Generating recombinant DNA clones with Anza T4 DNA Ligase Master Mix

Abstract

Recombinant DNA methodology often employs the introduction of DNA of interest into a circular plasmid vector, using DNA ligase to covalently join the ends of the DNA fragments. T4 DNA ligase, from the *E. coli* bacteriophage T4, is the enzyme most frequently used for this purpose in laboratory research. Many reaction parameters are important for maximizing ligation efficiency and obtaining the desired recombinant plasmid. Invitrogen™ Anza™ T4 DNA Ligase Master Mix, part of the Invitrogen™ Anza™ Restriction Enzyme Cloning System, can be used to join DNA fragments with overhanging ends or blunt ends. The T4 DNA ligase is incorporated into a 4X concentrated master mix that contains the necessary enzyme, cofactors, and buffer composition for blunt- or cohesive-end ligation, minimizing pipetting and enhancing ease of use.

Introduction

T4 DNA ligase, the ~68 kDa product of gene 30 of the *E. coli* bacteriophage T4, catalyzes the formation of a phosphodiester bond between the 3'-hydroxyl end of a double-stranded DNA fragment and the 5'-phosphate end of the same or another DNA fragment (Figure 1). It can catalyze a reaction between blunt-end DNA fragments or between cohesive ends with 3' or 5' complementary single-stranded overhangs. T4 DNA ligase activity is dependent on Mg^{2+} and ATP, and requires 5'-phosphorylation of one or both fragments. Anza T4 DNA Ligase is formulated in a 4X concentrated master mix (Cat. No. IVGN2108) containing the necessary cofactors for optimal ligase activity.

T4 DNA ligase is widely used for generating recombinant DNA molecules in which DNA fragments of interest are covalently inserted into vector molecules. The ligated DNA can then be used to transform the desired bacterial strain for propagation and further processing. When transforming bacteria with plasmid ligation products, the goal is to maximize the number of colonies containing recombinant plasmids with single inserts. This requires ligation reaction conditions that maximize the number of monomeric, circular molecules, since concatemers or linear molecules do not transform as efficiently. A number of ligation reaction parameters are important, including DNA concentration, the molar ratio of insert to vector, temperature, buffer composition, and enzyme concentration. Traditionally, different protocols have been recommended for cloning into circular vectors with cohesive ("sticky") ends and those with blunt ends; because ligating blunt ends is less efficient, the process requires higher concentrations of enzyme and DNA, a longer incubation time, and a lower reaction temperature than are used for cohesive ends. Anza T4 DNA Ligase Master Mix is formulated to improve the efficiency of blunt-end ligations, and can effectively join DNA fragments with either cohesive ends or blunt ends using the same convenient protocol. Ligation can be performed using DNA in water, TE, elution buffer, or 1X Anza buffers.

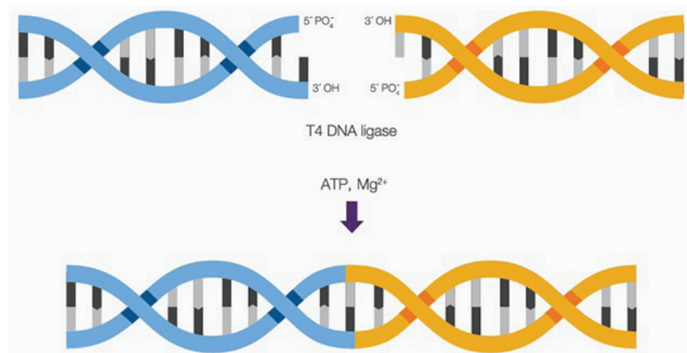

Figure 1. Joining of two double-stranded DNA ends by T4 DNA ligase.

When ligation reaction products are used for bacterial transformation, a high background of colonies containing nonrecombinant plasmids may result from the religation of the vector ends, particularly with vectors digested with restriction enzymes that produce complementary or blunt ends. This background can be reduced by treating the linear vector DNA with alkaline phosphatase prior to ligation with the insert. The phosphatase removes the 5'-phosphate groups from each strand of the vector molecule, preventing T4 DNA ligase from forming phosphodiester bonds between the two ends of the vector. Please visit **thermofisher.com/anza** to see our white paper on dephosphorylation using Invitrogen™ Anza™ Alkaline Phosphatase (Cat. No. IVGN2204).

Here, we describe conditions for using Anza T4 DNA Ligase for direct cloning into plasmids.

DNA ligation protocol

The Anza T4 DNA Ligase Master Mix is used to perform 15-minute ligation of DNA (having either blunt or cohesive ends) into a vector for cloning. At least one of the DNA fragments must be 5'-phosphorylated at both ends for ligation to occur. Most often the insert DNA will contain 5'-phosphate groups, which are exposed after cleavage by restriction enzymes or added by Invitrogen™ Anza™ T4 Polynucleotide Kinase (PNK, Cat. No. IVGN2304) following PCR amplification, while the vector DNA will be dephosphorylated by alkaline phosphatase prior to ligation.

1. Prepare a reaction mix by adding the reagents listed in the following table to a clean microcentrifuge tube:

Reagent	Amount
Nuclease-free water	As required to reach final reaction volume
Linearized vector DNA*	10–100 ng
Insert DNA*	3:1 molar excess over vector DNA
Anza T4 DNA Ligase Master Mix (4X)	5 µL
Final reaction volume	**20 µL**

*Ligations can be performed with DNA in water, TE, elution buffer, or 1X Anza buffers.

- Multiple reactions with varying insert:vector molar ratios (in the range of 1:1 to 5:1) can be prepared to optimize ligation efficiency.
  - Use the CloningBench app at **thermofisher.com/cloningbench** as a convenient tool to calculate ratios.
  - Alternatively, use the following formula to calculate the amount of insert:

    (ng vector/vector size) x insert size x (insert:vector molar ratio)
    e.g., 100 ng vector/6 kb vector x 0.8 kb insert x 3/1 = 40 ng insert DNA

2. Mix the reagents by pipetting up and down.

3. Incubate at room temperature for 15 minutes. If you are ligating cohesive ends, 5 minutes at room temperature may be sufficient.

4. Use 1–5 µL of the ligation reaction mixture to transform competent cells.

   If using electrocompetent cells, perform column purification of the ligated DNA (e.g., use the Invitrogen™ PureLink™ Quick Gel Extraction and PCR Purification Combo Kit, Cat. No. K220001) prior to transformation to remove salts that will cause arcing and damage the cells during electroporation.

   The ligation reaction can be stored at 0–4°C until required for transformation.

Analysis of ligations

For cloning applications, transformation of competent cells is the best method to test a ligation reaction. Several controls are recommended to determine if the ligation and transformation steps are working properly.

- Uncut supercoiled vector (or control DNA provided with the competent cells): This will allow you to calculate the transformation efficiency of your cells. Control DNA provided with competent cells (usually pUC19) is a supercoiled monomer that will transform with higher efficiency than vector preparations, which usually contain other forms (relaxed circle, linear) that will not transform as efficiently.
- Linearized and dephosphorylated vector, without T4 DNA ligase: Use the same amount used in the transformations for the ligation reactions. The transformation should produce few or no colonies, indicating a complete restriction digest of the vector.
- Linearized and dephosphorylated vector, with T4 DNA Ligase: Prepare a ligation reaction with the same amount of vector DNA used in the experimental ligations, and use the same volume to transform competent cells.

The transformation should produce few or no colonies, indicating effective dephosphorylation of the digested vector and low background of religated vector.

- No-DNA transformation: No colonies should be seen, verifying that the antibiotic selection on the agar plate is functional, and that the competent cells are pure.

After colonies are obtained through transformation, there are multiple options for screening for the presence of inserts. The fastest is colony PCR, using primers that flank the insert region on the vector. Other options involve growing overnight cultures from colonies, followed by plasmid isolation via miniprep (Invitrogen™ PureLink™ Quick Plasmid Miniprep Kit, Cat. No. K210011). The plasmids can be evaluated by PCR, restriction enzyme digestion, or sequencing to verify the presence of the insert.

Ligation reaction products may also be evaluated by gel electrophoresis prior to transformation, though the low DNA amounts used for cloning require loading a significant portion of the ligation reaction in order to visualize the DNA. Furthermore, while the gel may indicate depletion of low molecular weight inserts and empty vector, and the generation of higher molecular weight forms, not all high molecular weight forms will transform efficiently.

Example

A DNA fragment was amplified by PCR and treated with one of three Invitrogen™ Anza™ DNA-modifying enzyme kits (Cat. No. IVGN2304, IVGN2404, IVGN2504) to generate blunt-end, 5'-phosphorylated inserts for ligation into the Invitrogen™ pZErO™-2 vector (Cat. No. K260001), which was linearized with the blunt-cutting restriction enzyme Invitrogen™ Anza™ 26 Eco32I (Cat. No. IVGN0266). Ligation reactions were set up according to the Anza T4 DNA Ligase Master Mix protocol and incubated at room temperature for 15 minutes.

Reagent	Volume
Nuclease-free water	To 20 µL
Linearized vector DNA (3.3 kb)	2 µL (50 ng)
Insert DNA (0.72 kb)	0 or 1.9 µL (0 or 18.9 ng*)
Anza T4 DNA Ligase Master Mix (4X)	5 µL

*1.7:1 insert:vector molar ratio.

A portion of each ligation mixture was run on a 1% agarose TAE gel and stained with ethidium bromide to visualize the ligation products (Figure 2). Lanes 2 and 3 of the gel demonstrate, respectively, the complete digestion of the vector with Anza 26 Eco32I and the inability to religate after dephosphorylation with Anza Alkaline Phosphatase. Lanes 4–6 show the products of ligation between the prepared vector and the three different insert preparations using Anza T4 DNA Ligase Master Mix.

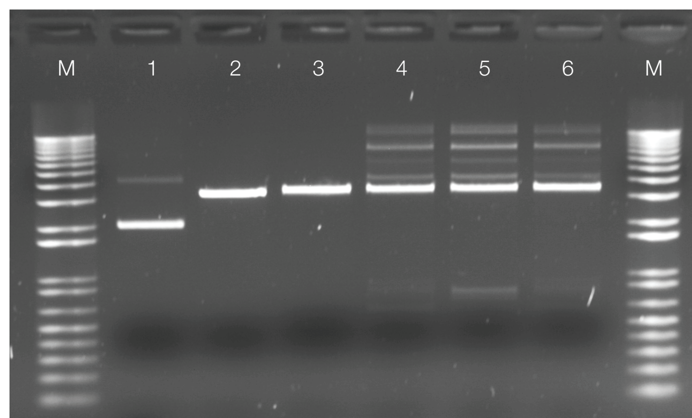

Figure 2. Gel analysis of products from ligation reactions using Anza T4 DNA Ligase. Invitrogen™ 1 Kb Plus DNA Ladder; lane 1: uncut pZErO-2 vector; lane 2: pZErO-2 vector, digested with Anza 26 Eco32I and dephosphorylated; lane 3: pZErO-2 vector, digested with Anza 26 Eco32I and dephosphorylated, plus ligase; lane 4: digested and dephosphorylated pZErO-2 vector, plus insert treated with Anza T4 PNK Kit, plus ligase; lane 5: digested and dephosphorylated pZErO-2 vector, plus insert treated with Anza DNA Blunt End Kit, plus ligase; lane 6: digested and dephosphorylated pZErO-2 vector, plus insert treated with Anza DNA End Repair Kit, plus ligase.

Subsequently, 1 µL of each ligation reaction was used to transform Invitrogen™ One Shot™ TOP10 Chemically Competent *E. coli* (Cat. No. C4040-03) using the standard protocol. Clones were screened for the presence of inserts by colony PCR. The vector-only controls, both without and with ligase, produced very few background colonies; the ligations of vector plus insert yielded 69- to 333-fold more colony-forming units (CFUs), and 75–100% of the colonies contained the insert (Figure 3).

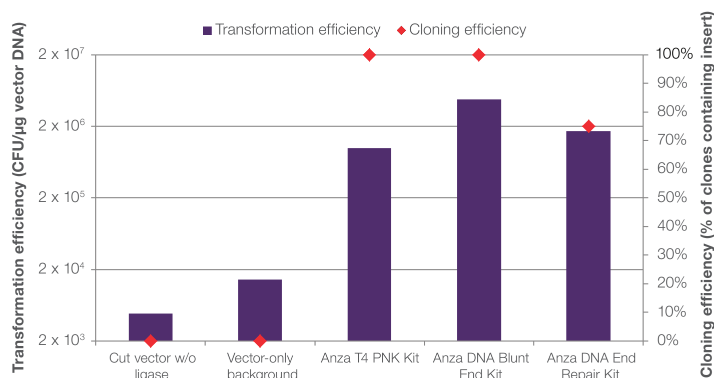

Figure 3. Transformation and cloning efficiencies of pZErO-2 vector ligated with the prepared insert using Anza T4 DNA Ligase Master Mix.

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Troubleshooting

As mentioned above, there are many reaction parameters that are important for achieving high ligation efficiency. Using Anza T4 DNA Ligase Master Mix simplifies many of these parameters by providing a single reagent vial with the correct enzyme concentration, enzyme cofactors, and buffer composition, with a single 15-minute room-temperature protocol for both blunt- and cohesive-end ligations. The Anza T4 DNA Ligase Master Mix protocol also provides guidelines for other factors, such as DNA concentration and insert:vector molar ratio, that will maximize the ligation efficiency.

The controls described here will help to distinguish between problems with the ligation reaction and problems with the competent cells, the selection medium, the restriction endonuclease digestion of the vector, and the phosphatase treatment of the vector. Some possible causes of unsuccessful ligation are listed below, with suggested solutions (Table 1).

Conclusion

T4 DNA ligase is an important tool for recombinant DNA technology. There are several reaction parameters that must be considered for optimal reaction efficiency. Anza T4 DNA Ligase, offered in a concentrated 4X master mix, simplifies many of these parameters by providing the correct concentrations of enzyme and cofactors required for successful ligation of blunt- or cohesive-end DNA fragments using a single 15-minute room-temperature protocol. Ligation can be performed using DNA previously suspended in water, TE, elution buffer, or 1X Anza buffers. Here we have shown the successful application of Anza T4 DNA Ligase Master Mix for blunt-end cloning of a DNA fragment into a plasmid vector. Please visit **thermofisher.com/anza** for additional practical examples of Anza T4 DNA Ligase in cloning workflows and for more information on the Anza Restriction Enzyme Cloning System.

Table 1. Possible causes of failed ligation reactions, and suggested solutions.

Possible cause	Suggested solution
Inhibitors of DNA ligase are present in the DNA samples (NaCl, KCl, NH_4^+, EDTA, proteins, phenol, ethanol, dATP, high glycerol)	Column- or gel-purify vector and insert DNA to remove inhibitors and contaminants (PureLink Quick Gel Extraction and PCR Purification Combo Kit)
DNA ligase is inactive	Verify master mix has not expired and has been stored properly at –20°C Use fresh master mix
ATP or DTT in reaction buffer has degraded	ATP and DTT are sensitive to freeze-thaw cycles; use fresh Ligase Master Mix, and make aliquots for future use
Insert and vector have incompatible ends	Make sure vector and insert are digested with the same restriction enzyme, or restriction enzymes that generate compatible ends Treat both vector and insert with Anza DNA-modifying kits to generate blunt-end fragments
Neither vector nor insert has 5'-phosphates	Make sure that either the vector or insert has 5'-phosphorylated ends; treat with Anza T4 PNK
Concentrations of DNA are incorrect	Check DNA concentrations by gel electrophoresis, or by absorbance after column purification Use an insert:vector molar ratio between 1:1 and 5:1 for most applications

Find out more at **thermofisher.com/anza**

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