volumes (5)

Since ligation efficiency benefits from high initial DNA concentrations, the reaction is set up ideally without any diluting H_2O. For a quick calculation of the optimally required vector and insert DNA solution volumes, a formula was devised by Cranenburgh (2004).

1)

$$V_v = \frac{T}{\left(\frac{(V_c \cdot I_l \cdot R)}{(I_c \cdot V_l)}\right) + 1}$$

Example:

Component:	Example Value:
Insert length (I_l)	1.8 kb
Insert concentration (I_c)	20 ng μl^{-1}
Insert/ vector ratio (R)	2
Vector length (V_l)	3.2 kb
Vector concentration (V_c)	50 ng μl^{-1}
Total DNA volume (T)	8 µl
Vector volume (V_v)	to determine

$$V_v = \frac{8\ \mu l}{\left(\frac{(50\ ng\ \mu l^{-1} \cdot 1.8\ kb \cdot 2)}{(20\ ng\ \mu l^{-1} \cdot 3.2\ kb)}\right) + 1} = \frac{8\ \mu l}{\left(\frac{180}{64}\right) + 1} = 2{,}10\ \mu l$$

2)

$$I_v = T - V_v$$

Example:

Component:	Example Value:
Vector volume (V_v)	2.10 µl
Total DNA volume (T)	8 µl
Insert volume (I_v)	to determine

$$I_v = 8\ \mu l - 2{,}10\ \mu l = 5{,}9\ \mu l$$

Notes:

1. **Prerequisites for efficient ligation:**
   - Clean, well-purified DNA solutions of linearized vector and of insert DNA.
   - High DNA concentrations of linearized vector and insert DNA solutions (recommended 5-50 ng/µl) favor intermolecular over intramolecular (self-) ligation. Extremely high DNA concentrations lead to undesired formation of very long linear DNA fragments.
   - Vector and insert DNA in molar ratios between 1:1 and 1:3 (recommended: 1:2). At vector/ insert ratios of 1:1, intermolecular ligation is favored. For ratios larger than 1:3, self-ligation is preferred.

2. **Reaction speed:** Velocity of the ligation reaction depends solely on the concentration of free, compatible DNA ends, regardless whether they are located on the same DNA strand (intramolecular ligation) or on different DNA strands (intermolecular ligation). Two factors favoring intermolecular ligation over self-ligation are high DNA concentrations and long DNA fragments. Contrary, low DNA concentrations and small DNA fragments lead to a preference for self-ligation. Under the latter conditions it is more likely that two ends from one single molecule, rather than from different DNA strands, will get into close spatial contact. For a detailed discussion see references (2, 3, 4).

3. **Dephosphorylated DNA:** An optional strategy to prevent self-ligation, is to remove 5'-phosphates from plasmid DNA (but not from insert DNA) prior to ligation. Bacterial and Calf Intestine Phosphatase (Cat. No. E1026 and E1025) catalyze the removal of 5'-phosphate groups from DNA and RNA (2). Dephosphorylation of plasmid DNA fragments efficiently prevents self-ligation, at the expense, that only two new phosphodiester bonds are formed during ligation (not four, as for phosphorylated DNA strands). Ligated molecules thus carry two nicks, which are repaired by the bacterial host following transformation.

4. **Condensing Agents:** Substances generating macromolecular crowding effects (e.g. Polyethylene Glycol or Hexamminecobalt Chloride) can be used for increasing from low to adequate DNA concentrations for blunt end cloning (2). Useful Polyethylene Glycol (PEG8000) final concentrations range between 5% and 15%. Excess condensing agent concentrations lead to preferred formation of large linear DNA, which may inhibit transformation.

5. **Optional Control Reactions:**
   - Cut vector, no insert - for estimating self-ligation background (blunt end: plus checking for ligatability).
   - Cut vector, no ligase - for estimating undigested vector background.
   - Uncut vector – for estimating efficiency of transformation.