The Anza Restriction Enzyme Cloning System simplifies directional cloning

Abstract

Despite many recent advances in molecular cloning, traditional cloning, involving the use of restriction endonucleases (REases) and other DNA-modifying enzymes, is still the most universal method employed to manipulate and propagate desired DNA fragments and plasmids. 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 select Anza components for the directional cloning of Emerald Green Fluorescent Protein (emGFP) into Invitrogen™ Champion™ pET302/NT-His vector. Our results demonstrate the simplicity, speed, and effectiveness of the Anza system and provide an example of the best way to incorporate these new reagents in the standard cloning workflow.

Introduction

REases and DNA-modifying enzymes have become part of the standard recombinant DNA technology “toolbox” over the past four decades. These enzymes enable the joining of DNA molecules into plasmids for vector construction, gene cloning, and protein expression experiments. To achieve maximal cloning efficiency, both the vector and insert must be carefully prepared for ligation and transformation.

In traditional cloning, vector preparation is accomplished by digestion of a plasmid with one or two REases to generate ends for targeted ligation of the insert. The DNA ends can be blunt, which is generally used when the orientation of the insert isn’t important, such as when generating a clone for sequencing (please visit **thermofisher.com/anza** for the Anza Restriction Enzyme Cloning System white paper on blunt-end cloning), or sticky, with either 3'- or 5'-overhangs depending on the restriction enzymes used. For directional cloning, a specific orientation of the ligated insert is desired, and two restriction enzymes generating non-compatible ends should be used. Additionally, the vector can be treated with alkaline phosphatase to remove 5'-phosphate groups. Dephosphorylation of the vector will prevent religation of the excised fragment, reducing the amount of vector background in the transformation.

Insert preparation for directional cloning is frequently accomplished through PCR amplification of a targeted DNA sequence using primers containing restriction sites compatible with the prepared vector ends. Cloning is achieved through ligation of the insert into the vector sequence 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.

Unfortunately, restriction enzymes can exhibit limitations in their functionality due to nonspecific cutting (star activity), incompatible buffers or temperatures for multiple-enzyme digests, and long reaction times to achieve complete digestion. Conducting simultaneous digestion with multiple REases may require the use of buffers that are suboptimal for one or more of the restriction enzymes, resulting in poor cutting efficiency. This is usually overcome by performing sequential digestions in the optimal buffers, but this adds time to the sample preparation and potential sample loss.

The next sections describe the advantages of the Anza system and how these REases and DNA-modifying enzymes can be used in a specific example of directional cloning of the emGFP gene into the Champion pET302/NT-His vector.

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Anza restriction enzymes

Invitrogen™ Anza™ restriction enzymes are Invitrogen's most advanced restriction enzyme system. The enzymes come with a single buffer that enables digestion with multiple 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, and lambda DNA). The proprietary universal Invitrogen™ 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 the 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 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 Buffer.

Materials and methods

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

Insert preparation

Emerald GFP (emGFP) was amplified from the Invitrogen™ pJTI™ R4 Exp CMV EmGFP pA vector (Cat. No. A14146) with primer pairs containing selected restriction sites using Thermo Scientific™ Phusion High-Fidelity PCR Master Mix (Cat. No. F-531S). 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 measurement with the Thermo Scientific™ NanoDrop™ spectrophotometer, and 200 ng of each PCR product were digested with selected Anza restriction enzymes (1µl of each) in 20 µL of 1X Anza Buffer for 15 minutes at 37°C. The enzymes were then heat inactivated at 80°C for 20 minutes.

Vector preparation

500 ng of Champion pET302/NT-His vector (Cat. No. K6302-03) were digested with selected Anza restriction enzymes (1µL of each). Additionally, 500 ng of vector were treated with the Anza one-step dephosphorylation protocol, where the vector is digested and simultaneously dephosphorylated with 1µL of Anza Alkaline Phosphatase in 20 µL of 1X Anza Buffer for 15 minutes at 37°C. The enzymes were then heat inactivated at 80°C for 20 minutes.

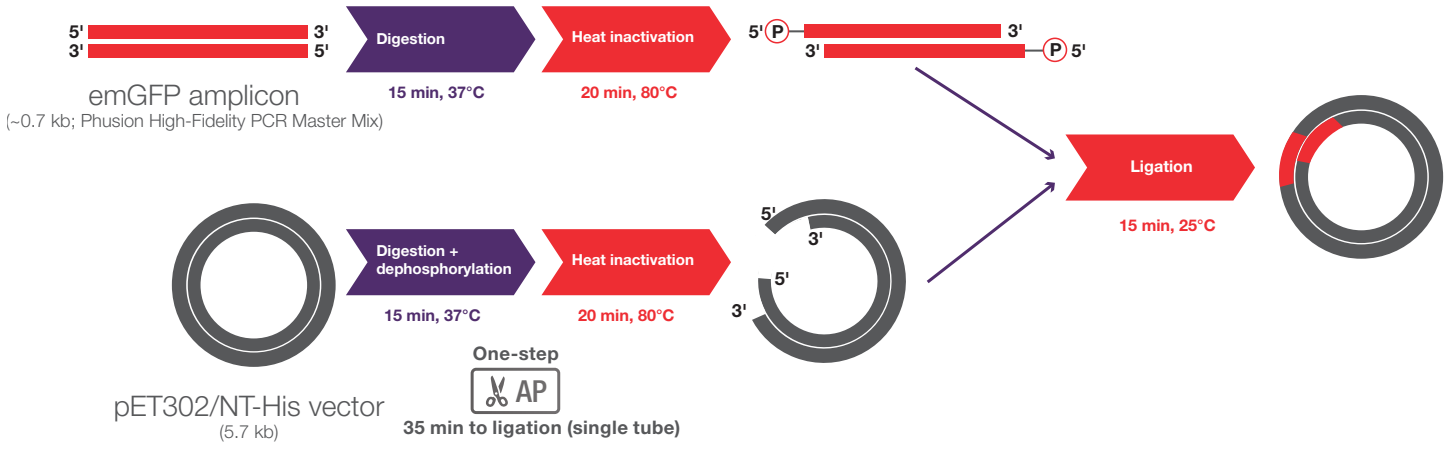

Figure 1. Experimental workflow for directional cloning with the Anza Restriction Enzyme Cloning System. The emGFP gene was amplified with Phusion High Fidelity PCR Master Mix, gel purified, and treated with Anza restriction enzymes in a double digest following the standard protocol. pET302/NT-His vector was dephosphorylated using the Anza one-step protocol. Prepared insert and vector with compatible overhangs generated by restriction enzyme digestion were ligated using Anza T4 DNA Ligase Master Mix prior to transformation.

Anza DNA ligation protocol

Digested and dephosphorylated vectors (10 ng per reaction) were treated with Anza T4 DNA Ligase Master Mix to check for self-ligation. The dephosphorylated vectors were also combined at a 1:3 molar ratio with prepared inserts and ligated with Anza T4 DNA Ligase Master Mix. After incubation of the 20 µL reaction for 15 minutes at room temperature, the reactions were placed on ice. Ligation products were analyzed on a 1% agarose TAE gel.

Transformation and insert verification

2 µL of each ligation reaction, containing 1 ng of linearized vector, were transformed into 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 were plated on LB agar plates containing 100 µg/mL ampicillin. Supercoiled pUC19 and pET302/NT-His vectors were also transformed as positive controls. 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 T7 promoter and T7 reverse primers, inserts were amplified with Thermo Scientific™ DreamTaq™ Green PCR Master Mix (Cat. No. K1081) in 25 µL reactions. PCR products were diluted 4-fold in water and were visualized on Invitrogen™ E-Gel™ 48-well 2% agarose gels (Cat. No. G8008-02). Positive hits were noted by samples producing an approximately 900 bp amplicon, while empty vectors produced a band around 220 bp. Transformation efficiency (CFU/µg vector DNA) and cloning efficiency (percentage of colonies containing the cloned insert) were calculated for each reaction.

Results

Ligation analysis

Gel analysis of ligation products is shown in Figure 2 for DNA fragments prepared with two REase pairs: Anza 8 XhoI/Anza 15 XmaJI and Anza 12 XbaI/Anza 8 XhoI. Supercoiled and relaxed circle forms of the pET302/NT-His plasmid are visible in the undigested sample (U). The vector was completely digested by Anza restriction enzymes in the 15-minute reaction, as seen by the single linearized band in lanes 1 (without Anza Alkaline Phosphatase) and 3 (with Anza Alkaline Phosphatase). Without dephosphorylation, the plasmid readily ligates with the short excised fragment and can form vector concatemers, visible in lane 2. When treated with the Anza one-step DNA digestion and dephosphorylation protocol, the vector fragments are unable to religate and remain in the linearized form (lane 4 in Figure 2). Anza T4 DNA Ligase was able to join the dephosphorylated vector with emGFP inserts containing compatible overhangs generated by Anza restriction enzyme double-digestion (lane 5).

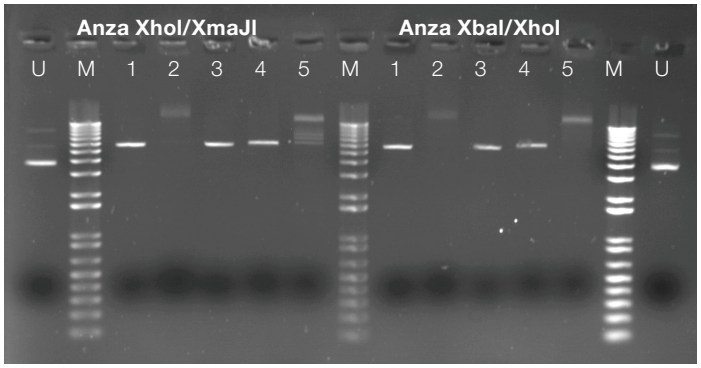

Figure 2. Digestion and ligation products of pET302/NT-His vector and emGFP inserts. Lane U: undigested Champion pET302/NT-His plasmid; lane M: Invitrogen™ 1 Kb Plus DNA Ladder (Cat. No. 10787018); **lane 1:** pET302/NT-His plasmid double-digested with the indicated Anza restriction enzyme pair; **lane 2:** product of lane 1 treated with Anza T4 DNA Ligase Master Mix; **lane 3:** pET302/NT-His plasmid double-digested and dephosphorylated with the indicated Anza restriction enzyme pair and Anza Alkaline Phosphatase using the one-step DNA digestion and dephosphorylation protocol; **lane 4:** product of lane 3 treated with Anza T4 DNA Ligase Master Mix; **lane 5:** product of lane 3 and corresponding Anza enzyme–digested emGFP insert treated with Anza T4 DNA Ligase Master Mix.

Transformation and cloning efficiency

Transformation efficiencies between 4.8 x 10^5 and 9.6 x 10^6 were obtained for the vector and insert ligation products prepared with the Anza Restriction Enzyme Cloning System. Figure 3 compares the transformation efficiencies for vector-insert ligations (purple) to the vector-only background (gray). For each vector-insert ligation reaction, 12 colonies were screened by colony PCR to determine the cloning efficiency. For both combinations of Anza restriction enzymes tested, over 90% of the colonies screened contained the insert (Figure 4).

Discussion

The experiments above illustrate how the Anza Restriction Enzyme Cloning System was evaluated for cloning of the emGFP gene into the Champion pET302/NT-His vector using a directional-cloning strategy. Two Anza restriction enzyme combinations were tested using the single proprietary Anza Buffer, achieving complete digestion in 15-minute reactions.

Vector preparation without alkaline phosphatase treatment allowed for the self-ligation of the vector, even when two different restriction enzymes were used (Figure 2, Lane 2 samples); the small excised fragment readily reinserted into the vector when treated with Anza T4 DNA Ligase

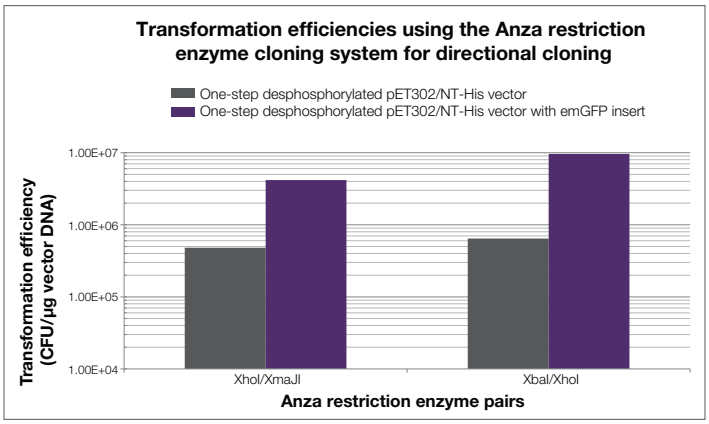

Figure 3. Transformation efficiencies of ligated pET302/NT-His vector and emGFP inserts prepared using the Anza Restriction Enzyme Cloning System.

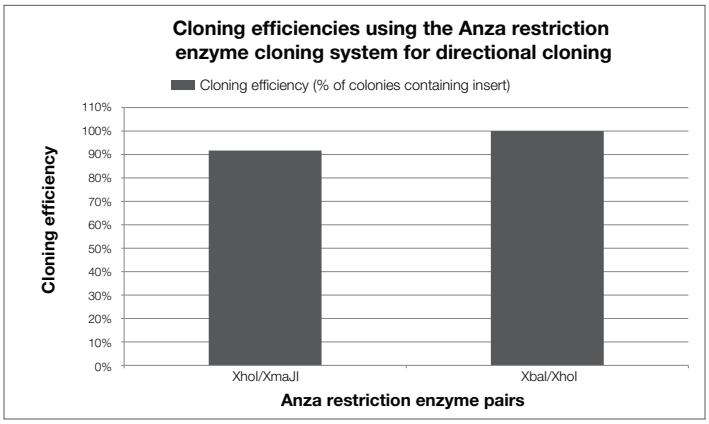

Figure 4. Cloning efficiencies for emGFP inserts ligated into pET302/NT-His vector prepared using the Anza Restriction Enzyme Cloning System.

Master Mix, as well as formed concatemers with the large vector fragments. Using vectors prepared by this method for cloning would lead to a very high background, requiring that the researcher screen significantly more colonies to find one containing the insert of interest. Gel purification is a traditional solution but adds processing time, may lead to sample loss, and increases the chance of contamination. Therefore, treatment with alkaline phosphatase is a preferred method, and the Anza cloning system allows for digestion and dephosphorylation in the same proprietary Anza Buffer. In most cases the 15-minute one-step DNA digestion and dephosphorylation protocol can be used, as illustrated above, compared to products from other suppliers that recommend digestion for 15–60 minutes (or longer if sequential digestion is required), followed by dephosphorylation for an additional 30–60 minutes. In cases where the Anza one-step protocol does not result in sufficient dephosphorylation, the Anza cloning system provides either the two-step/heat inactivation or the two-step/column purification protocol (see the Anza Restriction Enzyme Cloning System user guide for more details). Figure 2, sample lanes 3 and 4 demonstrate the effectiveness of the one-step dephosphorylation protocol. Anza Alkaline Phosphatase does not interfere with digestion, and vector religation upon treatment with Anza T4 DNA Ligase Master Mix is eliminated.

	Process	Traditional cloning	Anza system
		Time required	
Insert prep	Digestion with two restriction enzymes	Up to 2.5 hours	15 minutes
	Heat inactivation or column purification	20 minutes	
Vector prep	Digestion with two restriction enzymes	Up to 2.5 hours	15 minutes, including dephosphorylation
	Dephosphorylation	Up to 60 minutes	--
	Heat inactivation or column purification	20 minutes	
Ligation	Ligation	5 minutes to overnight	5 to 15 minutes
		Traditional cloning	**Anza system**
	Preparation time	**120 minutes to overnight**	**<60 minutes**

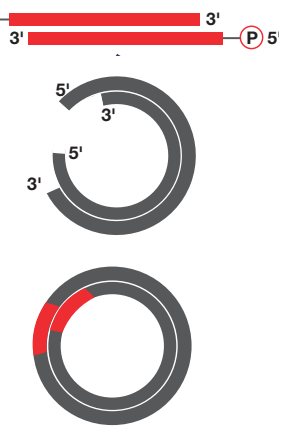

Figure 5. Comparison of workflows using traditional cloning methods and the Anza system.

Excellent transformation and cloning efficiencies were obtained (Figures 3 and 4) using the directional cloning workflow. Transformations with vector-plus-insert ligations produced approximately 9 to 15 times as many colonies as the vector-only background. Insert verification by colony PCR supported these results, with 11 or 12 of the 12 colonies screened containing the emGFP insert.

Figure 5 compares directional cloning workflows for the Anza Restriction Enzyme Cloning System against traditional methodology. Double digestion of the insert may require sequential digestion with multiple purification steps when using traditional enzymes with non-compatible buffers, while all Anza restriction enzymes are compatible with the proprietary Anza Buffer and require only 15 minutes for complete digestion. When using traditional enzymes, vector preparation may require sequential digestion as well, followed by a separate hour-long dephosphorylation step. A majority of Anza REases are compatible with the one-step DNA digestion and dephosphorylation protocol, which allows simultaneous digestion and dephosphorylation in as little as 15 minutes, followed by heat inactivation of the enzymes. When performed side-by-side, vector and insert preparation can be accomplished in less than 40 minutes using the Anza Restriction Enzyme Cloning System. Furthermore, Anza T4 DNA Ligase Master Mix provides efficient ligation of sticky-end fragments for directional cloning in less than 15 minutes, allowing the researcher to complete the cloning workflow in less than one hour.

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 directional 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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