Promega Notes Magazine **Number 58, 1996, p. 02**

pTARGET™ Vector: A New Mammalian Expression T-Vector

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Promega's pTARGET™ Vector has been constructed to improve the cloning and expression of DNA fragments amplified by PCR or modified by addition of deoxyadenosine tails. The presence of the alpha-peptide of beta-galactosidase in the pTARGET Vector allows detection of recombinants by blue/white screening, a feature not shared by other mammalian expression T-vectors. Comparison of the pTARGET™ Vector to another commercially available expression T-vector demonstrates enhanced mammalian expression of Renilla *luciferase from the pTARGET™ Vector by as much as 15- to 18-fold.*

Introduction

With the tremendous popularity of the polymerase chain reaction in the biological sciences, many investigators find cloning of PCR products to be a necessary procedure. The most common and arguably the easiest way to clone PCR products is the T-vector method (Figure 1; 1). This method utilizes the finding that many thermostable polymerases (e.g., *Taq*[(a)], *Tfl*[(a)], *Tth*[(a)]) add a single deoxyadenosine, in a template independent fashion, to the 3´-end of amplified products (2). The amplified product containing the 3´ adenosine is then directly ligated to a T-vector, which is a linearized vector containing a single thymidine at the 3´-ends. Thermostable polymerases that have a 3´-->5´ exonuclease activity generate blunt-ended products that also can be easily cloned into a T-vector by the addition of an "A-tailing" step following amplification (3,4). Blunt-ended fragments generated by restriction enzyme digests can also be efficiently subcloned into T-vectors after a simple A-tailing step.

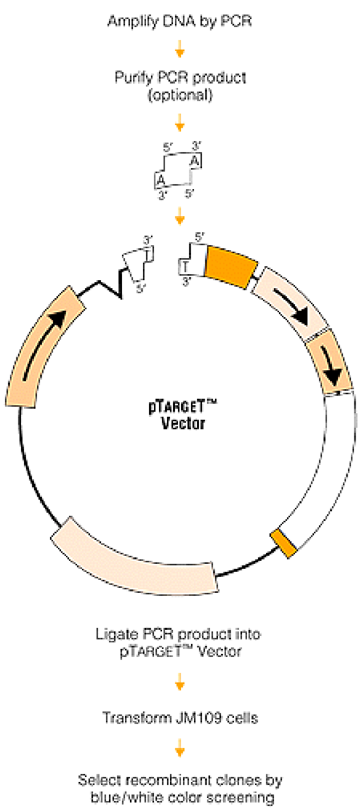

Figure 1. Flow diagram of preparation and cloning of DNA using the pTARGET™ Vector.

An application frequently used by investigators cloning PCR-amplified genes is the expression of the cloned genes in mammalian cells. Promega's pTARGET™ Vector (Figure 2) has been constructed so that it may be used both as a T-vector for cloning PCR products and as a mammalian expression vector. Having both the T-vector cloning and expression capability on one vector is advantageous because it eliminates a subcloning step. The pTARGET™ Vector can be used for strong constitutive expression in transient transfections, or for generating stable transfectants since it expresses the neomycin phosphotransferase gene, a selectable marker in mammalian cells.

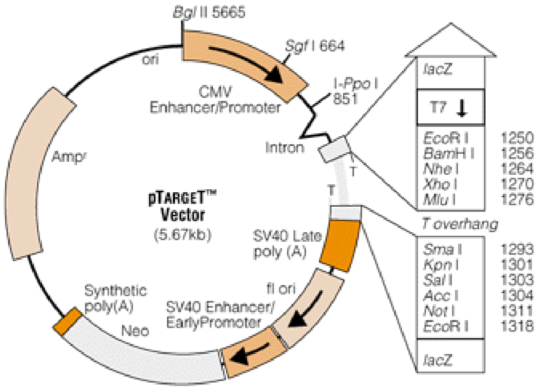

Figure 2. Circle map of the pTARGET™ Vector.

[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 this 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.*

Features and performance of the pTARGET™ Vector System

The pTARGET™ Vector was derived from the pCI-neo[b] Vector (5) and contains the identical elements for regulating expression of the cloned gene (Figure 2). The expression of the inserted gene in the pTARGET™ Vector is regulated by the human cytomegalovirus (CMV) enhancer/promoter[c], an extremely strong regulatory region active in a variety of cell types (6). Downstream from the CMV region is a chimeric intron composed of the 5´-donor splice site from the first intron of the human beta-globin gene and the branch and 3´-acceptor splice site from the intron of an immunoglobulin gene heavy chain variable region (7). The presence of an intron increases the expression of many cDNAs; this chimeric intron has been shown to increase expression of the chloramphenicol acetyltransferase (CAT) gene 21-fold and the luciferase gene 3-fold (8-11).

[b]*U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.*
[c]*The CMV Vector technology is the subject of U.S. Patent No. 5,168,062 assigned to the University of Iowa Research Foundation.*

The pTARGET™ Vector also contains the simian virus 40 (SV40) late polyadenylation signal downstream of the cloning site. The SV40 late polyadenylation signal is extremely efficient at inducing the polyadenylation of RNA transcripts, which is necessary for a high level of expression (12). For generation of stable transfectants, the pTARGET™ Vector contains the neomycin phosphotransferase gene under the regulation of the SV40 enhancer and early promoter region. Expression of neomycin phosphotransferase in mammalian cells confers resistance to the antibiotic G418 (13). The SV40 regulatory region also contains the SV40 origin of replication which induces transient, episomal replication of the pTARGET™ Vector in cells expressing the SV40 large T antigen (e.g., COS-1 or COS-7) (14).

The major differences between the pTARGET™ Vector and the pCI-neo Vector are that the pTARGET™ Vector is a T-vector, and that it contains the alpha-peptide sequence of beta-galactosidase surrounding the multiple cloning site. Expression of the alpha-peptide in *E. coli* strains that express an appropriate alpha-acceptor results in blue colonies when plated onto an appropriate indicator plate. This feature is extremely useful because insertional inactivation of the alpha-peptide sequence allows recombinant clones to be directly identified by blue/white screening on indicator plates (Table 1).

Table 1. Blue/White Color Selection of Recombinant Clones.

DNA in Ligation Mixture	# of Blue Colonies	# of White Colonies
pTARGET™ Vector (60ng)	41	10
pTARGET™ Vector (60ng) + Control Insert (8ng)	47	614

Ligation reactions were incubated overnight at 4°C. E. coli JM109 cells were transformed with the ligation reactions and 20% of the transformed mixture was plated onto LB/X-gal/IPTG/Ampicillin plates. The data from each reaction is representative of the number of colonies on a single plate. The background white colonies seen in transformation with the pTARGET™ Vector alone are the result of deletions in the alpha-peptide region. Deletions of plasmid DNA during the transformation of E. coli with linear plasmid molecules is a well documented phenomenon (15-18).

The sequence of the alpha-peptide in the pTARGET™ Vector is significantly different from that found in other cloning vectors. Because part of the alpha-peptide sequence will be present in both the 5´- and 3´-untranslated sequence of an expressed gene, major modifications to the alpha-peptide sequence were made to prevent it from having any deleterious effect on expression. One modification was the removal of an ATG sequence in the alpha-peptide sequence that is upstream of the cloning site. This sequence was changed because it has been demonstrated that upstream, out-of-frame ATGs frequently reduce the efficiency of translation (19).

A significant effort was also made to remove any potential 5´- and 3´-splice sites of the GT-AG class from the alpha-peptide coding sequences that are upstream or downstream of the multiple cloning site, respectively. Functional 5´-splice sites were eliminated because splicing might occur within the gene of interest, thereby disrupting the coding sequence and reducing functional expression.

Likewise, a similar situation would occur if a functional 3´-splice site was present in the alpha-peptide sequence downstream of the cloning site and the cloned gene contained a functional 5´-splice site. We also verified that the alpha-peptide coding sequence was free from the highly conserved sequences of the splice site of the minor class (AT-AC) of pre-mRNA (20), the AU-rich nonamer sequence that mediates mRNA degradation (21), and predicted hairpin structures within the transcribed RNA that would reduce translation efficiency (22).

Additional features of the pTARGET™ Vector include (i) a T7 RNA Polymerase promoter upstream of the multiple cloning region for synthesizing RNA corresponding to the sense strand of the cloned DNA insert; (ii) the origin of replication for the filamentous phage f1 for generating ssDNA; (iii) unique restriction sites flanking the CMV enhancer (*Bgl* II and *Sgf* I) and the CMV promoter (*Sgf* I and I-*Ppo* I) that make it easy to replace the CMV regulatory sequences with other regulatory sequences; and (iv) a high-copy bacterial origin of replication, which increases the yield of DNA from plasmid preparations.

Effects of the modified alpha-peptide on gene expression by pTARGET™ Vector

To determine whether the presence of the modified alpha-peptide sequence had an effect on the expression of genes, the pTARGET™ Vector was compared to the pCI-neo Vector in the expression of five reporter genes (Figure 3). The genes tested were a cDNA of *Renilla* luciferase (Figure 3A), a genomic clone of the human growth hormone gene (Figure 3B), the CAT gene (Figure 3C), a modified cDNA of firefly luciferase (Figure 3D), and the *lac*Z gene, which encodes for beta-galactosidase (Figure 3E). HeLa and NIH3T3 cells were transiently transfected with the pCI-neo and pTARGET™ Vector constructs. The pTARGET™ Vector was comparable to the pCI-neo Vector in the expression of the *Renilla* luciferase gene, the human growth hormone gene, the CAT gene and the firefly luciferase gene, indicating that the presence of the modified alpha-peptide sequence had no deleterious effect on the expression of these genes. The pCI-neo-CAT and pTARGET™-CAT constructs were also compared for stable transfection and were found to express the same level of CAT activity in pooled stable clones (data not shown). The expression of beta-galactosidase from the pCI-neo Vector was 2.4- to 2.7-fold higher than that seen from pTARGET™ Vector. One possible explanation for the reduced expression of this gene by the pTARGET™ Vector is that the mRNA transcribed from pTARGET™-beta-galactosidase has a stable secondary structure attributable to annealing of the alpha-peptide sequence in the transcript to the cloned *lac*Z gene, resulting in a reduction in the translation efficiency.

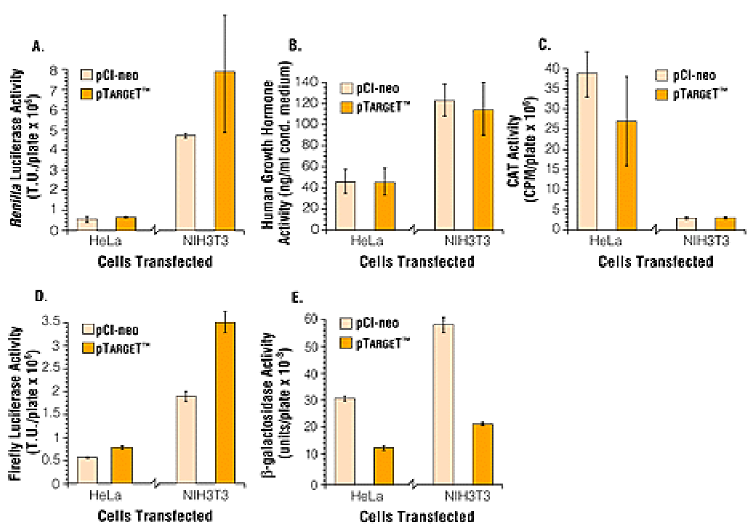

Figure 3. Comparison of pCI-neo and pTARGET™ Vectors in the expression of firefly luciferase, *Renilla* luciferase, CAT, human growth hormone and beta-galactosidase. To subclone the reporter genes into the pTARGET™ Vector, the gene-containing plasmid was digested with restriction enzymes flanking the gene, the restriction sites were blunt-ended with the Klenow fragment of DNA Polymerase I, the gene was A-tailed (3,4) and then ligated into the pTARGET™ Vector. HeLa and NIH3T3 cells were transfected as described (4). For each experiment the total amount of plasmid DNA transfected per dish was 5µg; 1µg from either the pTARGET™ construct or the pCI-neo construct, 3.8µg of pGEM®-3Zf(+) which served as carrier DNA, and 0.2µg of the transfection control. Untransfected HeLa and NIH3T3 cells served as negative controls. Cells or culture supernatant were assayed for reporter activity 48 hours post-transfection. Except where

otherwise stated, values are the mean ± the range of 3-4 culture plates. Except where indicated, the transfection control was pGL3 (a firefly luciferase-containing expression vector) and its activity was measured using Promega's Luciferase Assay System with Reporter Lysis Buffer[d] (Cat.# E4030). **Panel A:** *Renilla* luciferase activity was quantitated using Promega's Dual-Luciferase™ Reporter Assay System[e] (Cat.# E1910). **Panel B:** Human growth hormone activity was quantitated from culture supernatants by ELISA (Boehringer Mannheim). **Panel C:** CAT activity was determined with Promega's CAT Enzyme Assay System (Cat.# E1220). CAT activity in HeLa cells represents the mean of two culture plates ± the range, while in NIH3T3 cells values represent the mean ± the range of 3-4 culture plates. **Panel D:** Firefly luciferase activity was determined using Promega's Dual-Luciferase™ Reporter Assay System. The transfection control for this experiment was pCI-neo-*Renilla* luciferase. **Panel E:** beta-galactosidase activity was quantitated using Promega's beta-galactosidase Enzyme Assay System (Cat.# E2000).

[d]*U.S. Pat. No. 5,283,179 has been issued to Promega Corporation for a firefly luciferase assay method which affords greater light output with improved kinetics.*
[e]*Patent Pending.*

Comparison of the pTargeT™ Vector to another mammalian expression/T-vector

The expression capability of the pTargeT™ Vector was compared to another commercially available T-vector used in mammalian expression (Vector I from Vendor A). While both vectors are similar, Vector I does not have blue/white screening capabilities for detecting recombinants, does not contain an intron for increased expression of cDNAs, and has a different polyadenylation signal. For comparison, we tested the ability of both vectors to express *Renilla* luciferase. PCR primers flanking the *Renilla* luciferase cDNA[f] were generated and the Shine-Dalgarno sequence for efficient translation in *E. coli* was incorporated into the 5´ PCR primer such that it was positioned just upstream of the translational start codon. The *Renilla* cDNA was amplified with *Taq* DNA Polymerase using high fidelity PCR conditions (23-25). The amplified *Renilla* cDNA was purified using agarose gel electrophoresis and ligated into both the pTargeT™ Vector and Vector I. *E. coli* were transformed with the ligation mixtures and clones were screened for the presence and orientation of the *Renilla* luciferase cDNA.

To ensure that the clone chosen for expression in mammalian cells did not have PCR-induced mutations affecting the activity of *Renilla* luciferase, the expression of the clones was first checked in *E. coli* by using the T7 promoter present upstream of the multiple cloning site (MCS) in both clones. From five clones each, four pTargeT™-*Renilla* clones and three Vector I-*Renilla* clones expressed functional *Renilla* luciferase. The level of specific activity of *Renilla* luciferase between clones expressing functional *Renilla* luciferase differed less than 2-fold. Two clones of Vector I-*Renilla* and one clone of pTargeT™-*Renilla* expressing functional *Renilla* luciferase in *E. coli* were transiently transfected into HeLa and NIH3T3 cells. Figure 4 demonstrates that the level of *Renilla* luciferase activity in cells transfected with pTargeT™-*Renilla* was 15- to 18-fold higher than the level of *Renilla* activity found in cells transfected with Vector I-*Renilla*. The lower level of expression from Vector I-*Renilla* is presumably due to the lack of an intron in Vector I, the presence of which has been shown to significantly increase cDNA expression.

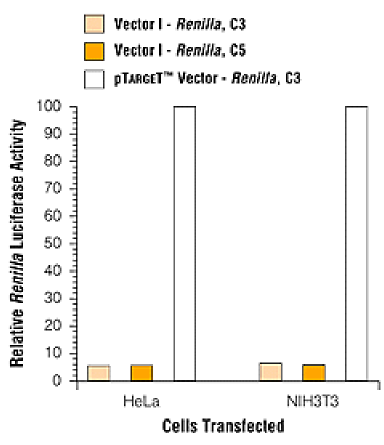

Figure 4. Comparison of *Renilla* luciferase expression from Vector I and pTargeT™ Vector. Transfections were performed as described in Figure 3. The total amount of plasmid transfected per 60mm culture plate was 5µg; 1µg of either pTargeT™-*Renilla* clone 3 (C3) or Vector I-*Renilla* clone 3 (C3) or Vector I-*Renilla* clone 5 (C5), plus 0.2µg of pGL-3 Control Vector (luciferase-containing expression vector), plus 3.8µg of pGEM®-3Z Vector as carrier DNA. 48 hours post-transfection cells were lysed and *Renilla* luciferase quantitated as described in Figure 3. Data represent the mean of four culture plates; the standard deviation was <8% of the mean.

[f]*The cDNA encoding luciferase from* Renilla reniformis *is the subject of U.S. Pat. No. 5,292,658 assigned to the University of Georgia and sublicensed from SeaLite Sciences, Inc., Bogart, GA.*

Summary

The pTARGET™ Vector System is a convenient method for cloning PCR-amplified genes and expressing them in mammalian cells. Identification of recombinant clones is made easy through the use of blue/white screening. The pTARGET™ Vector contains the necessary regulatory elements to promote strong, constitutive expression in mammalian cells. The pTARGET™ Vector can be used for either transient expression or stable transfection by utilizing the neomycin phosphotransferase selectable marker.

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Ordering Information

Product	Cat.#
pTARGET™ Mammalian Expression Vector System	A1410
