Cloning Blunt-End *Pfu* DNA Polymerase-Generated PCR Fragments into pGEM®-T Vector Systems

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Pfu *DNA Polymerase*[a]*-generated blunt-end PCR fragments can be ligated into the pGEM®-T and pGEM®-T Easy Vectors*[b,c] *if the fragments are first A-tailed using* Taq *DNA Polymerase*[a]*. We describe a method for A-tailing these PCR fragments and demonstrate its utility by cloning two different* Pfu *DNA Polymerase-generated fragments into the pGEM®-T Easy Vector, using the new 2X Rapid Ligation Buffer. We found that, depending upon the size and yield of the amplified fragment, the tailing protocol must be adjusted to optimize the insert:vector ratio for ligation. With some inserts we found that the* alpha*-peptide based blue/white screening results in both white and pale blue recombinant colonies. One PCR product tested here gives such a mixture of recombinant colonies. With this particular PCR fragment we found that the two different colony colors resulted from opposite orientations of the insert.*

INTRODUCTION

When PCR is used for applications where accurate replication of the template is extremely important (e.g., cloning), it is essential that amplification be performed using a DNA polymerase that exhibits the highest possible fidelity. *Pfu* DNA Polymerase possesses an intrinsic 3´-->5´ exonuclease activity that serves a proofreading function. It is a superior enzyme in applications such as high fidelity PCR, because it exhibits the lowest error rate of any commercially available thermostable DNA polymerase (13).

Taq DNA Polymerase, the enzyme most commonly used in PCR, adds a nontemplate-dependent A residue to the 3´-end of the PCR products. In contrast, the exonuclease activity of *Pfu* DNA Polymerase results in the generation of amplification products with blunt ends (4). Therefore, protocols for cloning *Taq* DNA Polymerase-generated PCR products cannot be used for cloning *Pfu* DNA Polymerase-generated PCR products unless modified. Among the options for cloning *Pfu* DNA Polymerase-generated fragments are: 1) blunt-end cloning; 2) A-tailing the PCR product followed by ligation into a T-vector and; 3) incorporating restriction enzyme sites into the amplification primers. For this third option, the amplified product and cloning vector are digested with the appropriate restriction enzymes to achieve cohesive-end ligation.

There are several advantages to the A-tailing approach. Only one insert will be ligated into the vector as opposed to multiple insertions that can occur with blunt-end cloning. In addition, with T-vector cloning there is no need to dephosphorylate the vector, and there is a low number of recombinants due to religated vector. In this study we describe an updated A-tailing procedure for *Pfu* DNA Polymerase-generated PCR fragments. This protocol is updated from earlier *Promega Notes* articles (5,6) for use with the new 2X Rapid Ligation Buffer now provided with Promega's pGEM®-T and pGEM®-T Easy Vector Systems. In this article we examine the A-tailing and ligation of two different inserts into the pGEM®-T Easy Vector.

A-TAILING PROTOCOL FOR BLUNT-END FRAGMENTS

The protocol described in Figure 1 can be used to add a nontemplate-dependent A-residue to the 3´-ends of *Pfu* DNA Polymerase-generated products with blunt ends, thereby allowing for ligation into the pGEM®-T and pGEM®-T Easy Vectors. To use this A-tailing procedure successfully, it is essential to first purify the PCR product from the polymerase. If *Pfu* DNA Polymerase is not removed, the exonuclease activity of the enzyme will remove the 3´ A-tail and can potentially degrade the fragment during the tailing procedure. Heat inactivation of *Pfu* DNA Polymerase is not practical because incubation at 95°C for one hour causes no appreciable decrease in the activity of this thermostable polymerase. The Wizard® PCR Preps DNA Purification System[d] efficiently removes the *Pfu* DNA Polymerase and has the additional advantage of removing primer-dimers that can be present after amplification. To remove extraneous PCR products as well as the polymerase, gel purification of the desired amplification product can be performed.

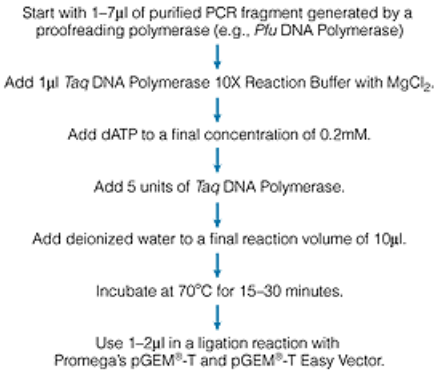

Figure 1. An A-tailing procedure for blunt-end *Pfu* DNA Polymerase-generated PCR fragments purified with Wizard® PCR Preps DNA Purification System and used in T-vector cloning.

The A-tailing protocol and subsequent ligation of two different PCR-generated fragments were tested for this report. As with most cloning procedures, this investigation includes insert:vector ratio optimization. To optimize cloning results we found that the amount of DNA in the A-tailing reaction and ligation volumes must be adjusted depending on the molar yield of the purified PCR product. When molar concentrations are high due to small fragment size or good amplification, then small volumes of the fragment are needed for A-tailing and ligation reactions. However, when the molar concentrations are low due to large fragment size or poor amplification, then large volumes of the PCR fragment are needed for A-tailing and ligation reactions.

CLONING OF A 542BP PCR FRAGMENT

The pGEM®-T and pGEM®-T Easy Vector Systems contain a 542bp Control Insert DNA for use as a positive control. This 542bp insert DNA (0.2ng) was used as a template in PCR containing *Pfu* DNA Polymerase (Table 1). Gel analysis of the 542bp PCR fragment revealed a single band. The fragment was purified using the Wizard® PCR Preps and the concentration determined by spectrophotometry (data not shown). Due to the high molar concentration, only a small volume (12µl) of the purified PCR product was used in the A-tailing reaction (Figure 1). To verify that A-tailing was occurring in the reactions, no-tail control experiments were performed. In the no-tail reactions *Taq* DNA Polymerase was omitted. The A-tailed fragments and the no-tail controls were ligated into the pGEM®-T Easy Vector and transformed as described in the *pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual*, #TM042 (7). Ligation of the A-tailed fragment into the pGEM®-T Easy Vector resulted in a high percentage of recombinants and in good cloning efficiency (Table 1). In the no-tail control reactions for each insert:vector ratio and ligation condition, there were <=10% white colonies in the transformed cells, indicating that the A-tailing procedure worked well. When recombinants from these ligations (10 recombinants/ligation) were tested for the presence of the correct insert size, >=90% contained a fragment of the correct size. As Frackman and Kephart report in this issue (8), the percentage of recombinants and cloning efficiency was greater for the 16-hour ligation at 4°C than for the one-hour ligation at 24°C. In addition, there was an increase in the percent recombinants and cloning efficiency with increasing insert:vector ratios.

Table 1. Cloning Efficiency of a 542bp Insert DNA Generated with *Pfu* DNA Polymerase in PCR.

Insert: Vector	1-Hour Ligation, 24°C		16-Hour Ligation, 4°C	
	% Recombinants	Cloning Efficiency	% Recombinants	Cloning Efficiency
1.3:1	65	273	81	543
2.6:1	78	297	95	1,092
5.3:1	84	267	93	1,192

Data is from one ligation for each insert:vector ratio. Percent recombinants is the percent of white colonies. Cloning efficiency is the number of white colonies/ng vector from a ligation containing 50ng pGEM®-T Easy Vector.

The 542bp insert (0.2ng) was amplified in reactions as directed in the Product Insert (9) with 120pmol of each primer. The hot-start amplification scheme involved an initial denaturation cycle (94°C for 2 minutes), then 30 cycles of denaturation (94°C for 30 seconds), annealing (65°C for 1 minute) and extension (72°C for 1 minute) and was concluded by a final extension (68°C for 7 minutes). Ligations were performed and the products were transformed into High Efficiency JM109 Competent Cells (7).

CLONING OF THE 1.8KB PCR FRAGMENT

To determine the effect of a larger insert size on the A-tailing and ligation of blunt-end *Pfu* DNA Polymerase-generated amplification products, the experiment was repeated with a 1.8kb fragment from the human familial *adenomatous polyposis coli* (*APC)* gene (10). A-tailing and ligation of the 1.8kb fragment required increased volumes of the purified PCR fragment, due to the lower molar concentrations of PCR product. Two to seven microliters of Wizard® PCR Preps-purified product were added to the A-tailing reactions. The no-tail control reactions were performed as described above. Ligations of the A-tailed and no-tailed control reactions (2µl/ligation) were performed at either 24°C for one hour or 4°C for 16 hours (Table 2) and transformed into High Efficiency JM109 Competent Cells (Cat.# L2001; 7).

Table 2. Cloning Efficiency of a 1.8kb Fragment Generated with *Pfu* DNA Polymerase in PCR and After 1-Hour and 16-Hour Ligations.

Insert:Vector Molar Ratio	1-Hour Ligation, 24°C				16-Hour Ligation, 4°C			
	% White	% Pale Blue	% Recombinants	Cloning Efficiency	% White	% Pale Blue	% Recombinants	Cloning Efficiency
0.8:1	9.7	6.5	16	92	17	11	27	141
1.2:1	22	9	31	162	32	18	50	318
2.0:1	23	16	39	233	42	24	66	342
2.8:1	31	24	55	420	43	33	75	395

Data is from one ligation for each insert:vector ratio. Percent recombinants is the percent of white and pale blue colonies. Cloning efficiency is the number of white and pale blue colonies/ng vector from a ligation with 50ng pGEM®-T Easy Vector.

The 1.8kb APC fragment (bases 1,9783,808) was amplified in reactions using 50pmol of each primer and 100ng Human Genomic DNA (Cat.# G3041) as a template (9). The hot-start amplification scheme involved an initial denaturation cycle (94°C for 2 minutes), then 35 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 1 minute) and extension (72°C for 4 minutes) and concluded with a final extension (72°C for 10 minutes). Two to seven microliters of purified PCR product were added to the A-tailing reactions to compensate for the four different insert amounts tested. Ligations were performed and the products were transformed into High Efficiency JM109 Competent Cells (7).

Transformed cells were grown on LB agar plates containing ampicillin, IPTG and X-gal, and exhibited three colony colors: white, pale blue and blue. Similar results were observed when the 1.8kb fragment was amplified using *Taq* DNA Polymerase and cloned into the pGEM®-T Easy Vectors (8). The pale blue colonies were present in significant numbers (4177% of the white colonies). In addition, there was an increased number of blue colonies present on the plates compared to the ligation/transformation of the positive control insert DNA experiments and the ligation control.

A total of 10 colonies per ligation for each colony type were screened for the presence of the 1.8kb insert using the T7 and SP6 primers (Figure 2 and Figure 3, Panel A). As expected, none of the blue colonies tested contained an insert (Figure 3, Panel A). The 194bp amplification product that is seen in Figure 3 arises from amplification of the multiple cloning site of the pGEM®-T Easy Vector. We have no explanation for the increased number of blue colonies seen here; in other investigations with the pGEM®-T Vectors this increase in blue colonies was not seen (8).

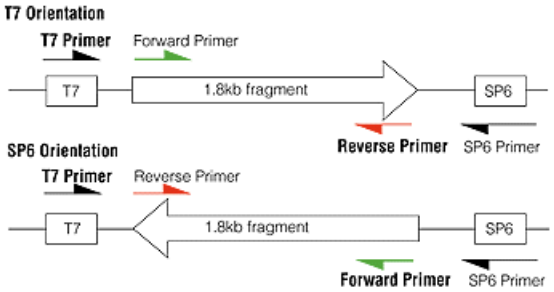

Figure 2. PCR primers used to establish the presence and orientation of the cloned 1.8kb fragment of the *APC* gene. T7 and SP6 primers were used to check for the presence of the 1.8kb insert. Gene-specific forward and reverse primers were used in concert with the T7 primer to establish orientation of the inserts in specific clones. Orientation is in reference to the T7 primer.

Both the white and pale blue colonies contained recombinant plasmids with inserts. In most ligations the majority of the inserts were the correct size (Figure 3, Panel A). The percentage of colonies with the correct insert size was lower for the low insert:vector ratios (40% at

the 0.8:1 ratio) but increased with the higher insert:vector ratios (6080% at the 2.8:1 ratio) for the one-hour ligation. The percentage of colonies with the correct insert size increased with the 16-hour ligation and ranged from 6080% with the 0.8:1 ratio to 80100% with the 2.0:1 ratio.

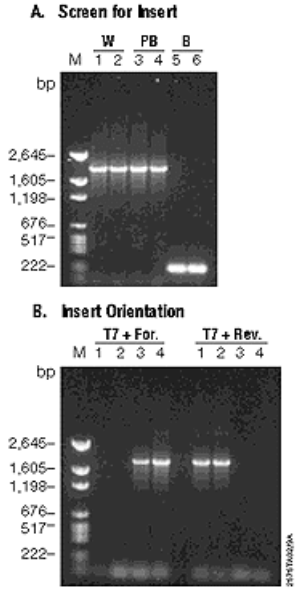

Figure 3. Screening of white, pale blue and blue colonies for insert of the correct size and orientation. Colonies were suspended in 50µl sterile water, boiled for 10 minutes, centrifuged at 16,000 x *g* for 5 minutes, and 5µl of the supernatant was used in each amplification. The DNA was amplified by PCR in 50µl volumes with 50pmol of each primer and 1.25 units of Promega's *Taq* DNA Polymerase (Cat.# M1661). After an initial denaturation of 2 minutes at 94°C, the amplification profile was 35 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 1 minute) and extension (72°C for 2.5 minutes); PCR reaction concluded with 1 cycle of 72°C for 10 minutes. Amplification products (8µl) were analyzed on a 1% agarose gel containing ethidium bromide. **Panel A:** Screen for the presence of the correctly sized insert. Primers used were T7 and SP6, illustrated in Figure 2. W = white, PB = pale blue and B = blue colonies. **Panel B:** Screen of colonies from Panel A (white, lanes 1 and 2; pale blue, lanes 3 and 4) for orientation of the insert; primers are those depicted in Figure 2 (T7 in concert with forward or reverse primer as indicated). Lanes M, Promega's pGEM® DNA Markers[(b)] (Cat.# G1741).

One possible explanation for the difference in colony color of recombinants (white versus pale blue) is that the inserts are ligated into the vector in different orientations. To investigate this possibility, the white and pale blue colonies from both the one-hour and 16-hour ligation experiments were screened for insert orientation, using the primers illustrated in Figure 2. The majority (36/37) of the white colonies tested had inserts in the T7 orientation and all (40/40) of the pale blue colonies had inserts in the SP6 orientation (Figure 2 and Figure 3, Panel B). These orientations for the two colony colors were confirmed on plasmid DNA from two pale blue colonies and one white colony by sequencing. Since the sequence for the 1.8kb fragment is known, we checked this sequence in both orientations of the pGEM®-T Easy Vector to see if there were any obvious explanations for the difference in colony color, such as new start codons in the 1.8kb fragment that would result in initiation of the downstream *lac*Z alpha-peptide. None were found.

The ligation of the A-tailed fragment into the pGEM®-T Easy Vector resulted in a good percentage of recombinants and in good cloning efficiency (Table 2). High insert:vector ratios gave much better results (percent recombinants, cloning efficiency and percent colonies with the correct-sized insert) than did low insert:vector ratios. This illustrates the need to optimize the A-tailing and ligation reactions to give a suitable insert:vector ratio. In no-tail control reactions for each insert:vector ratio and ligation condition, there were <=4% recombinant colonies in the transformed cells, indicating that the A-tailing procedure was working. As seen with the 542bp PCR fragment, the 16-hour ligation resulted in increased percentage of recombinants and in most cases, increased cloning efficiency and percentage of colonies with the correct insert size when compared to the one-hour ligation (Table 2). With some insert:vector ratios, particularly with the one-hour ligation, the percentage of colonies with the correct insert size was somewhat low. The majority of the colonies screened that did not have the correct-sized insert had smaller inserts. To increase the percentage of recombinants with the correct inserts, the 1.8kb PCR fragment could have been gel-purified. Finally, the lower percentage of recombinants and cloning efficiency for the 1.8kb fragment compared to the 542bp fragment may be due to fragment size.

SUMMARY

The A-tailing procedure used in conjunction with the pGEM®-T Vector Systems is an effective method for cloning of *Pfu* DNA Polymerase-generated blunt end PCR fragments. The new 2X Rapid Ligation Buffer in the T-Vector Systems allows for quick cloning results. Overnight ligations can be used to improve the percentage of recombinants and cloning efficiency if necessary. When using these procedures it may be necessary to optimize the insert:vector ratio by adjusting the volume of the purified fragment added to the A-tailing reaction, as well as the volume added to the ligation. With some PCR products, such as the 1.8kb fragment investigated here, it might be particularly important to note the colony color when selecting recombinant colonies. Recombinant colonies can be white or

pale blue, depending on insert orientation, and the orientation can be investigated using the procedures described here.

ACKNOWLEDGEMENTS

We thank Mike Slater and Susan Frackman for their helpful discussions during this project.

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Ordering Information

Product	Size	Cat.#
Pfu DNA Polymerase	100u	M7741
	500u	M7745
Taq DNA Polymerase	100u	M1661
	500u	M1665
	2,500u	M1668
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
Wizard® PCR Preps DNA Purification System	50 preps	A7170

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[b]U.S. Pat. No. 4,766,072.

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