PCR Subcloning

Introduction

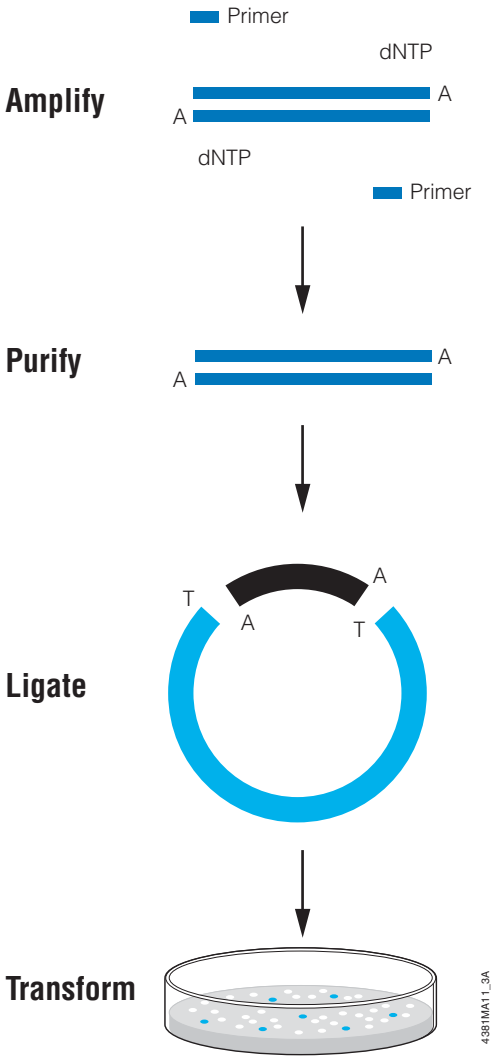
You may wish to subclone your PCR product into a plasmid cloning vector. When PCR was in its infancy, researchers found that subcloning PCR products by simple blunt-ended ligation into blunt-ended plasmid cloning vectors was not easy. Thermostable DNA polymerases, like *Taq* DNA polymerase, add a single nucleotide base extension to the 3′ end of blunt DNA in a template-independent fashion (1,2). These polymerases usually add an adenine, leaving an "A overhang."

Historically, researchers have used several approaches to overcome the cloning difficulties presented by the presence of A overhangs on PCR products. One method involves treating the product with the Klenow fragment of *E. coli* DNA Polymerase I to create a blunt-ended fragment for subcloning. However this technique is not particularly efficient.

Another method commonly used by researchers is to add restriction enzyme recognition sites to the ends of the PCR primers (3). The PCR product is then digested and subcloned into the desired plasmid cloning vector in a desired orientation. Care must be exercised in primer design when using this method, as not all REs cleave efficiently at the ends of DNA, and you may not be able to use every RE you desire (4). Some REs require extra bases outside the recognition site (see page 40), adding further expense to the PCR primers as well as risk of priming to unrelated sequences in the genome.

A method of choice for cloning PCR products is T-Vector cloning. In essence, the plasmid cloning vector is treated to contain a 3′ T overhang to match the 3′ A overhang of the amplicon (5). The A-tailed amplicon is directly ligated to the T-tailed plasmid vector with no need for further enzymatic treatment of the amplicon other than the action of T4 DNA ligase. Promega has systems based on this technology for routine subcloning, and direct mammalian expression.

4 Simple Steps to Success



References

1. Clark, J.M. (1988) Novel non-template nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucl. Acids Res.* **16**, 9677–86.
2. Mole, S.E., Iggo, R.D. and Lane, D.P. (1989) Using the polymerase chain reaction to modify expression plasmids for epitope mapping. *Nucl. Acids Res.* **17**, 3319.
3. Scharf, S.J., Horn, G.T. and Erlich, H.A. (1986) Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* **233**, 1076–8.
4. Kaufman, D.L. and Evans, G.A. (1990) Restriction endonuclease cleavage at the termini of PCR products. *BioTechniques* **9**, 304–6.
5. Mezei, L.M. and Storts, D.R. (1994) Cloning PCR Products. In: *PCR Technology Current Innovations*. Griffin, H.G. and Griffin, A.M. (eds). CRC Press, 21–7.

T-Vector Systems

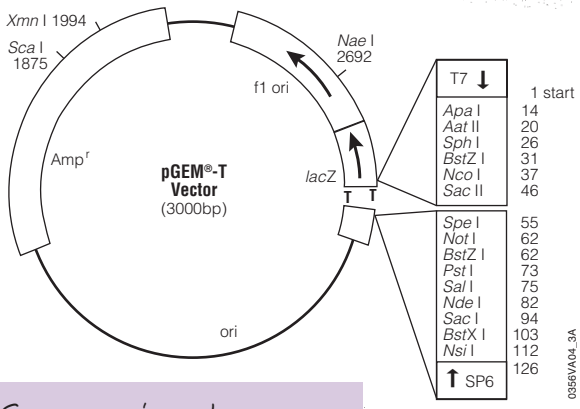
pGEM®-T and pGEM®-T Easy Vector Systems

The most basic need in PCR subcloning is a simple, general cloning vector. The pGEM-T and pGEM-T Easy Vector Systems[e,f,g] are designed for just that purpose. The vectors are based on the pGEM-5Zf(+) Vector[g] backbone. Each provide convenient T7 and SP6 promoters to serve as sequencing primer binding sites or for in vitro transcription of either strand of the insert with the appropriate RNA polymerase. The vectors have the *lac*Zα, allowing easy blue/white screening of the inserts with an appropriate bacterial strain (e.g., JM109, DH5α™, XL1 Blue, etc). To speed your research, these vectors are provided with 2X Rapid Ligation Buffer, allowing efficient ligation in just 1 hour with the supplied T4 DNA Ligase. You can either supply your own favorite *E. coli* cells or purchase the system with Promega JM109 Competent Cells. The choice is yours.

pGEM®-T Vector System I
(you supply competent cells)
Cat.# A3600 20 reactions

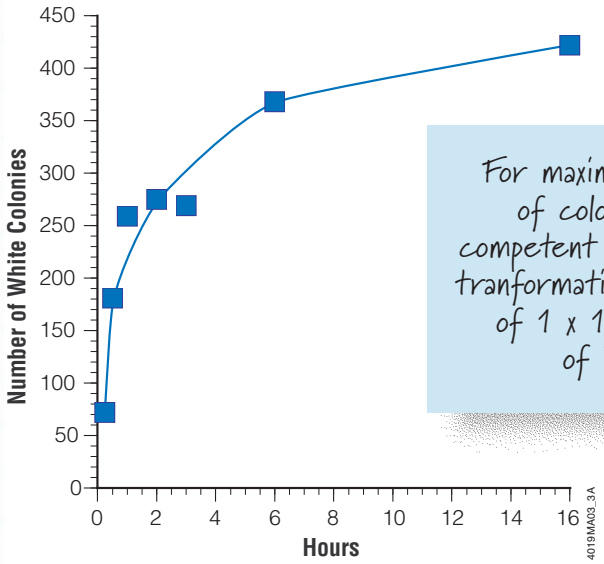
pGEM®-T Vector System II
(supplied with High Efficiency JM109 Competent Cells)
Cat.# A3610 20 reactions

Protocol available at:
www.promega.com/tbs/tm042/tm042.html



Select recombinants by blue/white selection.

Sequence inserts with the following:
- SP6 Promoter Primer
- T7 Promoter Primer
- M13 Forward Primer
- M13 Reverse Primer



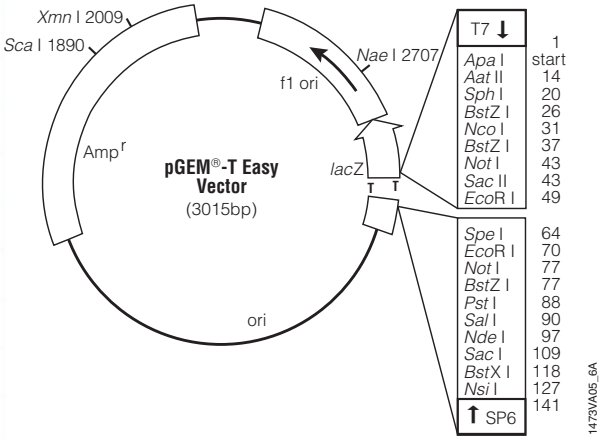
For maximum number of colonies, use competent cells with a tranformation efficiency of 1 x 10^8 cfu/µg of DNA.

Drop out insert with a single BstZ I digest.

Number of white colonies (transformants) versus time of ligation. Control pGEM-T Easy ligation reactions were set up at room temperature (24°C) and allowed to proceed from 0.25 to 16 hours using the methods described in Technical Manual #TM042. Graph was adapted from Table 2 in Frackman, S. and Kephart, D. (1999) Rapid ligation for the pGEM-T and pGEM-T Easy Vector Systems. *Promega Notes* **71,** 8–10.

PCR Subcloning

T-Vector Systems



pGEM®-T Easy Vector System I
(you supply competent cells)
Cat.# A1360 20 reactions

pGEM®-T Easy Vector System II
(supplied with High Efficiency JM109 Competent Cells)
Cat.# A1380 20 reactions

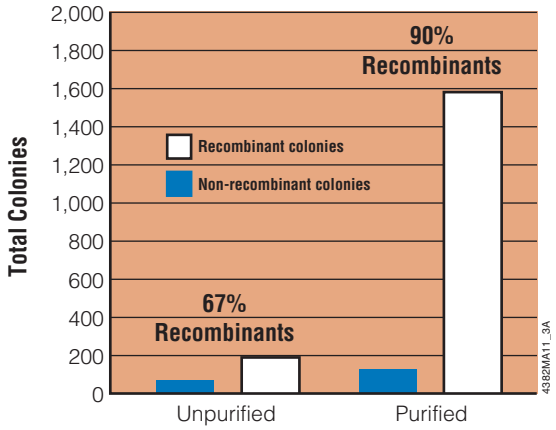
Protocol available at:
www.promega.com/tbs/tm042/tm042.html

Sequence inserts with the following:
- SP6 Promoter Primer
- T7 Promoter Primer
- M13 Forward Primer
- M13 Reverse Primer

Drop out insert with a single BstZ I, EcoR I or Not I digest.

For maximum subcloning efficiency, purify the PCR product before subcloning. The presence of PCR primers and primer dimers can reduce subcloning efficiency.

Purification achieved with Wizard® SV Gel and PCR Clean-Up System. For more information, see page 28.

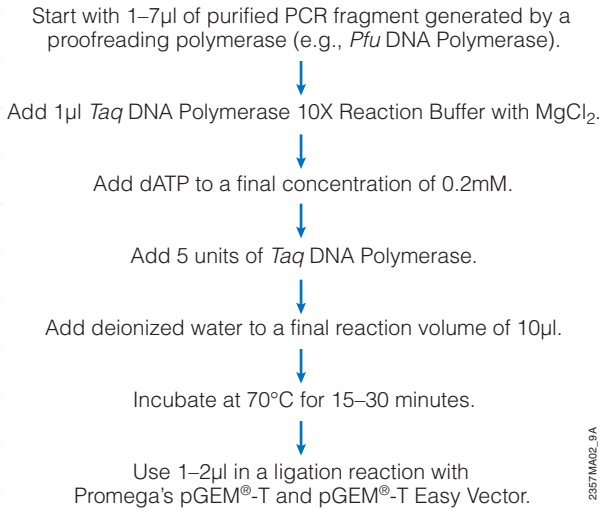


Cloning efficiency of a purified PCR product and an unpurified PCR product.

PCR Subcloning

Giving Blunt-Ended DNA an A-tail for T-Vector Subcloning

PCR amplicons generated with proofreading polymerases like *Pfu* or *Tli* DNA Polymerase are blunt-ended. Promega has developed an easy method to add an A-Tail to the DNA so that it can be used for T-Vector cloning.



Ends Left on PCR Products by Thermostable Enzymes.

Polymerase	Type of End*
Taq DNA Polymerase	3′ A overhang
GoTaq® DNA Polymerase	3′ A overhang
Tfl DNA Polymerase	3′ A overhang
Tth DNA Polymerase	3′ A overhang
Pfu DNA Polymerase	Blunt end
Tli DNA Polymerase	Blunt end
Long PCR mixes	Blunt end
Other Proofreading Polymerases	Blunt end

*All bases may be found at 3′ overhang; adenine tends to be encountered most often.

Full details of the protocol are available in the *pGEM®-T* and *pGEM®-T Easy Vector Systems Technical Manual*, TM042. The proofreading enzyme must be removed using a system like the Wizard® SV Gel and PCR Clean-Up System prior to the A-tailing procedure. Any remaining proofreading enzyme in the PCR will remove the A-overhangs created by the *Taq* DNA polymerase.

For more information and techniques for cloning PCR DNA, check out Promega Frequently Asked Questions for the T-Vector cloning systems at: **www.promega.com/faq**

Promega recommends a guanidine-based purification method, like the Wizard SV Gel and PCR Clean-Up System, to remove proofreading polymerases.

Subcloning with RE Sites

What PCR Cloning Controls Can Do for You

Each Promega PCR cloning system is provided with a control insert. The ligation and subsequent transformation of this positive control can give you a lot of information with regard to the ligation and transformation of your insert.

Typical Results

	Efficiency*	% White
Control insert	1110	92%
Control insert	1125	92%
No insert	92	—
No insert	109	—

*cfu/ng control insert DNA; JM109 cells at 1.5 × 10^8 cfu/µg; pGEM®-T Easy Vector System II using room temperature ligation for 1 hour.

The total number of blue colonies obtained with positive control insert and no-insert controls should be approximately equal. The negative control may have some white colonies as well.

Interpreting Results from T-Cloning

Experimental insert looks like control insert in efficiency and percent white colonies.

Successful experiment. Greater than 80% of the white colonies should contain inserts

Experimental insert and control insert look like negative control.

Ligation has failed. Avoid multiple freeze/thaws of the ligation buffer. Ligase buffer contains ATP and could be damaged by freeze/thaw cycles. You may need to dispense the ligase buffer into smaller aliquots for your experimental needs.

No colonies with experimental insert, control insert or negative control.

Transformation has failed. Reassess the competent cells with an intact, supercoiled plasmid and determine the transformation efficiency. Use cells >1 × 10^8 cfu/µg to insure >100 colonies from the control insert ligation.

Experimental insert has more blue colonies than control insert or negative control and fewer white colonies than control insert.

In-frame insertion, no interruption of α-fragment. Although the pGEM®-T Vector Control DNA will produce recombinants that generate white colonies, the insertion of other DNA fragments into the *lac*Z coding sequence may not result in white colonies unless the fragments disrupt the *lac*Z reading frame. Although this tends to occur most frequently with PCR products of 500bp or less, inserts of up to 2kb can result in blue colonies. Moreover, some insert DNAs can also result in pale blue colonies or "bull's-eye" colonies with a blue center and a white perimeter. In one case in particular, we found that a 1.8kb insert when oriented in one direction produced white colonies and in the other produced bull's-eye colonies [Knoche, K. and Kephart, D. (1999) Cloning blunt-end *Pfu* DNA polymerase-generated PCR fragments into pGEM®-T Vector Systems. *Promega Notes* **71**, 10–13.].

Subcloning Using PCR Primers Containing Restriction Sites

Frequently, the ends of insert DNA do not contain a suitable restriction enzyme site. The problem can be solved by using PCR to generate a site at the desired location. For this technique, the restriction enzyme site is designed into the 5′-end of the PCR primer. Because certain restriction enzymes inefficiently cleave recognition sequences located at the end of a DNA fragment, it is advisable to include at least four additional bases in front of the restriction recognition site. For the majority of restriction enzymes this will result in efficient cleavage.

Success in digesting PCR products can depend on the purity of the PCR product. Primers and primer dimers are present in overwhelming quantities when compared to the actual PCR product. Your PCR product will be competing with primers and primer dimers for the attention of the restriction enzyme, resulting in conditions favoring partial restriction digest. A simple clean-up of the reaction with the Wizard® SV Gel and PCR Clean-Up System can improve RE cleavage.

If you encounter a situation where the PCR product will not subclone, the digest may be adversely affected by proximity to the end of the PCR product. To improve the "placement" of the restriction site, the PCR product can be subcloned into the pGEM®-T Easy Vector. The restriction site should be readily cleavable in the context of the vector.

Ability of Restriction Enzymes to Cut PCR Products With RE Sites Near the End of the Fragment.

Enzyme	Distance (in bp) from the end of the PCR Fragment			
	0	1	2	3
Apa I	–	–	+/–	+
*Bam*H I	–	+/–	+	+
*Bst*X I	–	+/–	+	+
Cla I	–	+/–	+	+
*Eco*R I	–	+/–	+	+
*Eco*R V	–	+	+	+
*Hin*d III	–	–	+	+
Not I	–	–	+	+
Pst I	–	–	+/–	+
Sac I	–	+/–	+	+
Sal I	+	+	+	+
Sma I	–	+/-	+	+
Spe I	+	+	+	+
Xba I	–	+/–	+	+
Xho I	–	–	+/–	+

PCR products in which the end of the restriction enzyme recognition sequence was flush with the end of the product or 1, 2, or 3 base pairs away from the end of the product were digested with a variety of enzymes. Purified PCR fragments (10–50ng) were digested with 0.5units of RE in 10µl of the appropriate reaction buffer for 45 minutes. Digestion is indicated as follows: Cleavable (+), not cleavable (–) and not reproducible (+/–). Data are the result of at least duplicate experiments and are reproduced by permission of Eaton Publishing. Taken from Simmermann, K. *et al.* (1998) Digestion of terminal restriction endonuclease recognition sites on PCR products. *BioTechniques* **24**, 582–4.

PCR Subcloning: Ordering Information

Basic PCR Cloning Systems

Product	Size	Cat.#
pGEM®-T Vector System I[e,f,g]	20 reactions	A3600
Supplied with linearized, ligation-ready pGEM®-T Vector, 2X Rapid Ligation Buffer, T4 DNA Ligase and Positive Control Insert.		
pGEM®-T Vector System II[e,f,g]	20 reactions	A3610
Same contents as System I with 6 × 200µl JM109 High Efficiency Competent Cells.		
pGEM®-T Easy Vector System I[e,f,g]	20 reactions	A1360
Supplied with linearized, ligation-ready pGEM®-T Easy Vector, 2X Rapid Ligation Buffer, T4 DNA Ligase and Positive Control Insert.		
pGEM®-T Easy Vector System II[e,f,g]	20 reactions	A1380
Same contents as System I with 6 × 200µl JM109 High Efficiency Competent Cells.		

For Laboratory Use.

Sequencing Primers

Product	Conc.	Size	Cat.#
T7 Promoter Primer [5′-d(TAATACGACTCACTATAGGG)-3′]	10µg/ml	2µg	Q5021
SP6 Promoter Primer [5′-d(TATTTAGGTGACACTATAG)-3′]	10µg/ml	2µg	Q5011
pUC/M13 Primer, Forward (24 mer) [5′-d(CGCCAGGGTTTTCCCAGTCACGAC)-3′]	10µg/ml	2µg	Q5601
pUC/M13 Primer, Reverse (22 mer) [5′-d(TCACACAGGAAACAGCTATGAC)-3′]	10µg/ml	2µg	Q5421

Thermostable DNA Polymerases

Product	Conc.	Size	Cat.#
PCR Master Mix[h]	2X	100 reactions	M7502
	2X	1,000 reactions	M7505
PCR Master Mix contains dNTPs, buffer, Mg^{2+} and *Taq* DNA Polymerase. A standard reaction contiains 25µl of PCR Master Mix giving 1.5mM Mg^{2+}, 200µM each dNTP and 1.25u of *Taq* DNA Polymerase in the final 50µl reaction.			
GoTaq® DNA Polymerase[i]	100u	5u/µl	M3001
	500u	5u/µl	M3005
	2,500u	5u/µl	M3008
Supplied with 5X Green and 5X Colorless GoTaq® Reaction Buffer. Each contain 1.5mM $MgCl_2$ in the final 1X concentration. Use the Green Buffer for direct gel analysis of amplification reactions. Use the Colorless Buffer for any reaction requiring absorbance or fluorescence measurements without prior PCR clean-up.			
Taq DNA Polymerase in Storage Buffer B[i]	5u/µl	100u	M1661
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$ Solution.)	5u/µl	500u	M1665
	5u/µl	2,500u	M1668
Taq DNA Polymerase in Storage Buffer B[i]	5u/µl	100u	M2661
(Supplied with 10X Thermophillic Reaction Buffer containing 15mM $MgCl_2$.)	5u/µl	500u	M2665
	5u/µl	2,500u	M2668
Taq DNA Polymerase in Storage Buffer A[i]	5u/µl	100u	M1861
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$ Solution.)	5u/µl	500u	M1865
	5u/µl	2,500u	M1868
Taq DNA Polymerase in Storage Buffer A[i]	5u/µl	100u	M2861
(Supplied with 10X Thermophilic Reaction Buffer containing 15mM $MgCl_2$.)	5u/µl	500u	M2865
	5u/µl	2,500u	M2868

For Laboratory Use.

PCR Subcloning: Ordering Information

Thermostable DNA Polymerases

Product	Size	Conc.	Cat.#
*Taq*Bead™ Hot Start Polymerase[f]*			
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$ Solution.)	100 reactions	1.25u/bead	M5661
Tfl DNA Polymerase[f]*	100u	5u/µl	M1941
(Supplied with *Tfl* 10X Reaction Buffer and 25mM $MgSO_4$ Solution.)	1,000u	5u/µl	M1945
Tth DNA Polymerase[f]*	100u	5u/µl	M2101
(Supplied with 10X Reverse Transcription Buffer, 10X Chelate Buffer, 10X Thermophillic Reaction Buffer, 25mM MgCl2 and 25mM $MnCl_2$.)	500u	5u/µl	M2105
Pfu DNA Polymerase[f]*	100u	2–3u/µl	M7741
(Supplied with *Pfu* 10X Reaction Buffer containing $MgSO_4$. Not available in North America.)	500u	2–3u/µl	M7745
Tli DNA Polymerase[f]*			
(Supplied with 10X Thermophillic Reaction Buffer and 25mM $MgCl_2$.)	50u	3u/µl	M7101

*For Laboratory Use.

PCR-qualified Nucleotides

Product	Conc.	Size	Cat.#
Set of dATP, dCTP, dGTP, dTTP[f]	100mM	10µmol each	U1330
	100mM	25µmol each	U1420
	100mM	40µmol each	U1240
	100mM	200µmol each	U1410
PCR Nucleotide Mix[f]	10mM	200µl	C1141
	10mM	1,000µl	C1145

Equal mixture of dATP, dCTP, dGTP and dTTP. Use 1µl per 50µl reaction for a final dNTP concentration of 200µM each.

For Laboratory Use.