Optimal dephosphorylation protocols using Anza Alkaline Phosphatase

Abstract

A basis of recombinant DNA technology since the 1970s, traditional cloning utilizing restriction enzymes and a DNA ligase is still the most widely used method to manipulate and propagate desired DNA fragments and plasmids. To improve cloning efficiency in traditional cloning, additional DNA-modifying enzymes have become an important part of the workflow. One DNA-modifying enzyme routinely used in vector preparation for cloning is alkaline phosphatase, an enzyme that removes 5'-phosphate groups from DNA and RNA ends. Invitrogen™ Anza™ Alkaline Phosphatase (Cat. No. IVGN2208), newly introduced with the Invitrogen™ Anza™ Restriction Enzyme Cloning System, is fully compatible with the Invitrogen™ Anza™ Buffer and thoroughly characterized for use with each Anza restriction enzyme. Here we describe the features of Anza Alkaline Phosphatase in three dephosphorylation protocols to highlight the most streamlined and effective methods for its use.

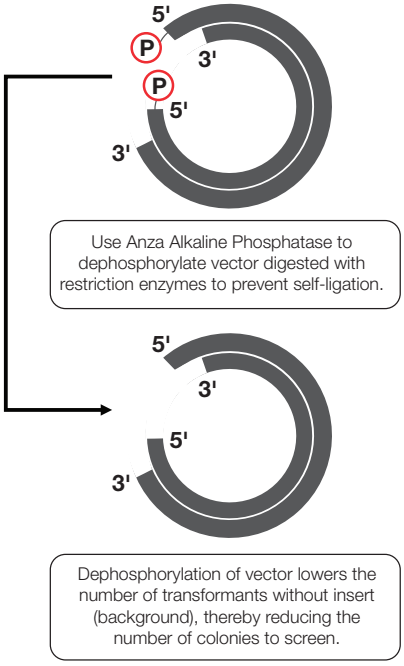

Figure 1. Dephosphorylation of plasmid DNA by alkaline phosphatase.

Introduction

Vector preparation is a critical step in successful cloning. Inefficient digestion or self-ligation of plasmid DNA can result in lower cloning efficiency and higher colony background from the empty vector. Using a vector with a positive or blue/white selection marker can alleviate screening a large number of colonies and improve the probability of picking colonies with a DNA insert. However, self-ligated vectors will compete with insert-carrying vectors during transformation and can lower the number of transformants with the desired insert.

When plasmid DNA is digested with two restriction enzymes with non-compatible ends, dephosphorylation of the vector is often not necessary due to low probability of self-ligation and the abundance of the insert relative to the vector. The standard guideline for an insert:vector molar ratio in a ligation reaction is 3:1.

However, when plasmid DNA is digested with one restriction enzyme or with two restriction enzymes that result in compatible ends (such as blunt ends, or protruding ends as in XhoI and SalI digestion), the vector DNA should be dephosphorylated to prevent self-ligation. Dephosphorylation is commonly carried out by the enzyme alkaline phosphatase that removes the phosphate groups from 5'-ends of the DNA (Figure 1). Without dephosphorylation, ligation between compatible phosphorylated ends of the same molecule (i.e., self-ligation) would be more efficient than ligation between the vector and insert, resulting in high background from the self-ligated plasmid DNA.

Here we describe the methodology used to establish the three dephosphorylation protocols using Anza Alkaline Phosphatase with Anza restriction enzymes. The optimal Anza Alkaline Phosphatase protocol for digested DNA is highly dependent upon the nature of the Anza restriction enzyme used in digestion. We provide three dephosphorylation protocols to offer the fastest options that still ensure complete dephosphorylation to prevent vector self-ligation that leads to lower cloning efficiency (Figure 2). The first protocol is the one-step protocol, which permits the simultaneous digestion and dephosphorylation of DNA. Of the three protocols, the one-step protocol is the fastest with the fewest steps. The second protocol is the two-step/heat protocol, and it requires heat inactivation following restriction digestion, but prior to dephosphorylation. The two-step/heat protocol minimizes the number of tubes needed and sample lost when performing sequential digestion and dephosphorylation. The third protocol, the two-step/column protocol, requires column purification following restriction digestion. The two-step/column protocol is the most thorough method, but requires purification columns and may result in some loss of DNA sample.

Materials and methods

Detailed experiments were performed to evaluate the simultaneous DNA digestion and dephosphorylation using the one-step protocol with all Anza restriction enzymes. For Anza restriction enzymes with which the one-step protocol did not result in complete dephosphorylation, evaluation of the two-step/heat protocol was performed. Finally, when Anza Alkaline Phosphatase failed to completely dephosphorylate DNA using either the one-step or the two-step/heat protocols, those Anza restriction enzymes were evaluated for complete dephosphorylation using the two-step/column protocol. The workflows for each protocol are outlined below.

One-step protocol: simultaneous digestion and dephosphorylation

1 µg of Invitrogen™ pFastBac™1 DNA was digested with selected Anza restriction enzymes and dephosphorylated with Anza Alkaline Phosphatase in 20 µL of 1X Anza Buffer (Cat. No. IVGN2008) for 15 minutes at 37°C. Following simultaneous digestion and dephosphorylation, all enzymes in the reaction mixture were heat inactivated at 80°C for 20 minutes. Each reaction mixture was cooled to room temperature, followed by the addition of 6.7 µL of Invitrogen™

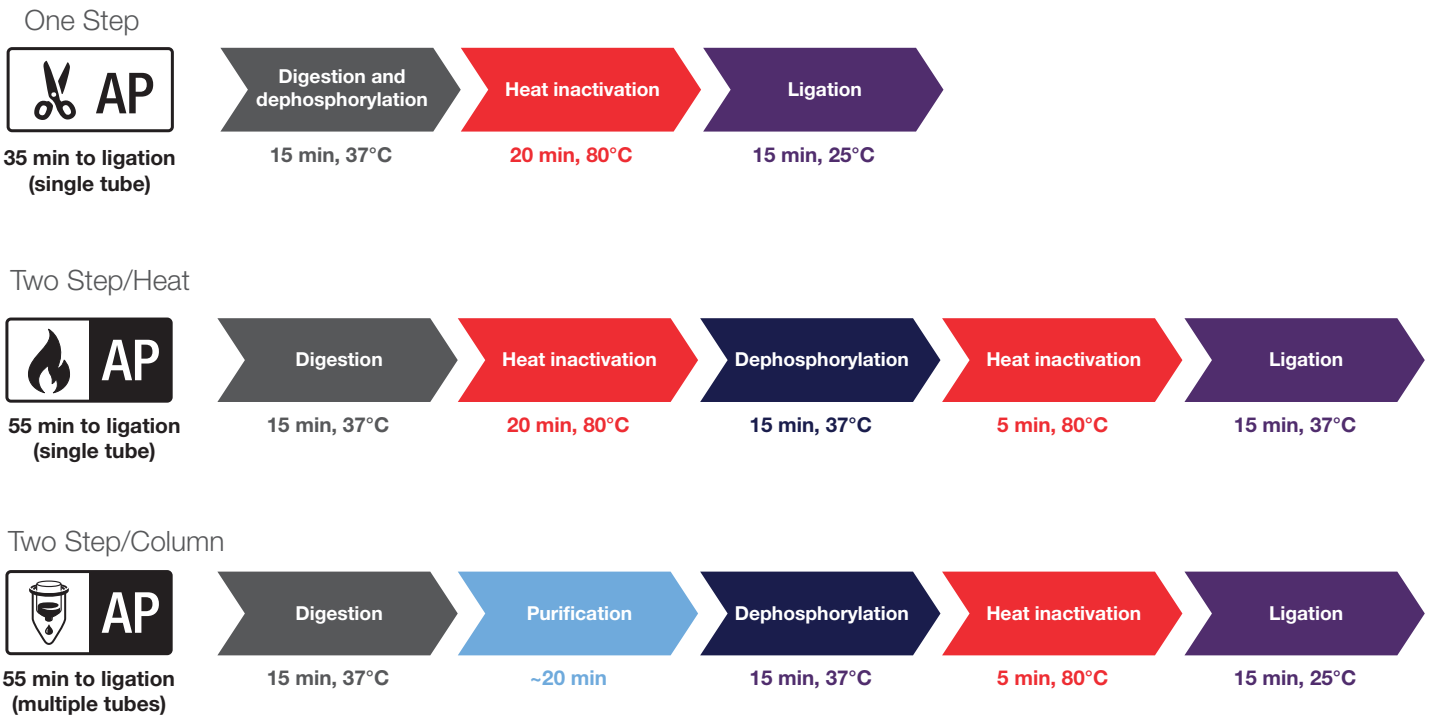

Figure 2. Using Anza Alkaline Phosphatase in three dephosphorylation protocols.

Anza™ T4 DNA Ligase Master Mix (Cat. No. IVGN2104) and incubated at room temperature for 15 minutes. The ligation reaction was stopped with an SDS-containing loading dye and heat (80°C for 10 minutes) and separated in a 1% TAE agarose gel.

Two-step/heat protocol: Heat inactivation of restriction enzymes, followed by DNA dephosphorylation
Further evaluation was performed with the Anza restriction enzymes in which DNA was not completely dephosphorylated with Anza Alkaline Phosphatase using the one-step protocol. Each of these restriction enzymes was used to digest 1 µg of pFastBac1 DNA in 20 µL of 1X Anza Buffer for 15 minutes at 37°C. The restriction enzyme digestion was heat inactivated at 80°C for 20 minutes. After cooling down the reaction mixture to the room temperature, 1 µL of Anza Alkaline Phosphatase was added to each reaction mixture and incubated for 15 minutes at 37°C. The alkaline phosphatase was heat inactivated for 5 minutes at 80°C. Each reaction mixture was allowed to cool and 7 µL of Anza T4 DNA Ligase Master Mix was added and incubated at room temperature for 15 minutes. The ligation reaction was stopped with an SDS-containing loading dye and heat (80°C for 10 minutes) and separated in a 1% TAE agarose gel.

Two-step/column protocol: Column purification of restriction enzymes, followed by DNA dephosphorylation:
Final evaluation was performed for the remaining Anza restriction enzymes for which the DNA was not completely dephosphorylated using Anza Alkaline Phosphatase in either the one-step or the two-step/heat protocols. These Anza restriction enzymes were used to digest 1 µg of pFastBac1 DNA in 20 µL of 1X Anza Buffer for 15 minutes at 37°C. The digested DNA samples were purified with the Invitrogen™ PureLink™ PCR Purification Kit (Cat. No. K310001) without heat inactivation of the restriction enzymes. 2 µL of 10X Anza Buffer and 1 µL of Anza Alkaline Phosphatase were added to 17 µL of each eluted purified sample and incubated for 15 minutes at 37°C. The alkaline phosphatase was heat inactivated at 80°C for 5 minutes. After cooling down the reaction mixture to the room temperature, 6.7 µL of Anza T4 DNA Ligase Master Mix was added to the reaction mixture and incubated at room temperature for 15 minutes. The ligation reaction was stopped with an SDS-containing loading dye and heat (80°C for 10 minutes) and separated in a 1% TAE agarose gel.

Results

One-step protocol: Simultaneous restriction digestion and dephosphorylation

Anza 1 NotI, Anza 8 XhoI, Anza 12 XbaI, or Anza 14 SalI restriction enzymes demonstrate the evaluation of the one-step protocol with simultaneous restriction enzyme digestion and dephosphorylation with Anza Alkaline Phosphatase (Figure 3).

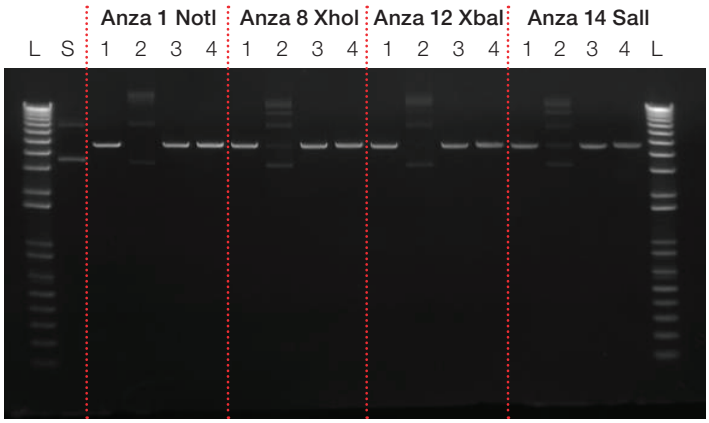

Figure 3. One-step protocol results with representative restriction enzymes.
Lane L: Invitrogen™ 1 Kb Plus DNA Ladder (Cat. No. 10787018)
Lane S: Supercoiled pFastBac1 plasmid
Lane 1: Linearized plasmid
Lane 2: Linearized plasmid, followed by ligation
Lane 3: One-step protocol
Lane 4: One-step protocol followed by ligation

Lane 1 contains pFastBac1 plasmid DNA digested separately with Anza 1 NotI, Anza 8 XhoI, Anza 12 XbaI, or Anza 14 SalI. A single, linearized band was present, demonstrating complete digestion with the tested Anza restriction enzymes in 15 minutes.

Lane 2 contains pFastBac1 plasmid DNA digested separately with each of the Anza restriction enzymes followed by addition of Anza T4 DNA Ligase Master Mix. The presence of the 5'-phosphates on the digested plasmid allowed plasmid self-ligation, as evidenced by supercoiled DNA and concatemers of the pFastBac1 plasmid.

Lane 3 contains pFastBac1 plasmid DNA digested separately with each of the Anza restriction enzymes and simultaneously dephosphorylated with Anza Alkaline Phosphatase. All samples show complete digestion in 15 minutes as in Lane 1, without any nonspecific digestion from the addition of the Anza Alkaline Phosphatase.

Lane 4 contains pFastBac1 plasmid DNA digested separately with each of the Anza restriction enzymes and simultaneously dephosphorylated with Anza Alkaline Phosphatase. All enzymes were heat inactivated prior to addition of Anza T4 DNA Ligase Master Mix. A single, linearized band was present on the gel, indicating that the plasmid was incapable of self-

ligation due to removal of the 5'-phosphates of the digested DNA ends by the alkaline phosphatase.

Two-step protocol: Restriction digestion, followed by heat inactivation or column purification, prior to dephosphorylation

Anza 11 EcoRI and Anza 20 SacI are used to demonstrate the evaluation process for the two-step/heat and two-step/column protocols using heat inactivation or column purification following restriction enzyme digestion prior to addition of Anza Alkaline Phosphatase for complete dephosphorylation (Figure 4).

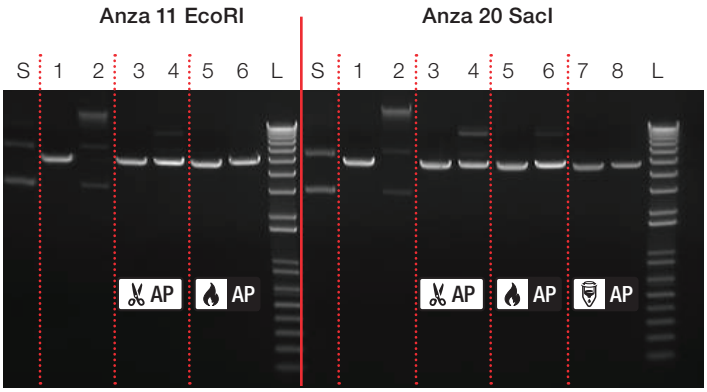

Figure 4. Two-step protocol results with representative restriction enzymes.
Lane L: 1 Kb Plus DNA Ladder
Lane S: Supercoiled undigested DNA
Lane 1: Linearized pFastBac1 plasmid
Lane 2: Linearized plasmid followed by ligation
Lane 3: One-step protocol
Lane 4: One step protocol followed by ligation
Lane 5: Two-step/heat protocol
Lane 6: Two-step/heat protocol followed by ligation
Lane 7: Two-step/column protocol
Lane 8: Two-step/column protocol followed by ligation

Lanes 1–3 are the same as in the one-step protocol above.

Lane 4 demonstrates incomplete plasmid DNA dephosphorylation using the one-step protocol. Some self-ligated plasmid DNA is visible on the gel.

Lane 5 contains pFastBac1 plasmid DNA following independent digestion reactions with Anza restriction enzymes followed by heat inactivation and then dephosphorylation with Anza Alkaline Phosphatase. These lanes with the DNA digested with the Anza restriction enzymes demonstrate complete digestion in 15 minutes as in Lane 1.

Lane 6 contains pFastBac1 plasmid DNA following independent digestion reactions with Anza restriction enzymes and subsequent heat inactivation of the restriction enzymes. This is followed by dephosphorylation with Anza Alkaline Phosphatase. The Alkaline Phosphatase was heat inactivated followed by addition of Anza T4 DNA Ligase Master Mix. With Anza 11 EcoRI, heat inactivation is sufficient to allow for the subsequent dephosphorylation with the Anza Alkaline Phosphatase. However, upon the addition of the Anza T4 DNA Ligase with the heat-inactivated Anza 20 SacI, there is resulting self-ligation as evidenced by the shift from the linearized DNA.

Lane 7 contains pFastBac1 plasmid DNA following digestion with Anza 20 SacI followed by column purification and subsequent dephosphorylation with Anza Alkaline Phosphatase. The DNA digested with the Anza restriction enzyme demonstrates complete digestion in 15 minutes as in Lane 1.

Lane 8 contains pFastBac1 plasmid DNA following digestion with Anza 20 SacI followed by column purification and subsequent dephosphorylation with Anza Alkaline Phosphatase. The Alkaline Phosphatase was heat inactivated followed by addition of Anza T4 DNA Ligase Master Mix. The plasmid DNA is completely dephosphorylated as no additional DNA banding is seen on the gel, only the linearized DNA.

Discussion

The experiments above illustrate how the Anza DNA dephosphorylation protocols were examined with each Anza restriction enzyme to fully characterize their use with Anza Alkaline Phosphatase. In cases where the one-step protocol did not result in sufficient dephosphorylation, the two-step protocols were tested, and the two-step/column protocol was recommended only if the two-step/heat method failed to sufficiently dephosphorylate the digested DNA. The dephosphorylation results suggest that the nature of Anza restriction enzymes dictates dephosphorylation efficiency with respect to the recommended protocols.

Overall, the one-step protocol of Anza Alkaline Phosphatase works effectively with 60% of Anza restriction enzymes (Figure 5). The one-step protocol allows simultaneous vector digestion and dephosphorylation in a single tube with minimal handling in just 15 minutes.

In the two-step protocols, DNA dephosphorylation is performed after either heat inactivation or column purification of the Anza restriction enzymes. The two-step/heat protocol works successfully with 17% of Anza restriction enzymes, and dephosphorylation works optimally with the remaining

23% of the restriction enzymes using the two-step/column purification protocol. While both two-step protocols take approximately 20 minutes for restriction enzyme removal, heat inactivation allows performing all steps in a single tube with minimal chances of sample loss and contamination. As a supplemental resource, Table 1, listing each restriction enzyme and its optimal dephosphorylation protocol, is included at the end of this technical paper.

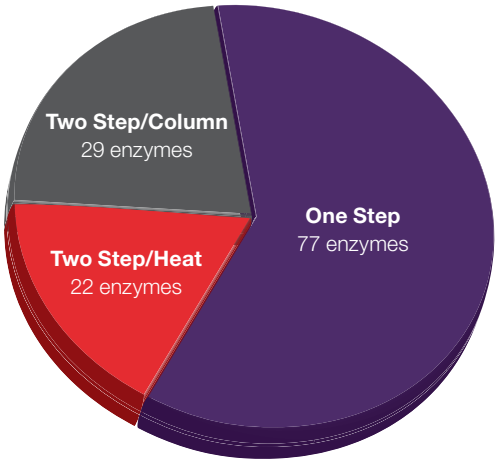

Figure 5: Dephosphorylation protocols

Conclusion

With the three dephosphorylation protocol options outlined, all Anza restriction enzymes have individually been tested to determine the optimal protocol when using Anza Alkaline Phosphatase, and show that this enzyme is compatible with all Anza restriction enzymes and Anza Buffers. These three protocols have been outlined here in detail so Anza Restriction Enzyme Cloning System users do not have to dedicate time and resources to determine optimal conditions through trial and error. Finally, these recommended protocols for using Anza Alkaline Phosphatase show a practical utility of the unique system approach of the Anza Restriction Enzyme Cloning System. For more practical examples of how to use the Anza Restriction Enzyme Cloning System, please visit **thermofisher.com/anza** and look for the Anza Restriction Enzyme Cloning System white papers on other cloning topics.

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Table 1. Anza restriction enzymes aligned to optimal Anza Alkaline Phosphatase dephosphorylation protocols.

With these Anza restriction enzymes, simultaneous digestion and dephosphorylation is recommended		With these Anza restriction enzymes, heat inactivation is recommended prior to dephosphorylation with Anza Alkaline Phosphatase	With these Anza restriction enzymes, column purification is recommended prior to dephosphorylation with Anza Alkaline Phosphatase
Anza 1 NotI	Anza 62 MlsI	Anza 10 DpnI	Anza 7 BshTI
Anza 2 NcoI	Anza 63 CpoI	Anza 11 EcoRI	Anza 13 Esp3I
Anza 3 BcuI	Anza 65 MspI	Anza 28 MluI	Anza 16 HindIII
Anza 4 BpiI	Anza 70 NsbI	Anza 35 Eco47III	Anza 17 KpnI
Anza 5 BamHI	Anza 71 HinfI	Anza 43 Eco105I	Anza 18 PacI
Anza 6 NheI	Anza 72 HincII	Anza 45 PteI	Anza 20 SacI
Anza 8 XhoI	Anza 73 BclI	Anza 50 KspAI	Anza 23 PstI
Anza 9 NdeI	Anza 75 Alw44I	Anza 57 Bpu1102I	Anza 25 PaeI
Anza 12 XbaI	Anza 76 VspI	Anza 60 Kpn2I	Anza 32 ApaI
Anza 14 SalI	Anza 79 PdiI	Anza 61 PfoI	Anza 37 Mph1103I
Anza 15 XmaJI	Anza 80 FspBI	Anza 68 BsuRI	Anza 46 AatII
Anza 19 BglII	Anza 82 Eco72I	Anza 77 DraI	Anza 51 BspTI
Anza 21 SgsI	Anza 84 FspAI	Anza 78 AdeI	Anza 56 Hin1II
Anza 22 SmaI	Anza 85 MreI	Anza 81 Eco91I	Anza 64 SaqAI
Anza 24 MssI	Anza 86 PdmI	Anza 87 Eco47I	Anza 66 BstXI
Anza 26 Eco32I	Anza 88 Bsp119I	Anza 90 Eco88I	Anza 67 RruI
Anza 27 PvuI	Anza 91 Acc65I	Anza 99 XagI	Anza 69 BglI
Anza 29 KfiI	Anza 92 EheI	Anza 111 XapI	Anza 74 CsiI
Anza 30 Bsu15I	Anza 93 HpaII	Anza 112 BseGI	Anza 83 Eco81I
Anza 31 MunI	Anza 94 BfmI	Anza 117 EcoO109I	Anza 89 Mva1269I
Anza 33 LguI	Anza 95 MauBI	Anza 123 Hin6I	Anza 96 XmiI
Anza 34 Pfl23II	Anza 97 Bsp143I	Anza 129 BshNI	Anza 98 XceI
Anza 36 Eco31I	Anza 100 Bsh1236I		Anza 102 CaiI
Anza 38 ScaI	Anza 101 BoxI		Anza 105 Hin1I
Anza 39 Bsp1407I	Anza 103 Psp1406I		Anza 106 Van91I
Anza 40 SfaAI	Anza 104 MboII		Anza 108 SatI
Anza 41 HpyF3I	Anza 107 BspLI		Anza 125 HpyF10VI
Anza 42 RsaI	Anza 109 Alw26I		Anza 126 Alw21I
Anza 44 AluI	Anza 113 BcnI		Anza 128 PspFI
Anza 47 Eco52I	Anza 114 Hpy8I		
Anza 48 MnlI	Anza 115 MbiI		
Anza 49 SmiI	Anza 116 Cfr13I		
Anza 52 PvuII	Anza 118 BseDI		
Anza 53 AanI	Anza 119 BmsI		
Anza 54 Eco147I	Anza 120 NmuCI		
Anza 55 MboI	Anza 121 Bsp120I		
Anza 58 PagI	Anza 122 Csp6I		
Anza 59 HhaI	Anza 124 PfeI		
	Anza 127 RseI		

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