

Cloning Blunt-End DNA Fragments Into the pGEM[®]-T Vector Systems



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Blunt-end DNA fragments can be ligated into Promega's T-Vectors if they are first "tailed" using dATP and Taq DNA Polymerase^(a) (1). In this article, the results of two different A-tailing protocols were evaluated using a pGEM[®]-T^(b,c) Easy Vector System. A standard A-tailing protocol was compared to an abbreviated alternative protocol using both PCR products generated by thermostable DNA polymerases that have proofreading activity, and modified blunt-end DNA fragments generated by restriction digestion. The efficiency of cloning long PCR^(d) fragments into the pGEM[®]-T Vector Systems was also evaluated.

^(a)Some applications in which this product may be used are covered by patents issued and applicable in certain countries. Because purchase of this product does not include a license to perform any patented application, users of the product may be required to obtain a patent license depending upon the particular application and country in which the product is used. For more specific information, please contact Promega.

^(b)Licensed under one or both of U.S. Pat. No. 5,487,993 and European Pat. No. 0 550 693.

^(c)U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

^(d)The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

Introduction

Certain thermostable polymerases, including *Taq*, *Tfl* and *Tth* DNA Polymerase^(a), add a single nucleotide, generally adenine, to the 3'-ends of amplified DNA fragments (2,3). Promega's T-Vector Systems (the pGEM[®]-T, pGEM[®]-T Easy and pTARGET^{™(b)} Vectors) are convenient systems for cloning PCR products generated by such thermostable polymerases. These linearized vectors contain single 3' terminal thymidines (T's) at each end which complement the A overhang added by the polymerase. Thus, PCR products can be directly subcloned into these vectors without further manipulation.

Thermostable DNA polymerases that have a "proofreading" function, such as *Pfu* and *Tli*^(a) DNA Polymerases, exhibit 3'→5' exonuclease activity (4,5) and produce greater than 95% blunt-end fragments. PCR fragments generated by proofreading enzymes can be ligated into the pGEM[®]-T Vectors if they are first tailed with dATP using *Taq* DNA Polymerase (1). This A-tailing protocol can also be used to subclone DNA fragments generated by restriction enzymes which produce blunt ends (1).

Blunt-end PCR fragments

To determine how efficiently blunt-end PCR amplification products could be tailed, two fragments of 1,200bp and 500bp were amplified using both *Pfu* DNA polymerase and *Tli* DNA Polymerase. Both of these DNA polymerases generate blunt-end fragments. After amplification, the fragments were purified using the Wizard[®] PCR Preps DNA Purification System (Cat.# A7170) (6). One to two microliters of each purified fragment were then added to a reaction mixture containing MgCl₂, dATP, *Taq* DNA Polymerase and buffer as shown in [Figure 1A](#). After a 15 minute incubation at 70°C, the resulting 3' A-tailed fragment was ligated into Promega's pGEM[®]-T Easy Vector according to recommended procedures (7). Following this standard protocol, 80-90% recombinants were obtained ([Table 1](#)).

The standard A-tailing protocol outlined in [Figure 1A](#) requires purification of the PCR product. When working with a large number of different amplified fragments this tailing procedure may prove cumbersome. To determine whether the protocol can be abbreviated in any manner, we performed several A-tailing reactions according to the procedure shown in [Figure 1B](#). In this shortened protocol, the PCR product is not purified and *Taq* DNA Polymerase and dATP are added directly to the 50µl PCR reaction mix after the amplification profile is complete.

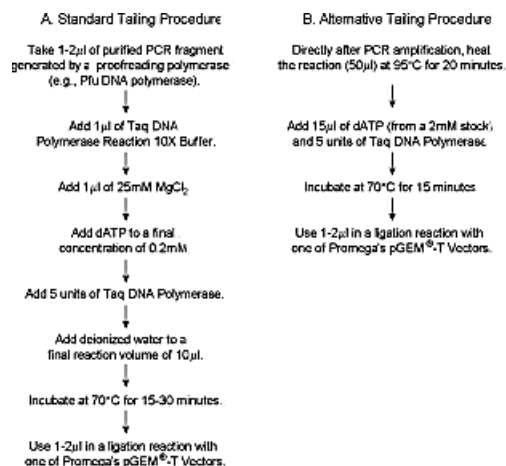


Figure 1. A-Tailing procedures. Panel A: Standard tailing procedure for blunt-end PCR fragments purified with the Wizard[®] PCR Preps DNA Purification System. **Panel B:** Alternative tailing procedure for blunt-end PCR fragments.

Using this alternative procedure, 20-30% recombinants were obtained when *Pfu* DNA polymerase was used in the amplification profile (Table 1). Recombinants were identified by blue/white screening and were confirmed by restriction digestion analysis (Figure 2). Few positives were observed in the control reaction, in which the PCR fragment was not tailed. These control results confirm that the majority of the pGEM[®]-T Easy Vector used contains 3'-terminal deoxythymidine. Although the efficiency of this shortened protocol is reduced compared to the standard tailing procedure shown in Figure 1A, the low background exhibited by the pGEM[®]-T Vectors means that this procedure is a useful way to obtain clones when processing large numbers of samples.

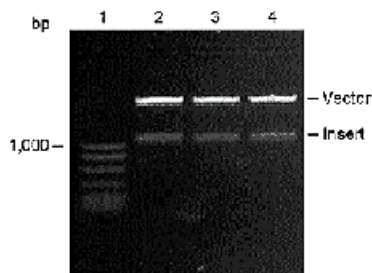


Figure 2. Analysis of recombinants obtained after cloning of an A-tailed PCR fragment into the pGEM[®]-T Easy Vector. A 1,200bp fragment was generated by amplification with *Pfu* DNA polymerase. This fragment was tailed using the alternative procedure described in Figure 1B. Two microliters (from a total reaction volume of 65µl) was then ligated into the pGEM[®]-T Easy Vector. The ligation reaction was transformed into JM109 Competent Cells (Cat.# L2001), and screened on indicator plates containing IPTG (Cat. # V3955) and X-Gal (Cat. #V3941) (7). Three recombinant colonies (white) were selected and plasmid DNA was isolated using the Wizard[®] Plus SV Minipreps DNA Purification System (Cat.# A1330). The plasmid DNA was then digested with *Not* I to release the insert from the vector (7). The DNA was analyzed on 1% agarose gel containing ethidium bromide. Lanes: lane 1, Promega's 100bp DNA Ladder (Cat.# G2101); lanes 2-4, *Not* I-digested recombinant plasmid DNA.

The shortened tailing protocol outlined in Figure 1B cannot be used with all thermostable DNA polymerases. The DNA polymerase used in the amplification is a critical factor. The results in Table 1 show that no recombinants were obtained using the alternative tailing procedure when *Tli* DNA Polymerase was used in the amplification profile. This may be due to the greater thermostability of *Tli* DNA Polymerase compared with *Pfu* DNA polymerase (8). However, greater than 90% recombinants were obtained using the standard tailing procedure (Figure 1A) in conjunction with a *Tli* DNA Polymerase-generated amplification fragment (Table 1).

Table 1. Comparison of A-Tailing Procedures Used With Different DNA Polymerases.

Polymerase	Percent Recombinants	A-Tailing Procedure
<i>Pfu</i> DNA polymerase	85-90%	Standard (Figure 1A)
<i>Pfu</i> DNA polymerase	20-30%	Alternative

		(Figure 1B)
<i>Tli</i> DNA Polymerase	80-90%	Standard (Figure 1A)
<i>Tli</i> DNA Polymerase	0%	Alternative (Figure 1B)

DNA fragments generated by restriction enzymes that leave 3' overhangs

The A-tailing protocol outlined in [Figure 1](#) can be modified so that DNA fragments generated using restriction enzymes that leave a 3' overhang can be cloned into T vectors such as the pGEM[®]-T and pGEM[®]-T Easy Vectors. The method outlined in [Figure 3](#) uses the 3'→5' exonuclease activity of T4 DNA Polymerase to remove the 3' overhang and generate a blunt-end. After treatment with T4 DNA Polymerase, *Taq* DNA Polymerase, reaction buffer and dATP are added. The reaction is incubated at 70°C for 15 minutes. The DNA can then be ligated directly into the pGEM[®]-T Vector according to standard protocols (7). Following this procedure we obtained greater than 80% recombinants based on blue/white screening and restriction digestion.

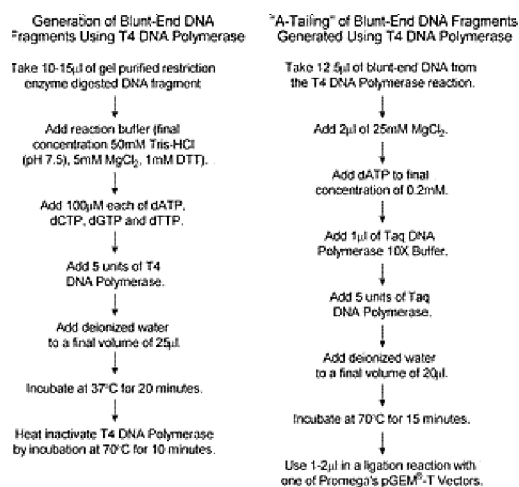


Figure 3. A-Tailing DNA fragments generated by restriction enzymes that leave 3' overhangs.

Secondary tailing of eLONGase[™]-amplified products

A novel PCR approach for generating long amplification products uses a mixture of two thermostable polymerases (9,10). One polymerase lacks a 3'→5' exonuclease activity; the second polymerase is present at a reduced concentration and contains 3'→5' exonuclease activity. When the first enzyme misincorporates a nucleotide, extension of the newly synthesized DNA strand proceeds very slowly or stops. The addition of the proofreading enzyme allows removal of the misincorporated nucleotide, and extension of the strand continues.

The presence of these two types of polymerase in a single reaction results in the amplification of a heterogeneous population of PCR fragments that are either blunt ended or have a 3'-A tail. We conducted experiments to determine if an additional incubation in the presence of dATP and *Taq* DNA Polymerase would increase the number of recombinants obtained from this type of reaction.

A 4.0kb PCR fragment was amplified using eLONGase[™] (Life Technologies, Inc.) - a mixture of *Taq* and *Pyrococcus species* GB-D DNA polymerases. Products were purified and tailed according to the protocol detailed in [Figure 1A](#). Samples were then ligated into the pGEM[®]-T Vector and transformed as described (7). Results indicated that this additional A-tailing step increased the number of recombinants obtained by 2-8 fold (data not shown).

Primer design

Several recent publications have indicated that adjacent nucleotide(s) influence the ability of *Taq* DNA Polymerase to add a 3'-A deoxynucleotide to the ends of amplified DNA fragments (11-13). We conducted several experiments to determine whether different 3'-ends would affect the cloning of a DNA fragment into the pGEM[®]-T Vector. The 3'-end combinations evaluated were T and G, A and G, A and C, A and G, and T and G.

All amplified products ranged in size from 600-1,400bp. After amplification, these PCR products were purified using the Wizard[®] PCR Preps DNA Purification System, ligated into the pGEM[®]-T Vector at a 1:1 vector:insert molar ratio and transformed into JM109

Competent Cells as recommended (7).

Yields of 20-86% recombinants were obtained, depending on the target DNA amplified. Our results showed that PCR fragments containing a 3' terminal A nucleotide gave lower percentages (20-40%) of recombinants than those with other nucleotides at this position. The presence of other nucleotides at the 3'-end of the amplified fragment did not affect the addition of a 3'-A deoxynucleotide and so equally high percentages of recombinants were obtained for fragments having a terminal T, C or G.

To design primers and cycling profiles that optimize conditions for *Taq* DNA Polymerase to add a 3'-A deoxynucleotide to the ends of amplified DNA fragments, consult references 11 and 12. However, in most cases where the pGEM[®]-T and pGEM[®]-T Easy Vectors are used, it is not necessary to adjust experimental parameters in order to obtain positive recombinants because of the low background of recircularized vector obtained with these systems.

Cloning of large DNA fragments into the pGEM[®]-T Vector

Amplification of long DNA fragments is desirable for numerous applications such as physical mapping, subcloning fragments from cosmids or bacteriophage, and direct genomic cloning. Further manipulation of these large PCR fragments requires that they be ligated into a suitable vector.

To determine if large DNA fragments can be cloned successfully into Promega's pGEM[®]-T Vectors, a 7.5kb PCR fragment was generated. This fragment was purified using the Wizard[®] PCR Preps DNA Purification System, and cloned into the pGEM[®]-T Easy Vector using a vector:insert molar ratio of 1:1. Subsequent transformations were performed according to standard protocols (7).

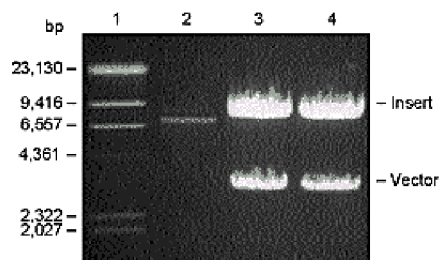


Figure 4. Analysis of recombinants obtained from cloning of a 7.5kb fragment into the pGEM[®]-T Easy Vector. A 7.5kb amplified fragment was purified using the Wizard[®] PCR Preps DNA Purification System (6). The fragment was ligated into the pGEM[®]-T Easy Vector at a 1:1 vector:insert molar ratio and then transformed into JM109 Competent Cells as described (7). Several positive recombinants were selected and plasmid DNA isolated using the Wizard[®] Plus SV DNA Purification System. The isolated DNA was then digested with *Not* I to release the insert and the DNA was analyzed on a 1% agarose gel containing ethidium bromide. Lanes: lane 1, Lambda DNA/*Hind* III Markers (Cat.# G1711); lane 2, 7.5kb insert; lanes 3 and 4, *Not* I-digested recombinant plasmid DNA.

Using this procedure, 20-30% recombinants were obtained. Recombinant clones were verified by digestion with the restriction enzyme *Not* I. The pGEM[®]-T Easy Vector contains two *Not* I restriction sites in the multiple cloning region flanking the insert site, so a single digest with this enzyme results in release of the cloned fragment (Figure 4).

Summary

Standard A-tailing protocols require purification of PCR products prior to incubation with dATP and *Taq* DNA Polymerase. The alternative, shorter protocol described here proved to be an effective way of processing multiple PCR amplification reactions when used in conjunction with *Pfu* DNA polymerase and the pGEM[®]-T Vector Systems. Although the percentage of recombinants obtained using the alternative protocol was reduced, the low levels of background (i.e., religated vector DNA) seen with the pGEM[®]-T Vector Systems made it easy to identify recombinants. A-tailing reactions occurred with reduced efficiency if the primers used in the PCR reaction had a 3' terminal A nucleotide. Primers designed with a 3' terminal C, T or G performed with equal efficiency in A-tailing reactions. The pGEM[®]-T Vector Systems were also used successfully to clone large PCR products generated using the eLONGase[™] enzyme blend.

References

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Ordering Information

Product	Size	Cat.#
pGEM [®] -T Easy Vector System I	20 reactions	A1360
pGEM [®] -T Easy Vector System II	20 reactions	A1380
pGEM [®] -T Vector System I	20 reactions	A3600
pGEM [®] -T Vector System II	20 reactions	A3610
T4 DNA Polymerase	100 units	M4211
	500 units	M4215

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