

Crossing the “T”s of Cloning: T-Vector Cloning

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Abstract

Promega offers four different T-Vector systems designed for the efficient cloning of PCR products and subsequent use in a variety of downstream applications. This article introduces these systems and offers answers to a number of common questions and troubleshooting tips that have proven useful for identifying and solving performance problems.

Promega has four T-vector systems that are compatible with numerous downstream applications.

What are the differences between Promega’s T-Vector Cloning Systems?

Promega’s pGEM[®]-T Vector Systems^(a,b) (Cat.# A3600 and A3610) and pGEM[®]-T Easy Vector Systems^(a,b) (Cat.# A1360 and A1380) are designed for most general cloning applications. The pGEM[®]-T Vector and pGEM[®]-T Easy Vector are high-copy number plasmids that contain T7 and SP6 RNA polymerase promoters flanking the multiple cloning region. The promoters are conveniently placed for sequencing of an insert as well as for *in vitro* transcription of both sense and anti-sense RNAs. The multiple cloning region for each vector is present within the α -peptide coding region of the enzyme β -galactosidase to allow for blue/white screening. A variety of restriction sites convenient for subcloning or other applications are included in the multiple cloning site. Both the pGEM[®]-T and the pGEM[®]-T Easy Vectors contain flanking *Bst*Z I restriction sites for release of a cloned insert by a single restriction digest; the pGEM[®]-T Easy Vector has additional flanking *Eco*R I and *Not* I restriction sites for this same purpose.

Promega’s PinPoint[™] Xa-1 T-Vector System^(a,b,c) (Cat.# V2610) is specifically designed for cloning and bacterial expression of PCR products. Coding sequences are cloned in frame with a purification tag sequence that becomes biotinylated in *E. coli* and may subsequently be used for affinity purification. The PinPoint[™] Xa-1 T-Vector contains an endoproteinase Factor Xa cleavage sequence for convenient separation of the purification tag from the expressed protein of interest. This vector also contains a variety of restriction sites for convenient subcloning of inserts, and the system includes a positive control insert that allows the use of blue/white screening to verify system function.

Promega’s pTARGET[™] Mammalian Expression Vector System^(b,d) (Cat.# A1410) is designed specifically for expression of cloned PCR products in mammalian cells. The pTARGET[™] Vector contains the human cytomegalovirus (CMV) immediate-early enhancer/promoter to promote high levels of expression in a large variety of cell types. The vector also contains the neomycin phosphotransferase gene (Neo), which can be used to select for stably transfected cells using the antibiotic G-418. A variety of restriction sites in the multiple coding region and blue/white screening are standard features of the pTARGET[™] Vector as well.

For more information on Promega’s T-Vector Systems, please refer to the product technical manuals (1–3).

What effect does choice of DNA polymerase have on cloning efficiency of T-vectors?

It is important to note whether the DNA polymerase has 3’→5’ exonuclease activity, commonly referred to as “proofreading” activity (Table 1). Thermophilic DNA polymerases that do not have proofreading activity will add an adenosine residue at the 3’ end of the PCR product in a template-independent manner (4). These 3’ adenosines are complementary to the 3’ terminal thymidine residues on the T-vectors. This base-pair interaction provides the basis for efficient ligation. In contrast, an enzyme with proofreading activity will remove the 3’ A-overhang and generate a PCR product with blunt ends, which will not clone effectively. Blunt-ended PCR products and even blunt-ended products from other enzymatic manipulations can be efficiently cloned into T-vectors as detailed in the *pGEM[®]-T and pGEM[®]-T Easy Technical Manual*, TM042 (1).

Table 1. Thermostable DNA Polymerases.

Thermostable DNA Polymerases leaving A overhangs (i.e., non-proofreading)

Taq DNA Polymerase^(e)
Tfi DNA Polymerase^(e)
Tth DNA Polymerase^(e)

Thermostable DNA Polymerases leaving blunt-ends (i.e., proofreading)

Pfu DNA Polymerase^(e)
Tli DNA Polymerase^(e)
Vent[®] DNA Polymerase
Pwo DNA Polymerase
Tgo DNA Polymerase

I'm using your T-vector system to clone a PCR product, but I'm only getting blue colonies. Why isn't it working?

The first step to troubleshooting a T-vector cloning problem is to use a positive control. All of Promega's T-Vector cloning systems include a positive control insert DNA to allow the user to determine the ligation efficiency. When competent cells with a transformation efficiency of 1×10^8 cfu/ μ g DNA are used, approximately 100 colonies should be generated. Of these colonies, 10–30% will be blue and 60% or greater should be white, and thus recombinant. If the positive control does not perform as expected, check the following:

- Efficiency of competent cells: Ensure that your competent cells have an efficiency of at least 1×10^8 cfu/ μ g DNA. You can determine the efficiency of your cells by transforming 0.1–1.0ng of circular plasmid DNA and counting the number of colonies after overnight growth on medium containing the appropriate antibiotic. Plating several dilutions of the transformed cells (i.e., 1:10, 1:100) will ensure that you obtain a number of colonies that can be easily counted. Promega's high-transformation efficiency JM109 Competent Cells (Cat.# L2001) are ideal for most T-vector cloning applications.
- Has the 2X Rapid Ligation Buffer or the T4 DNA Ligase 10X Buffer been subjected to multiple freeze-thaw cycles? These buffers contain ATP, which will degrade during temperature fluctuations. Dispense the buffers into single-use aliquots to avoid loss of activity. ATP may also be supplemented at a final concentration of 1mM to compensate for loss of activity.
- Is the T4 DNA Ligase active? A separate ligation using a vector that has been digested with a single restriction enzyme to leave a sticky end can be used to test ligase activity. It is also important to use only the T4 DNA Ligase that is provided with the system because the enzyme has minimal exonuclease activity. Nucleases will degrade the T- and A-overhangs, resulting in many more blue colonies than white as the vector religates to encode an intact, functional β -galactosidase protein.

The positive control works, but I'm not getting white colonies with my PCR products. What is the problem?

There are a variety of factors that can affect the ligation efficiency of a PCR product. These may include the size and sequence of your insert, insert purity, and molar ratio of vector to insert DNA. Although inserts as large as 12kb have been successfully cloned into Promega's T-vectors, very large inserts may be intrinsically more difficult to ligate and more prone to rearrangements or bacterial

deletions. Transformation of a ligated vector into a strain that is designed to minimize DNA rearrangement may be useful in these cases. Very small inserts may fail to disrupt the reading frame of the *LacZ* gene and result in a high number of blue colonies as compared to a vector-only (background) control. With regard to purity, many components of a typical PCR amplification such as salts, dNTPs, primers and primer-dimers, can adversely affect cloning. Purification of PCR products using the new Wizard® SV Gel and PCR Clean-Up System⁽¹⁾ (Cat.# A9281 and A9282) (5) can greatly enhance cloning efficiency.

References

1. *pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual #TM042*, Promega Corporation.
2. *PinPoint™ Xa-1 T-Vector Systems Technical Bulletin #TB234*, Promega Corporation.
3. *pTARGET™ Mammalian Expression Vector System Technical Manual #TM044*, Promega Corporation.
4. Clark, J.M. (1988) *Nucl. Acids Res.* **16**, 9677–86.
5. *Wizard® SV Gel and PCR Clean-Up System Technical Bulletin #TB308*, Promega Corporation.

Protocols

- ◆ *pGEM®-T Vector System Technical Manual #TM042*, Promega Corporation.
(www.promega.com/tbs/tm042/tm042.html)
- ◆ *pGEM®-T Easy Vector System Technical Manual #TM042*, Promega Corporation.
(www.promega.com/tbs/tm042/tm042.html)
- ◆ *PinPoint™ Xa-1 T-Vector Systems #TB234*, Promega Corporation.
(www.promega.com/tbs/tb234/tb234.html)
- ◆ *pTARGET™ Mammalian Expression Vector System #TM044*, Promega Corporation.
(www.promega.com/tbs/tm044/tm044.html)

^(a) U.S. Pat. No. 4,766,072.

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

^(c) For research purposes only. Not for diagnostic or therapeutic use. For nonresearch uses of the portion of the vector encoding the biotinylation sequence, please contact Promega Corporation for licensing information. For bulk purchases of the SoftLink™ Resin, contact TosoHaas, 156 Keystone Drive, Montgomeryville, PA 18936, 1-800-366-4875 or 215-283-5000.

^(d) The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

^(e) Certain applications of this product 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.

^(f) U.S. Pat. Nos. 5,658,548, 5,808,041, Australian Pat. No. 689815 and other patents pending.

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