

DNA Ligases

UNIT 3.14

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ABSTRACT

The DNA ligase enzyme family catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA. This activity can seal nicks in duplex DNA or join double-stranded DNA fragments having either blunt or cohesive ends. DNA ligases are central enzymes in molecular biology, nucleic acid research, and in next-generation sequencing applications. Reaction conditions and applications for T4 DNA ligase, *E. coli* DNA ligase, and thermostable DNA ligases are described in this unit. These enzymes differ in their cofactor requirements, substrate specificity, and thermal stability. *Curr. Protoc. Mol. Biol.* 94:3.14.1-3.14.7. © 2011 by John Wiley & Sons, Inc.

Keywords: T4 DNA ligase • *Taq* DNA ligase • DNA ligation • blunt-end ligation • cloning

INTRODUCTION

DNA ligases are critical DNA replication and repair enzymes and, since their discovery in 1967 (Weiss and Richardson, 1967), have found widespread use in molecular biology and biotechnology applications such as cloning and next-generation DNA sequencing (Eun, 1996). DNA ligases catalyze the joining of adjacent 3'-hydroxyl and 5'-phosphorylated DNA termini in duplex DNA. All DNA ligases accept nicked dsDNA (Fig. 3.14.1A) and homologous, cohesive ends (Fig. 3.14.1B) as substrates, though the minimum length of overlap required for efficient ligation varies widely. Some ligases, most notably T4 DNA ligase, accept fully base-paired (blunt end) substrates as well (Fig. 3.14.1C).

DNA ligases share a common mechanism and a high degree of structural similarity with other members of the nucleotidyl transferase superfamily, i.e., RNA ligases (UNIT 3.15) and RNA-capping enzymes (Pascal, 2008). The ligation reaction is known to proceed in three steps: (1) reaction of the enzyme with a cofactor to form an enzyme-AMP covalent intermediate; (2) transfer of the AMP to a 5'-phosphorylated DNA terminus; and (3) joining of a 3' hydroxyl DNA terminus with the adenylated 5' strand accompanied by the release of AMP (Fig. 3.14.2) (Shuman, 2009). All ligases require the formation of the enzyme-AMP covalent complex; mammalian and viral ligases (including T4) use ATP as the AMP donor, whereas bacterial ligases (including those from *Escherichia coli* and *Thermus aquaticus*) use NAD⁺. Successful ligation also requires divalent metal cations (generally Mg²⁺) and appropriate termini: 3' hydroxyls and 5' phosphates. Restriction endonucleases (UNIT 3.1) produce the desired phosphorylation state for ligation. If synthetic oligonucleotide fragments are used, they must either be synthesized with a 5' phosphate, or the DNA fragments must be phosphorylated with a DNA kinase (UNIT 3.10), typically T4 polynucleotide kinase, prior to ligation. Most DNA ligases are used at 16°C or room temperature and are inactivated by heat, although heat-stable ligases active at higher temperatures (such as *Taq* DNA ligase) are now also commonly used.

Enzymatic
Manipulation of
DNA and RNA

3.14.1

Supplement 94

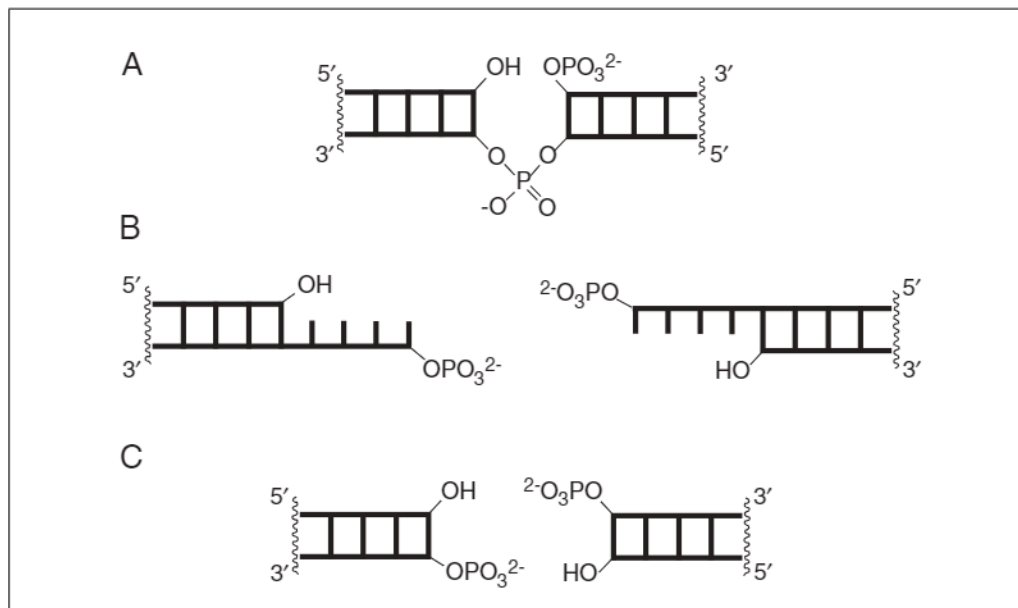


Figure 3.14.1 Ligation substrates: (A) nicked dsDNA, (B) cohesive ends, and (C) blunt ends.

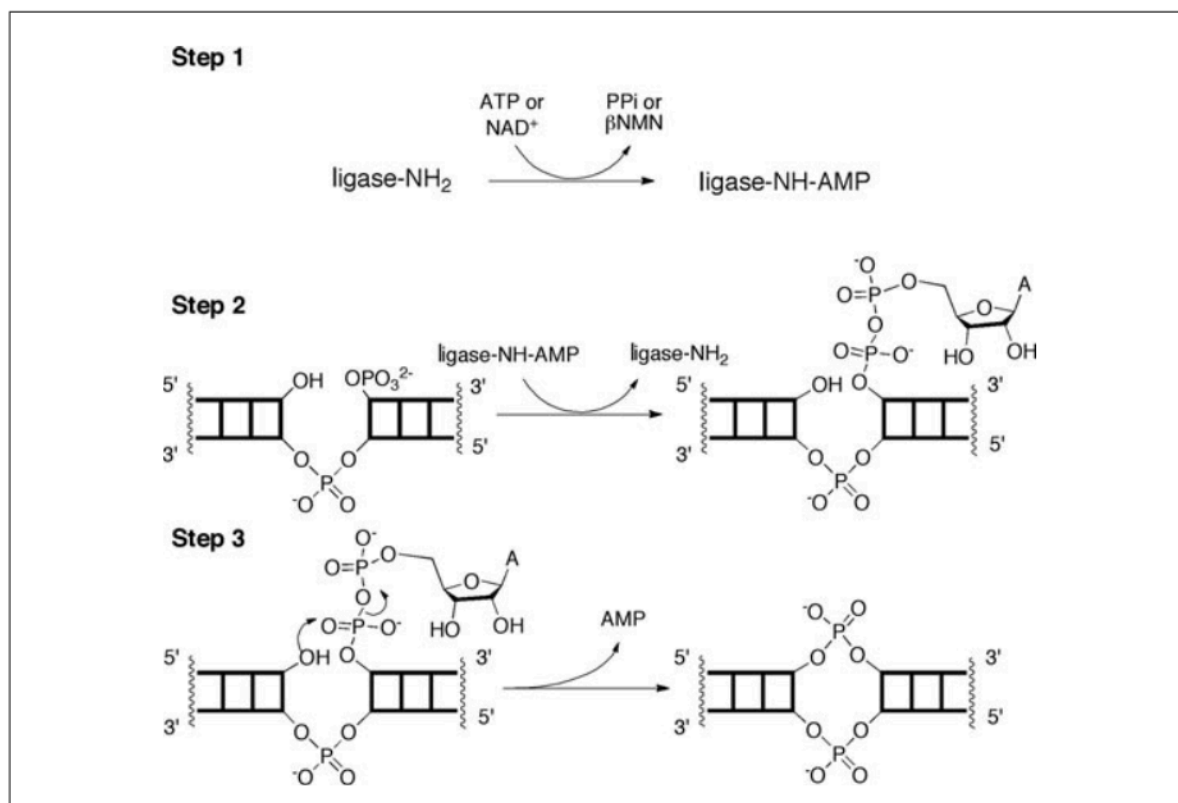


Figure 3.14.2 The mechanism of DNA ligase.

ENZYME: T4 DNA LIGASE

T4 DNA ligase, the product of phage T4 gene 30, was originally purified from phage-infected cells of *E. coli* (Weiss et al., 1968). The phage T4 gene 30 has been cloned and the recombinant enzyme is now widely available. T4 DNA ligase catalyzes the repair of single-stranded nicks in duplex DNA and joins duplex DNA fragments having either blunt or cohesive ends (Fig. 3.14.1). T4 DNA ligase will accept some hybrid RNA-DNA helices as substrates, generally those with RNA strands providing the 3'

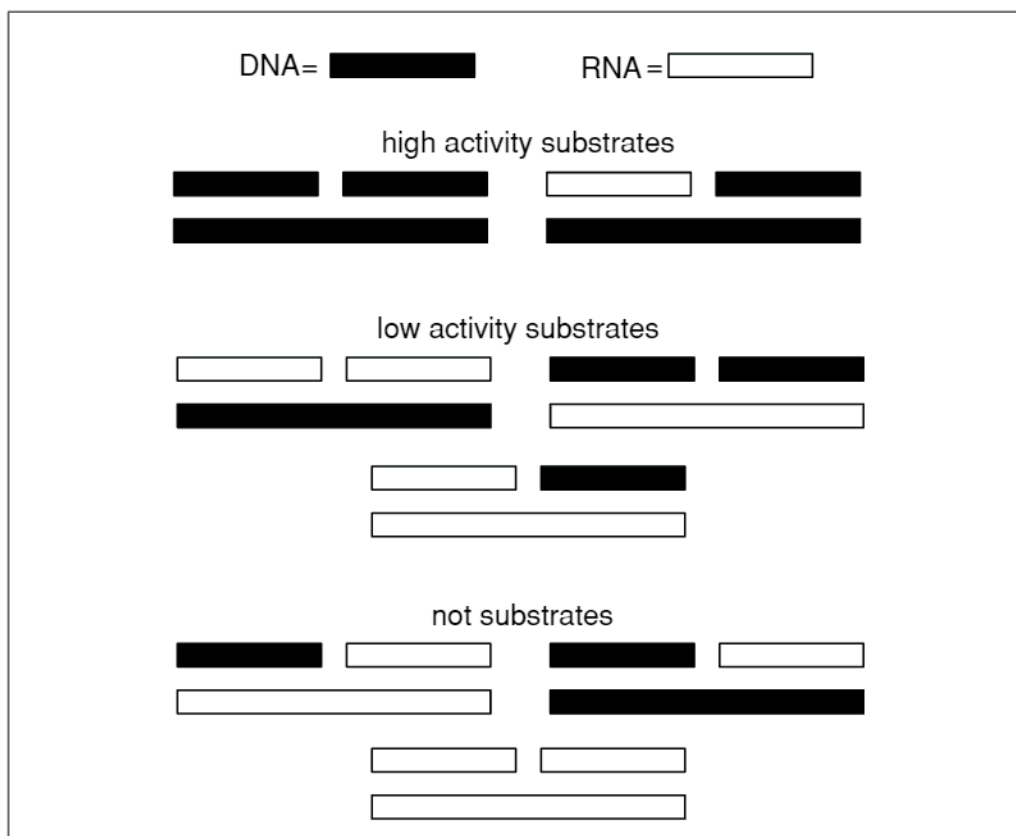


Figure 3.14.3 RNA/DNA hybrid helices accepted by T4 DNA ligase.

hydroxyl terminus but not the 5' phosphorylated terminus (Bullard and Bowater, 2006; see Fig. 3.14.3 for a schematic of hybrid helices that are known substrates for T4 DNA ligase).

This enzyme has a high tolerance for mismatches at either side of the ligation junction and will efficiently ligate all mismatches except A:G, G:A, and C:C pairs (Kim and Mrksich, 2010). T4 DNA ligase is the only commercially available ligase that efficiently joins blunt-end termini under normal reaction conditions. Macromolecular exclusion molecules (e.g., PEG 8000) have been shown to greatly increase the rate of both cohesive-end and blunt-end joining by T4 DNA ligase (Pheiffer and Zimmerman, 1983). Macromolecular exclusion encourages intermolecular ligation over circularization; nevertheless, the acceleration of ligation rate has resulted in widespread use of PEG in the preparation of circular DNA constructs (Hayashi et al., 1986).

T4 DNA ligase is inhibited by salts of monovalent cations (Li^+ , Na^+ , K^+ , Cs^+ , NH_4^+) at concentrations >70 mM with total loss of activity at >200 mM salt (Rae et al., 1975). It is inhibited by dATP (66 μM causes 60% inhibition) but is not inhibited by other nucleotides or deoxynucleotides at similar concentrations (Weiss et al., 1968). Concentrations of ATP >3 mM also inhibit ligation, with reaction rates 20 times slower with 10 mM ATP than with 1 mM ATP (Cherepanov and de Vries, 2003).

Enzyme activity may be defined as either Weiss units or cohesive end units. One Weiss unit is defined as the amount of enzyme required to convert 1 nmol of ^{32}P -labeled inorganic pyrophosphate into a form absorbable onto activated carbon in 20 min at 37°C , using specified reaction conditions (Weiss et al., 1968). One cohesive end unit (CEU) is defined as the amount of enzyme required to give 50% ligation of *Hind*III fragments of λ DNA (5' DNA termini concentration of 0.12 μM , 300 $\mu\text{g}/\text{ml}$) in a total reaction

volume of 20 μ l in 30 min at 16°C under standard reaction conditions. One Weiss unit corresponds to ~150 to 200 CEU.

Reaction Conditions

For a 20- μ l reaction:

50 mM Tris-Cl, pH 7.5 (APPENDIX 2)
10 mM MgCl₂
10 mM DTT
20 to 200 ng DNA
1 mM ATP
400 to 2000 CEU T4 DNA ligase

Incubate 10 min to 2 hr at 16° to 25°C. Stop reaction by adding 2 μ l of 0.5 M EDTA or by heating 20 min at 65°C. The volume of reaction, concentration of DNA, temperature, and time of the reaction will vary depending upon the individual application and concentration of 5' termini. When circularization is desired (e.g., ligation of recombinant vectors), concentrations of 1 to 10 μ g/ml total DNA (linker + insert) are recommended. Higher concentrations of DNA may be used for intermolecular ligation applications, such as linker ligations. In many applications, addition of higher concentrations of T4 DNA ligase or PEG (to 10% to 15%) can decrease reaction time, but may reduce the efficiency of subsequent transformation into competent cells. Blunt-end ligations require ~10 to 100 times more enzyme than cohesive-end ligations to achieve a comparable ligation rate and efficiency. Ligation of cohesive ends is usually carried out at 16°C to maintain a good balance between annealing of the ends and activity of the enzyme. Higher temperatures make it difficult for the ends to anneal, whereas lower temperatures diminish ligase activity. Blunt-end ligations are typically performed at room temperature. Higher temperatures are not recommended as the enzyme is unstable >30°C.

Applications

T4 DNA ligase is by far the most commonly used DNA ligase, used for virtually any application requiring a DNA ligase. Key applications include:

1. Repair of nicks in dsDNA and ligation of dsDNA fragments with complementary cohesive ends.
2. Ligation of blunt-end dsDNA fragments. Unlike most other ligases, T4 DNA ligase carries out blunt-end ligations with reasonable efficiency. Nevertheless, this reaction typically requires higher concentrations of ligase and longer reaction times than nick or cohesive end joining. Use of PEG at 25°C can shorten blunt-end ligation reaction times to 5 to 10 min. The following buffer conditions are recommended: 66 mM Tris-Cl, pH 7.6, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP, and 15% PEG 6000. Note that the presence of PEG is incompatible with transformation through electroporation.
3. Ligation of single base overhangs. Similar to blunt-end joining, ligation of single base overhangs also requires high enzyme concentrations and longer reactions times. Up to 10,000 CEU of ligase with reaction times of 18 hr at 16°C are recommended.
4. Addition of inserts and circularization of DNA for cloning. See UNIT 3.16 for a detailed protocol.
5. Production of hybrid RNA-DNA duplexes (Fig. 3.14.3) (Bullard and Bowater, 2006).
6. The synthesis of 5'-pre-adenylated DNA substrates (APP-DNA) from 5'-phosphorylated substrates. Typically, a long 3' overhang on the opposing strand, a gapped substrate, or low pH is required to allow the buildup of APP-DNA (Patel et al.,

2008). APP-DNA can itself act as a substrate for DNA ligases, and can be ligated to a 3'-hydroxyl-donating strand in the absence of cofactor. APP-DNA is also used as a substrate for ssRNA/ssDNA ligation using T4 RNA ligase 2 (see UNIT 3.15).

7. Next-generation sequencing applications: ligation or circularization of genomic DNA fragments during sample preparation, and as the primary enzyme for the high-fidelity sequencing of the DNA. See UNITS 7.1 & 7.8 for detailed discussions of this topic.

ENZYME: *Escherichia coli* DNA LIGASE

DNA ligase from *E. coli* is the product of the *lig* gene. The *lig* gene has been cloned, and the enzyme is typically obtained from overproducing recombinant strains (Panasenkov et al., 1978). Like other ligases, *E. coli* DNA ligase catalyzes the repair of single-stranded nicks in duplex DNA and joins restriction fragments having homologous cohesive ends. Unlike T4 DNA ligase, *E. coli* DNA ligase does not join termini with blunt ends under normal reaction conditions, though addition of macromolecular exclusion molecules (PEG) has been reported to stimulate this activity (Hayashi et al., 1985). *E. coli* DNA ligase requires NAD⁺ as its AMP-donating cofactor. Due to its poor acceptance of RNA and its low blunt-end ligation efficiency, *E. coli* DNA ligase is utilized when a high specificity for cohesive end DNA joining or nick sealing is required.

Reaction Conditions

For a 20- μ l reaction:

30 mM Tris-Cl, pH 8 (APPENDIX 2)
4 mM MgCl₂
1 mM DTT
20 to 200 ng DNA
26 μ M NAD⁺
50 μ g/ml BSA
10 to 50 U *E. coli* DNA ligase

Incubate 2 to 16 hr at 16° to 25°C. Stop reaction by adding 2 μ l of 0.5 M EDTA or by heating 10 min at 75°C. The volume of reaction, concentration of DNA, temperature, and time of reaction will vary, depending upon the individual application. Units are given as cohesive end units, defined as described for T4 DNA ligase.

Applications

1. Used as an alternative to T4 DNA ligase when blunt-end ligations are not required. Use of *E. coli* DNA ligase can result in a lower rate of aberrant transformants, as T4 DNA ligase has a much lower specificity for the structure of the termini.
2. The production of cDNA libraries with preferential cloning of full-length sequences. *E. coli* ligase is used to circularize the initial cDNA/mRNA hybrid and to ligate the newly synthesized DNA strand formed after RNA digestion and polymerization over the cDNA template. *E. coli* DNA ligase is preferred due to its poor ability to ligate RNA to DNA, ensuring only DNA-DNA ligation takes place (Okayama and Berg, 1982).

ENZYME: *Taq* AND OTHER THERMOSTABLE DNA LIGASES

Taq (or *Tth*) DNA ligase was first isolated from an organism that was previously classified as *Thermus aquaticus* HB8 and is currently classified as *Thermus thermophilus* HB8 (Barany and Gelfand, 1991). Both names are in current use for commercial preparations of the enzyme. *Taq* DNA ligase catalyzes the repair of single-stranded nicks in duplex DNA and requires NAD⁺ as a cofactor. However, *Taq* DNA ligase functions at elevated

temperatures (~45° to 65°C), and efficiently ligates only when the oligonucleotides at the ligation junction are perfectly paired and have no gaps between them. *Taq* DNA ligase was the first ligase employed in high-temperature applications and can survive 30 or more temperature cycles. Currently, several additional DNA ligases active at elevated temperatures are commercially available, including Pfu DNA ligase, 9°N DNA ligase, and Ampligase.

Reaction Conditions

For a 20-μl nick-sealing reaction:

- 20 mM Tris-Cl, pH 7.6 (APPENDIX 2)
- 25 mM potassium acetate
- 10 mM magnesium acetate
- 10 mM DTT
- 1.0 mM NAD⁺
- 0.1% Triton X-100
- 20 to 200 ng DNA
- 40 to 200 U *Taq* DNA ligase

Incubate 15 min at 45°C. Stop reaction by adding 2 μl of 0.5 M EDTA. The volume of reaction, concentration of DNA, temperature, and time of reaction will vary, depending upon the individual application. *Taq* DNA ligase cohesive end units are defined as the amount of enzyme required to give 50% ligation of the 12-bp cohesive ends of 1 μg of *Bst*EII-digested λ DNA in a total reaction volume of 50 μl in 15 min at 45°C.

Applications

1. *Taq* and other thermostable DNA ligases can be used for any nick sealing or cohesive end ligation at elevated temperatures.
2. Ligase detection reaction (LDR) and ligase chain reaction (LCR) (Barany, 1991). In these applications, probes are used to detect single base changes in a DNA template with high specificity. These probes are designed such that the base being analyzed is at the ligation junction. If both probes anneal, they will be ligated, giving a positive result. In LDR, the target is amplified by PCR and one set of probes is used for a final detection step. In LCR, two complementary pairs of probes are used, which, if ligated, provide a template for further ligation reactions. The temperature stability of *Taq* DNA ligase allows the reaction to be cycled and the ligation products to be amplified exponentially.
3. One-step isothermal assembly of large DNA fragments (Gibson et al., 2009). At 50°C, DNA fragments with complementary ends are partially digested with a 5' exonuclease to reveal overlapping fragments. The annealed fragments are then repaired by a thermostable polymerase and *Taq* DNA ligase. DNA structures up to several hundred kilobases can be assembled using this method. The use of *Taq* DNA ligase takes advantage of both its high fidelity and temperature stability.
4. Synthesis and amplification of whole genes (Bang and Church, 2008). In this method, overlapping linear ssDNA fragments are synthesized for both the + and – strands such that they will form a circular construct when annealed. A high-fidelity thermostable ligase (Ampligase) is used to preferentially ligate correctly annealed sequences. Remaining linear DNA is digested with an exonuclease, leaving only full-length circular sequences.

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KEY REFERENCE

- Eun, H.-M, 1996. See above.
Provides a broader description of the ligation mechanism and an application-focused perspective.

