The Anza Restriction Enzyme Cloning System provides multiple options for blunt-end cloning

Abstract

Several recombinant DNA methods are available to introduce DNA of interest into a circular plasmid vector. Due to its relative simplicity and low cost, traditional cloning, which involves the use of restriction endonucleases and other DNA-modifying enzymes, is still the most universal method employed. The Invitrogen™ Anza™ Restriction Enzyme Cloning System further simplifies the traditional cloning workflow by providing a selection of 128 restriction enzymes and five DNA-modifying enzymes compatible with a single proprietary buffer, along with fast, easy-to-use protocols. Here we describe the features of the Anza Restriction Enzyme Cloning System and present how to use Invitrogen™ Anza™ restriction endonucleases (REases) and DNA-modifying enzyme kits for blunt-ended cloning of a gene of interest (GOI) into Invitrogen™ pZErO™-2 vector. Our results demonstrate the simplicity, speed, and effectiveness of the Invitrogen™ Anza™ system and provide an example of the best way to incorporate these reagents in the standard cloning workflow.

Introduction

Traditional cloning begins by preparing vector and insert DNA using restriction endonucleases (REases), then joining the digested fragments together with DNA ligase to form a new plasmid containing the insert of interest. This recombinant DNA methodology has been in use since the 1970s, and over the years has been improved by the discovery of new REases and other DNA-modifying enzymes. When used together, these enzymes enable the joining of DNA molecules into plasmids for vector construction, gene cloning, and protein expression experiments.

Some cloning applications require a specific orientation of the GOI within the vector, such as preparation of clones for heterologous protein expression. In such cases it is advisable to use a directional cloning workflow (please visit **thermofisher.com/anza** for the Anza Restriction Enzyme Cloning System white paper on directional cloning).

If the orientation of the insert isn't important, such as when generating a clone for sequencing, the vector can be digested with a single restriction enzyme that leaves blunt ends. Prior to ligation, the digested vector should be treated with alkaline phosphatase to remove 5'-phosphate groups, which will prevent self-ligation and reduce the amount of vector background in the transformation. A blunt-ended insert can ligate into the vector in either orientation to produce the desired clone.

Insert preparation for blunt-ended cloning can be accomplished by several methods usually starting with PCR amplification of a targeted DNA sequence. If non-phosphorylated primers are used to amplify the GOI, the blunt-ended PCR amplicon must be treated with a T4 polynucleotide kinase (PNK) to add 5'-phosphate groups for ligation into the prepared dephosphorylated vector. *Taq*-based DNA polymerases leave 3'-A overhangs on the amplified fragment, which need to be removed prior to ligation. If *Taq*-based DNA polymerases are used, the amplicon can be treated with a T4 DNA polymerase possessing 3' → 5' exonuclease activity and T4 PNK, which together will blunt the 3'-overhangs and add 5'-phosphate groups. Occasionally, an insert needs to be ligated into multiple vectors containing sticky or blunt ends. First, the amplified DNA fragment can be treated with REases that leave 5'-phosphorylated sticky ends for directional cloning. Subsequently, the amplicon can be treated with a T4 DNA polymerase or Klenow fragment, which will fill in 5'-overhangs with 5' → 3' polymerase activity preparing the insert for blunt-ended cloning.

The prepared DNA fragments are joined using T4 DNA ligase, which catalyzes the formation of phosphodiester bonds in the presence of ATP between double-stranded DNAs with 3'-hydroxyl and 5'-phosphate termini. The ligated DNA can then be transformed into the desired bacterial strain for propagation and further processing.

The next sections describe the advantages of Anza REases and DNA-modifying enzymes and how they are used in a specific example of blunt-ended cloning of a gene of interest into the pZErO-2 vector.

Anza restriction enzymes

Invitrogen™ Anza™ high fidelity restriction enzymes are our most advanced restriction enzyme system. The proprietary universal Invitrogen™ Anza™ Buffer enables digestion with up to three restriction enzymes simultaneously in as little as 15 minutes, showing no star activity even with overnight digests. Anza restriction enzymes require one simple protocol irrespective of the DNA substrate (e.g., plasmid, PCR product, genomic DNA, lambda DNA). The universal Anza Buffer is also offered with a novel tracking dye that allows users to directly load their digested sample on a gel and is compatible with most downstream processes.

Anza Alkaline Phosphatase

Invitrogen™ Anza™ Alkaline Phosphatase (Cat. No. IVGN2208), fully compatible with Anza Buffer, is intended for use in the removal of 5'-phosphate groups that remain after Anza restriction enzyme digestion, such as for the dephosphorylation of vectors prior to insert ligation. Treatment of vectors with Anza Alkaline Phosphatase prevents self-ligation and re-circularization, resulting in decreased vector background when cloning.

Anza T4 PNK Kit

Invitrogen™ Anza™ T4 PNK (polynucleotide kinase) Kit (Cat. No. IVGN2304) is used to perform 5'-phosphorylation of DNA and oligonucleotides. The T4 PNK enzyme catalyzes the transfer of the terminal phosphate of ATP to a 5'-hydroxyl group of a nucleic acid.

Anza DNA Blunt End Kit

Invitrogen™ Anza™ DNA Blunt End Kit (Cat. No. IVGN2404) is used to convert DNA with overhanging ends to blunt-ended DNA for blunt-end ligation. The Anza™ DNA Blunting Enzyme Mix contains T4 DNA polymerase and Klenow fragment. The 3' → 5' exonuclease activity of T4 DNA polymerase acts to remove 3'-overhangs, while the 5' → 3' polymerase activity of the enzymes act to fill in 5'-overhangs. The Invitrogen™ Anza™ 10X Blunting Buffer contains dNTPs to facilitate the synthesis of blunt ends.

Anza DNA End Repair Kit

Invitrogen™ Anza™ DNA End Repair Kit (Cat. No. IVGN2504) is used to convert DNA with overhanging ends to blunt-ended DNA, while also performing 5'-phosphorylation, for blunt-end ligation. The Anza™ DNA End Repair Mix contains T4 DNA polymerase, Klenow fragment, and T4 PNK. The Anza™ 10X End Repair Buffer contains ATP and dNTPs to facilitate the activity of the enzyme mix.

Anza T4 DNA Ligase Master Mix

Invitrogen™ Anza™ T4 DNA Ligase is formulated as a 4X concentrated Master Mix (Cat. No.# IVGN2108) that can be used to join DNA fragments with both sticky ends and blunt ends, and to repair nicks in double-stranded DNA with 3'-hydroxyl and 5'-phosphate ends. Ligation can be performed with DNA in water, TE, elution buffer, or 1X Anza Buffers.

Using the Anza Restriction Enzyme Cloning System to streamline blunt-end cloning

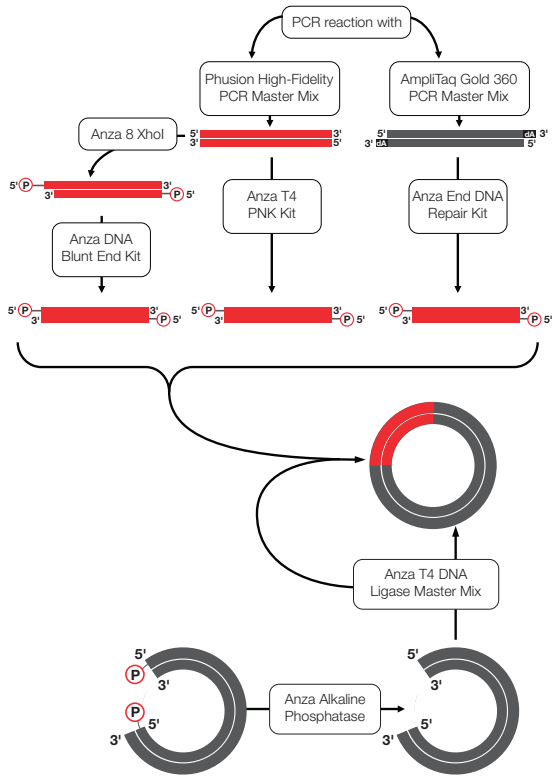

Figure 1. Experimental workflow with the Anza Restriction Enzyme Cloning System for blunt-end cloning. The gene of interest was amplified with Thermo Scientific™ Phusion™ High-Fidelity PCR Master Mix or Applied Biosystems™ AmpliTaq™ Gold 360 PCR Master Mix and gel purified. Fragments were treated with Anza restriction enzyme and Anza DNA modification enzymes using standard protocols as shown in the workflow. pZErO-2 plasmid DNA was treated with Anza 26 Eco32I REase and Anza Alkaline Phosphatase using the Anza one-step DNA digestion and dephosphorylation protocol. Prepared inserts and vector with blunt ends were ligated using Anza T4 DNA Ligase Master Mix prior to transformation.

Materials and methods

Experiments were performed to evaluate the streamlined cloning workflow of the Anza Restriction Enzyme Cloning System for blunt-end cloning of a gene of interest into a plasmid vector. The cloning workflow is shown in Figure 1.

Insert preparation
Fragments for cloning were amplified from plasmid DNA with primer pairs containing restriction sites for XhoI using Thermo Scientific Phusion High-Fidelity PCR Master Mix (Cat. No. F531S) or Applied Biosystems AmpliTaq Gold 360 PCR Master Mix (Cat. No. 4398901). Each PCR product was gel purified from a 1% agarose TAE gel using the Invitrogen™ PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Cat. No. K2200-01). DNA concentrations were determined by Thermo Scientific™ Nanodrop™ spectrophotometer.

Insert DNA end modification: Anza DNA Blunt End Kit
Insert generated by Phusion master mix was digested with Invitrogen™ Anza™ 8 XhoI (Cat. No. IVGN0088) to produce fragments with phosphorylated 5'-overhangs. The digestion was carried out in a 20 µL reaction using 0.4 µg of gel-purified PCR product, 2 µL Anza 10X Buffer, and 1µL of Anza 8 XhoI REase. The reaction was incubated at 37°C for 15 minutes, followed by heat inactivation of the restriction enzyme at 80°C for 20 minutes.

To convert the 5'-single-stranded overhangs generated by XhoI to blunt ends, the Anza DNA Blunt End Kit was used. 200 ng of digested DNA were incubated in 20 µL of 1X Anza Blunting Buffer with 1 µL of Anza DNA Blunting Enzyme Mix. The reaction was incubated at 20°C for 15 minutes and subsequently purified using the PureLink Quick Gel Extraction and PCR Purification Combo Kit. DNA concentration was determined by Nanodrop spectrophotometer.

Insert DNA end modification: Anza T4 PNK Kit
Gel-purified blunt-end PCR fragment amplified with Phusion High-Fidelity PCR Master Mix was subjected to the standard Anza T4 PNK 5'-Phosphorylation protocol. 200 ng of insert DNA were incubated in 20 µL of 1X Anza T4 PNK Buffer with 1 µL of Anza T4 PNK Enzyme. The reaction was incubated at 20°C for 15 minutes, followed by heat inactivation of the enzyme at 80°C for 5 minutes.

Insert DNA end modification: Anza DNA End Repair Kit
Gel-purified non-phosphorylated insert, containing 3'-A overhangs generated by amplification with AmpliTaq Gold 360 PCR Master Mix, was subjected to a standard Anza DNA End Repair Kit protocol. 200 ng of insert DNA were incubated in 20 µL of 1X Anza End Repair Buffer with 1 µL of Anza DNA End Repair mix. The reaction was incubated at 20°C for 15 minutes and subsequently purified using the PureLink Quick Gel Extraction and PCR Purification Combo Kit. DNA concentration was determined by Nanodrop spectrophotometer.

pZErO-2 vector preparation
500 ng of pZErO-2 vector (Invitrogen™ Zero Background™/ Kan Cloning Kit; Cat. No. K260001) were digested with Invitrogen™ Anza™ 26 Eco32I (Cat. No. IVGN0266) and treated with Anza Alkaline Phosphatase using the Anza one-step DNA digestion and dephosphorylation protocol in 20 µL of 1X Anza Buffer for 15 minutes at 37°C. The DNA was then column purified using the PureLink Quick Gel Extraction and PCR Purification Combo Kit. DNA concentration was determined by Nanodrop spectrophotometer.

Anza DNA ligation protocol
Prepared vector was treated with Anza T4 DNA Ligase Master Mix to check for self-ligation, and also combined at a 1:3 molar ratio with prepared inserts and ligated with Anza T4 DNA Ligase Master Mix. The 20 µL reactions were incubated for 15 minutes at room temperature and placed on ice.

Transformation and insert verification
Transformation reactions using 1 µL of each ligation, containing 2.5 ng of vector DNA, were performed with Invitrogen™ One Shot™ TOP10 Chemically Competent *E. coli* (Cat. No. C4040-03) using the standard recommended protocol. After recovery for one hour at 37°C, 100 µL of each transformation was plated on small LB agar plates containing 100 µg/mL ampicillin. Supercoiled pUC19 DNA was also transformed as a positive control. After incubating overnight at 37°C the colonies on each plate were counted and a selected number screened by colony PCR for insert verification. Using standard M13 primers, inserts were amplified in 25 µL reactions. PCR products were visualized on 1% agarose gels. Positive hits were noted by samples producing an approximately 900 bp amplicon, while empty vectors produced a band around 200 bp. Transformation efficiency (CFU/µg vector DNA) and cloning efficiency (percentage of colonies containing the cloned insert) were calculated for each reaction.

Results

Blunt-end cloning using the Anza Restriction Enzyme Cloning System resulted in 9.9 x 10^5 to 4.8 x 10^6 CFU/µg transformation efficiencies using ligated plasmids from vector digested with Anza REase and dephosphorylated with Anza Alkaline Phosphatase, and inserts prepared

with Anza DNA modification enzyme kits. Transformation efficiency of the self-ligated vector was at least 69 times lower than transformation efficiencies of vector-plus-insert ligations (Figure 2).

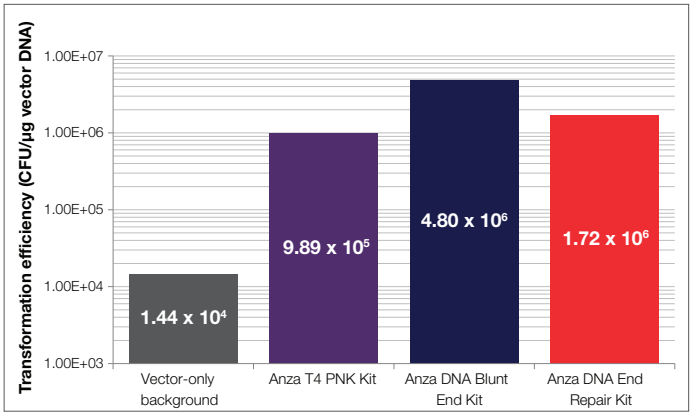

Figure 2. Transformation efficiencies of ligated blunt-end vector and inserts, prepared using the Anza Restriction Enzyme Cloning System. Transformation efficiency of supercoiled pUC19 was 2.5 x 10^8 CFU/µg DNA.

Cloning efficiency was calculated after colony PCR. In each case, 16 colonies were tested and at least 12 contained an insert of the correct length (Figure 3).

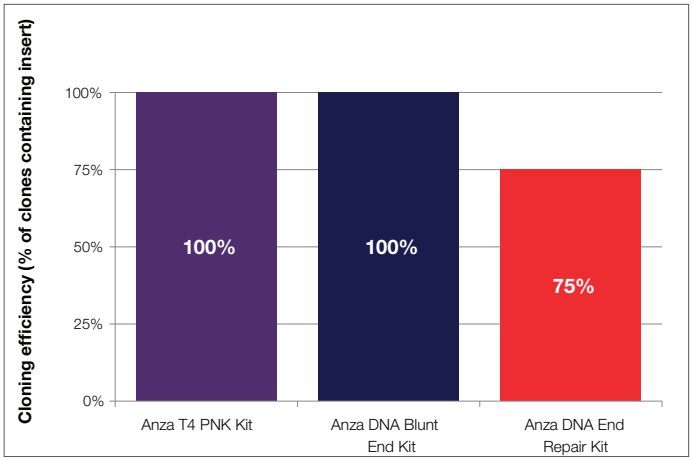

Figure 3. Blunt-end cloning efficiencies for inserts ligated into vector prepared using the Anza Restriction Enzyme Cloning System.

Discussion

The experiments above illustrate how the Anza Restriction Enzyme Cloning System was evaluated for cloning of an amplified gene of interest into pZErO-2 vector using a blunt-end cloning strategy.

The vector was treated with Anza 26 Eco32I and Anza Alkaline Phosphatase using the Anza one-step DNA digestion and dephosphorylation protocol, which enables vector preparation in a single proprietary buffer in 35 minutes. pZErO-2 contains the lethal *ccdB* gene fused to the C terminus of LacZα, which prevents growth of non-recombinants when expressed in cells that do not carry the *lacI*^{q} gene. Although vector dephosphorylation was not necessary using the pZErO-2 plasmid, many common cloning and expression vectors do not contain such negative selection markers and will require the rapid and efficient dephosphorylation provided by Anza Alkaline Phosphatase to minimize vector-only background.

The Anza Restriction Enzyme Cloning System provides several options for blunt-ended insert preparation, depending on the starting material. Here we have demonstrated three workflows using Anza DNA modification enzyme kits: (1) 5'-phosphorylation of blunt-ended PCR amplicons using the Anza T4 PNK Kit, (2) blunting the ends of restriction enzyme-digested DNA fragments with 5'-overhangs using the Anza DNA Blunt End Kit, and (3) simultaneous phosphorylation of 5'-ends and blunting of 3'-A overhangs, produced during amplification by *Taq* polymerase, using the Anza DNA End Repair Kit. Each kit required only 15 minutes of incubation at 20°C to modify the DNA insert, and for each treatment the insert DNA was successfully modified to allow for ligation into the blunt-ended pZErO-2 vector using the Anza T4 DNA Ligase Master Mix, with very good transformation and cloning efficiencies.

Conclusion

The Anza Restriction Enzyme Cloning System allows the researcher to progress from an amplified DNA fragment and supercoiled plasmid to a ligated product ready for transformation in less than 60 minutes. Here the flexibility of the Anza system for common blunt-end cloning workflows was shown. This example is just one way the Anza Restriction Enzyme Cloning System is ideal for all traditional restriction enzyme–based cloning workflows. For more practical examples please visit **thermofisher.com/anza** and look for the Anza Restriction Enzyme Cloning System white papers on other cloning topics.

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

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