

Cloning PCR DNA

Overview

Cloning PCR products into plasmid vectors is a common downstream application of PCR. When PCR was in its infancy, researchers found that it was not easy to clone PCR products by simple blunt-end ligation into blunt-ended plasmid vectors because some thermostable DNA polymerases, including *Taq* DNA Polymerase, add a single nucleotide base extension to the 3'-end of blunt DNA in a template-independent fashion (1,2). Most commonly the base added is adenine, leaving what is called an "A overhang." To overcome this, researchers had to treat PCR products to blunt-end the PCR fragments prior to cloning. Such experiments often suffered from low cloning efficiencies.

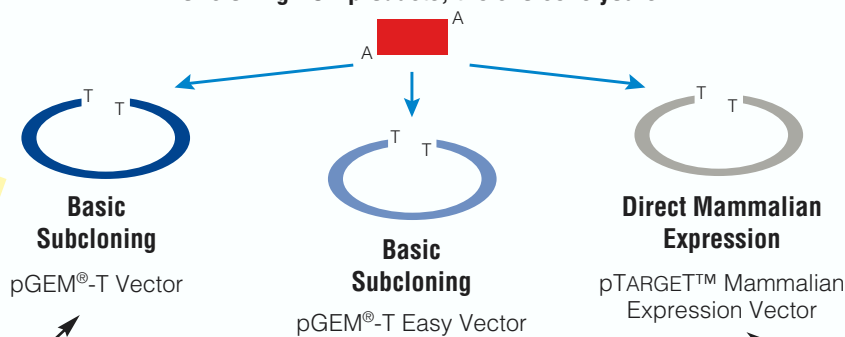
Another commonly used strategy for PCR cloning is to add restriction enzyme recognition sites to the ends of PCR primers (3). The PCR product is then digested and cloned into the desired vector. When using this method, care must be exercised in primer design because not all enzymes cleave efficiently at the ends of DNA fragments, and you may not be able to use every enzyme you desire (4,5). Some enzymes require extra bases outside the restriction enzyme recognition site, adding further expense to the PCR primers as well as increasing the risk of annealing to unrelated sequences in the genome.

The fact that amplicons generated with *Taq* DNA Polymerase typically have A overhangs led to the method referred to as T-vector cloning. In essence, the plasmid cloning vector is engineered to contain 3'-T overhangs that match the 3'-A overhang of the amplicon (6). The A-tailed amplicon is directly ligated to the T-tailed plasmid vector, and there is 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.

References

1. Clark, J.M. (1988) Novel non-template nucleotide addition reactions catalyzed by prokaryotic 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**, 1076-78.
4. Kaufman, D.L. and Evans, G.A. (1990) Restriction endonuclease cleavage at the termini of PCR products. *BioTechniques* **9**, 304-6.
5. Digestion of restriction sites close to the end of linear DNA. In: *Restriction Enzyme Resource Guide*. Promega Corporation. www.promega.com/guides/re_guide/chaptwo/2_6.htm
6. 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, pp. 21-7.

For cloning PCR products, the choice is yours



• Great for sequencing!
• Drop out insert with single *Bst*Z I digest

• Great for sequencing!
• Drop out insert with single *Eco*R I, *Not* I or *Bst*Z I digest

• CMV promoter
• *Neo*^R gene for G-418 Selection
• Drop out insert with single *Eco*R I digest

Cloning PCR DNA

Basic Subcloning

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

The most basic need in PCR cloning is for simple, general cloning vectors. The pGEM-T and pGEM-T Easy Vector Systems^(h,i) were designed for just that purpose. The vectors are based on the pGEM-5Zf(+)^{Vector}^(h) backbone. The pGEM-T and -T Easy Vectors provide convenient T7 and SP6 promoters that serve as sequencing primer binding sites and also allow in vitro transcription of either strand of the insert with the appropriate RNA polymerase. The vectors also have the *lacZ* α coding region, allowing easy blue/white screening of recombinants. The pGEM-T and -T Easy Vectors are provided with 2X Rapid Ligation Buffer, which allows efficient ligation in just 1 hour with the supplied T4 DNA Ligase. You can either supply your own favorite *E. coli* competent cells or purchase the system with Promega's high-efficiency JM109 Competent Cells. The choice is yours.

Select recombinants by blue/white selection.

What is Blue/White Selection?

The enzyme β -galactosidase, the product of the bacterial *lacZ* gene, can be separated into two domains—the α -fragment and the ω -fragment. The two fragments interact to form a functional β -galactosidase. For blue/white selection, the ω -fragment is expressed by the *E. coli* host strain, and the α -fragment is provided by the cloning vector. An intact, in-frame α -fragment will interact with the host strain ω -fragment, creating functional β -galactosidase. This is known as α -complementation. Bacteria capable of producing functional β -galactosidase will cleave the substrate X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), creating blue colonies when grown on indicator plates containing IPTG and X-Gal (see recipe on p. 40). Blue/white-capable cloning vectors have a multiple cloning site within the α -fragment coding sequence. When your sequence of interest is inserted within this region, the α -fragment is disrupted, α -complementation does not occur, and the colony is white. *E. coli* (e.g., JM109, DH5 α [™] or XL1-Blue) transformed with an insert-containing plasmid produce white colonies, while those containing empty or religated vector produce blue colonies.

pGEM[®]-T Vector System I

(you supply the competent cells)

Cat.#: A3600 (20 reactions)

pGEM[®]-T Vector System II

(supplied with High Efficiency JM109 Competent Cells)

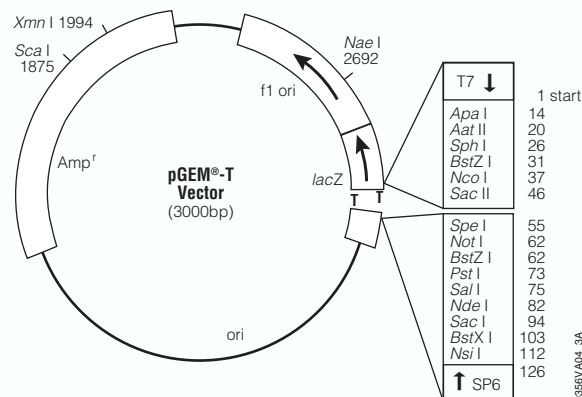
Cat.#: A3610 (20 reactions)

Protocol:

www.promega.com/tbs/tm042/tm042.html

Citations for use of the pGEM[®]-T System online at:

www.promega.com/citations/



Drop out your insert with a single Bst Z I digest.

Sequence inserts with:
SP6 Promoter Primer
T7 Promoter Primer
PUC/M13 Forward Primer
PUC/M13 Reverse Primer

Need sequencing-grade plasmid DNA?

Promega has the system.

Wizard[®] Plus SV Minipreps DNA Purification System^(a,r)

Simple spin preps for plasmid DNA. Guaranteed for fluorescent sequencing.

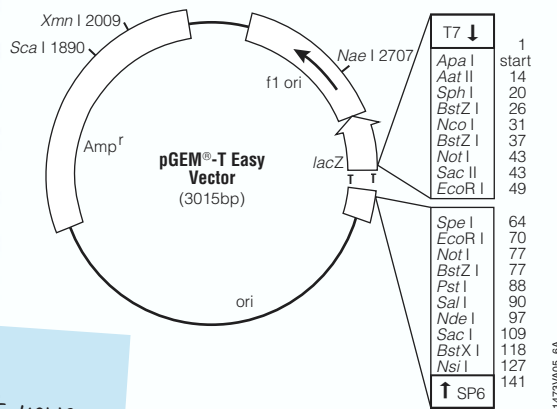
Cat.#: A1330 (50 preps)

Cat.#: A1460 (250 preps)

Protocol:

www.promega.com/tbs/tb225/tb225.html

Cloning PCR DNA



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

Sequence inserts with:
 SP6 Promoter Primer
 T7 Promoter Primer
 pUC/M13 Forward Primer
 pUC/M13 Reverse Primer

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

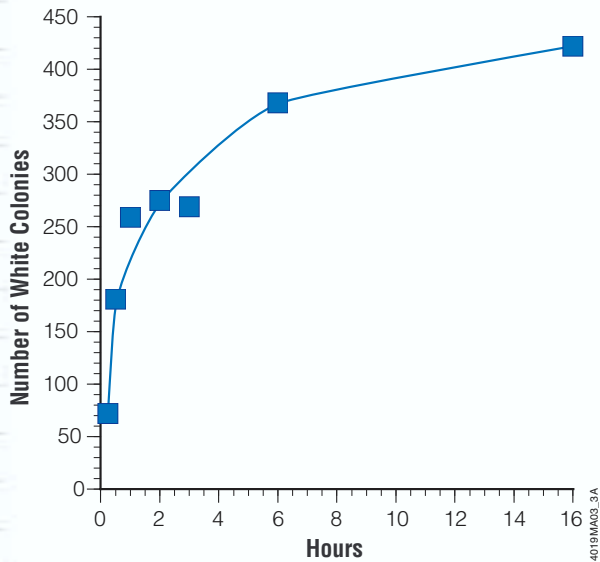
pGEM®-T Easy Vector System II
 (supplied with High Efficiency JM109 Competent Cells)
Cat.#: A1380 (20 reactions)

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

Citations for use of the pGEM-T Easy System online at:
www.promega.com/citations/

For maximum subcloning efficiency, purify the PCR product before subcloning. The presence of PCR primers and primer-dimers can reduce subcloning efficiency. See Chapter 3 for more information.

If you do not purify your PCR product, at least make the amplification as specific as possible. The cleaner the product, the better the ligation efficiency. Try to avoid production of primer-dimers by optimizing the amplification reaction conditions. See Chapter 2 for more information on optimizing PCR.



Relationship between incubation time and cloning efficiency using the 2X Rapid Ligation Buffer and the pGEM-T Easy Vector. The Control Insert DNA supplied with the pGEM-T Easy Vector was ligated into the vector using a 1:1 vector:insert molar ratio. The Rapid Ligation Buffer and T4 DNA Ligase were used in ligation reactions, which were set up at room temperature (24°C) and allowed to proceed from 0.25 to 16 hours. Number of white colonies (transformants) versus time of ligation are shown. This 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.

For maximum efficiency, use competent cells capable of at least 1×10^8 cfu/ μ g DNA.

Example of Transformation Efficiency Calculation:

After 100 μ l competent cells are transformed with 0.1ng uncut plasmid DNA, the transformation reaction is added to 900 μ l of SOC medium (0.1ng DNA/ml). A 1:10 dilution with SOC medium (0.01ng DNA/ml) is made, and 100 μ l is plated on each of two plates (0.001ng DNA/100 μ l). If 200 colonies are obtained (average of two plates), what is the transformation efficiency?

$$\frac{200\text{cfu}}{0.001\text{ng}} \times \frac{1\text{ng}}{10^{-3}\mu\text{g}} = 2 \times 10^8 \text{ cfu}/\mu\text{g DNA}$$

Cloning PCR DNA

Direct Mammalian Expression

pTARGET™ Mammalian Expression Vector System

The pTARGET Mammalian Expression Vector^(1,2) is designed to streamline your experiments, allowing you to go from PCR and T-vector cloning directly to expression analysis in a mammalian system. The pTARGET Vector is based on the popular pCI-neo Vector^(h,i) (Cat.# E1841) and delivers powerful mammalian expression through the CMV promoter. The vector also has the neomycin resistance necessary for G-418 Sulfate selection of stable transformants.

The pTARGET Vector is the only mammalian expression T-vector offering blue/white selection of recombinants. The vector contains the *lacZ* α -peptide fragment to complement the ω -fragment of *lacZ* that is expressed in common *E. coli* strains like JM109, DH5 α ™ and XL-1 Blue. See page 35 for more explanation of blue/white selection.

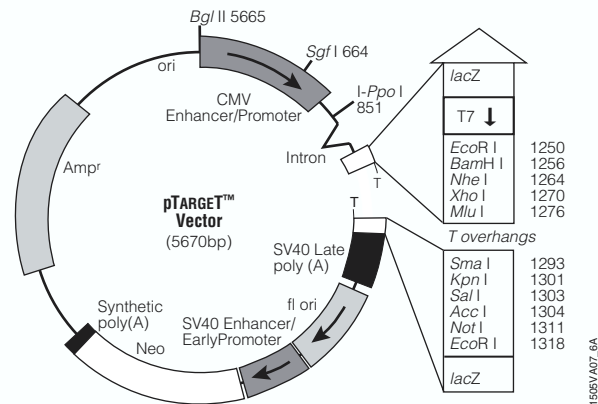
Sequence inserts with:
T7 Promoter Primer
pTARGET
Sequencing Primer

pTARGET™ Mammalian Expression Vector System
Cat.#: A1410 (20 reactions and 20 transformations with high-efficiency JM109 Competent Cells)

Protocol:
www.promega.com/tbs/tm044/tm044.html

Citations for use of the pTARGET System online at:
www.promega.com/citations/

The only mammalian expression T-vector capable of blue/white screening.



Drop out your insert with a single *EcoR I* digest.

pTARGET Mammalian Expression Vector has been used for **transient expression** in many cell lines including:

- COS-1 SV40-transformed monkey kidney
- COS-7 SV40-transformed monkey kidney
- H9c2 rat myoblast
- McA-RH7777 rat hepatoma
- Primary human melanoma

The pTARGET Mammalian Expression Vector has been used to create **stable transfectants** by G-418 Sulfate selection in many cell lines including:

- 1376 TCC human bladder transitional cell carcinoma
- 293 human embryonic kidney cell
- A549 human adenocarcinoma
- CHO Chinese hamster ovary
- NIH/3T3 mouse fibroblast
- J82 human bladder transitional cell carcinoma
- PS120 Chinese hamster lung fibroblast
- RAW264.7 mouse monocyte/macrophage cell line
- T24 human bladder transitional cell carcinoma
- U937 human leukemic cells

See Promega's online citation database for further examples and details: www.promega.com/citations/

The pTARGET Vector contains the **simian virus 40 (SV40) enhancer and early promoter region**

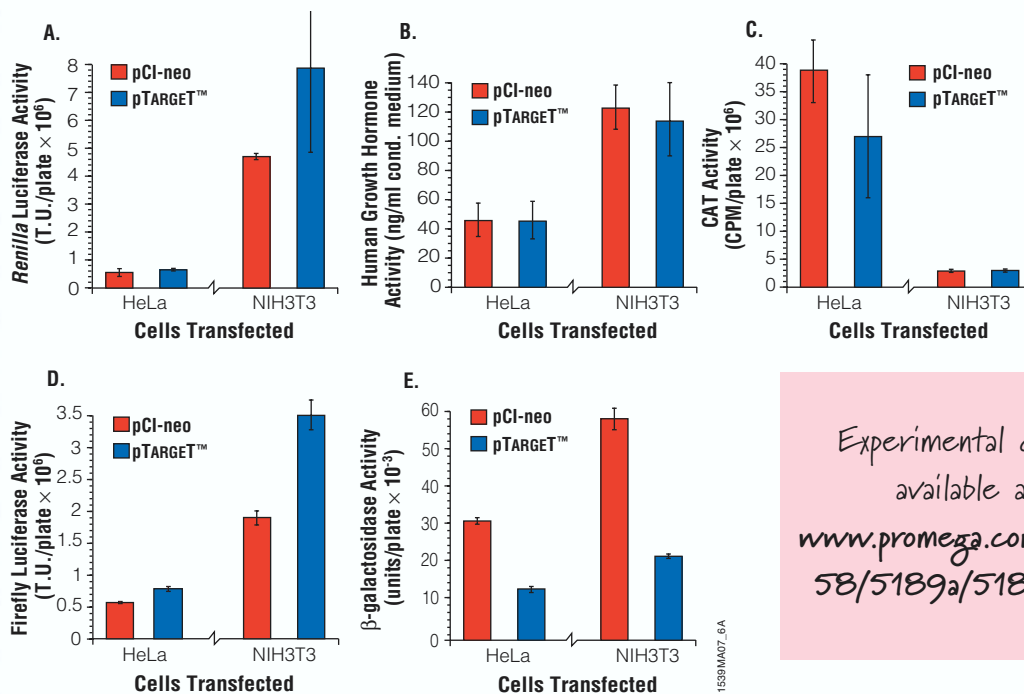
upstream of the neomycin phosphotransferase gene. The SV40 early promoter contains the SV40 origin of replication, which will induce transient, episomal replication of the pTARGET Vector in cells expressing the SV40 large T antigen such as COS-1 or COS-7 cells (1). Consequently, the copy number of the vector will increase in these SV40-transformed cell lines and give higher transient expression than in other cell types.

1. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175-82.

Cloning PCR DNA

Direct Mammalian Expression (continued)

pTARGET™ Mammalian Expression Vector System



Experimental details available at:
www.promega.com/pnotes/58/5189a/5189a.html

Expression of various reporter proteins using either the pTARGET Mammalian Expression Vector or the pCI-neo Mammalian Expression Vector. The pTARGET Vector was designed from the pCI-neo Vector (Cat.# E1841). Vector sequences for blue/white selection do not interfere with expression. T.U. = Turner light units. Details of this experiment may be found in Brondyk, B. (1996) pTARGET Vector: A new mammalian expression T-Vector. *Promega Notes* 58, 2-7.

For expression in mammalian systems, your amplicon should contain an initiation AUG codon and a stop codon. Ideally the AUG codon is in the context of a Kozak consensus sequence: (A or G)CCAUGG (1). Be sure the initiation codon is the first AUG codon encountered in the sequence.

1. Kozak, M. (1987) At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* 196, 947-50.

Need transfection grade plasmid DNA?

Promega has the system.

Wizard MagneSil Tfx™ System(s)

For automated 96-well transfection-grade plasmid DNA purification.

Cat.#: A2380 (4 × 96 preps)
A2381 (8 × 96 preps)

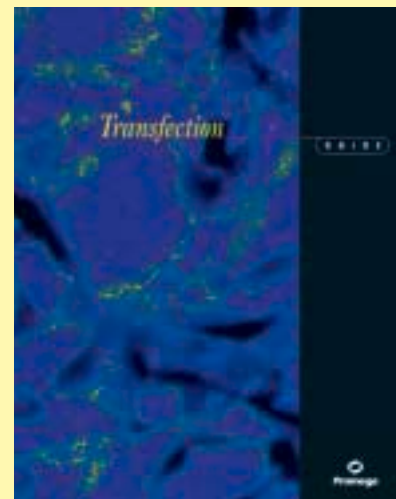
Protocol:

www.promega.com/tbs/tb314/tb314.html

For information on automated methods visit:

www.promega.com/automethods/

Learn about transfection and tools available from Promega in the Transfection Guide available online at: www.promega.com/guides/, or request literature #BR041 from your local Promega representative.



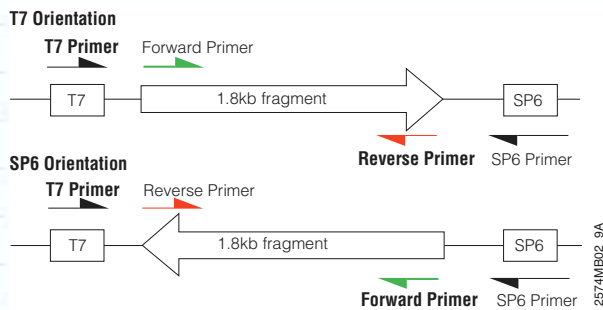
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Cloning PCR DNA

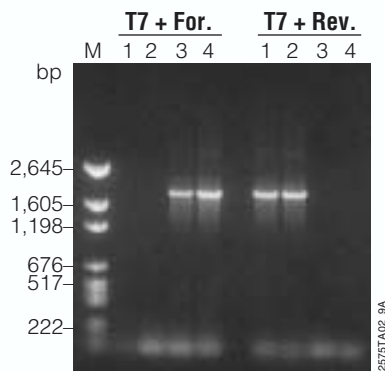
PCR Cloning Techniques

Rapid PCR-Based Screen for Orientation of Insert

All Promega PCR cloning vectors have some unique landmarks, including RNA promoter primer binding sites allowing easy sequencing of inserts. The primer binding sites can also be used to rapidly screen for insert orientation. For example, we cloned a 1.8kb insert into the pGEM[®]-T Easy Vector System (1). The insert could be oriented in one of two ways—toward the T7 promoter or toward the SP6 promoter:



To check for orientation, we performed colony PCR with the T7 Promoter Primer and either the gene-specific forward PCR primer or reverse PCR primer. Eight white colonies with inserts were chosen from the cloning experiment for orientation analysis. Clones with the T7 orientation will produce the fragment with only the reverse PCR primer, and clones in the opposite (SP6) orientation will only produce a product with the forward PCR primer as illustrated below.



Colony PCR. Colonies were suspended in 50 μ l sterile water, boiled for 10 minutes, centrifuged at 16,000 $\times g$ for 5 minutes, and 5 μ l of the supernatant was used in each amplification. The DNA was amplified by PCR in 50 μ l 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 $^{\circ}$ C, the amplification profile was 35 cycles of denaturation (94 $^{\circ}$ C for 30 seconds), annealing (55 $^{\circ}$ C for 1 minute) and extension (72 $^{\circ}$ C for 2.5 minutes); PCR was concluded with 1 cycle of 72 $^{\circ}$ C for 10 minutes. Amplification products (8 μ l) were analyzed on a 1% agarose gel containing ethidium bromide.

Reference

1. 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.

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 PCR products generated using these polymerases so that they will become suitable substrates for T-vector cloning. Full details of the protocol are available in the *pGEM-T and pGEM-T Easy Vector Systems Technical Manual #TM042*.

Start with 1–7 μ l of purified PCR fragment generated by a proofreading polymerase (e.g., *Pfu* DNA Polymerase).

Add 1 μ l *Taq* DNA Polymerase 10X Reaction Buffer with MgCl₂.

Add dATP to a final concentration of 0.2mM.

Add 5 units of *Taq* DNA Polymerase.

Add deionized water to a final reaction volume of 10 μ l.

Incubate at 70 $^{\circ}$ C for 15–30 minutes.

Use 1–2 μ l in a ligation reaction with Promega's pGEM[®]-T and pGEM[®]-T Easy Vector.

An A-tailing procedure for blunt-ended PCR fragments.

Ends Left by Various Thermostable Polymerases.

<i>Taq</i> DNA Polymerase	3'A overhang*
GoTaq [®] DNA Polymerase	3'A overhang*
<i>Tfi</i> DNA Polymerase	3'A overhang*
<i>Tth</i> DNA Polymerase	3'A overhang*
<i>Pfu</i> DNA Polymerase	Blunt
<i>Tli</i> DNA Polymerase	Blunt
Long PCR mixes	Blunt
Proofreading Polymerases	Blunt

* All bases may be found at 3' overhang. A tends to occur most often.

For more information and techniques for cloning PCR DNA, check out Promega's Frequently Asked Questions on the T-vector cloning systems at:

www.promega.com/faq/

Cloning PCR DNA

PCR Cloning Techniques (continued)

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 the Control Insert can give you a lot of information about your ligation and transformation reactions.

The total number of blue colonies in Control Insert and no-insert controls should be approximately equal. The negative control may have some white colonies.

Typical PCR Cloning Results Using pGEM[®]-T Easy Vector and JM109 Competent Cells (1.5×10^8 cfu/ng DNA).

	Efficiency (cfu/ng DNA)	% White Colonies
Control Insert	1,110	92%
Control Insert	1,125	92%
No insert	92	0%
No insert	109	0%

Ligations performed at room temperature for 1 hour.

Bacterial Plates for Blue/White Selection

LB medium (per liter)

10g Bacto[®]-tryptone
5g Bacto[®]-yeast extract
5g NaCl
Adjust pH to 7.0 with NaOH.

Ampicillin Stock Solution

Dissolve at 50mg/ml in water. Filter sterilize. Store in aliquots at -20°C

IPTG stock solution (0.1M)

1.2g IPTG (Cat.# V3951)
Add water to 50ml final volume. Filter-sterilize and store at 4°C .

X-Gal (2ml)

100mg X-Gal (Cat.# V3941)
Dissolve in 2ml N,N'-dimethyl-formamide. Cover with aluminum foil and store at -20°C .

LB plates with ampicillin/IPTG/X-Gal

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of $100\mu\text{g/ml}$, then supplement with 0.5mM IPTG and $80\mu\text{g/ml}$ X-Gal and pour the plates. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

Interpreting Results

Results with the experimental insert look like those with the Control Insert in terms of efficiency and % white colonies.

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

Results with the experimental insert and Control Insert look like the negative control.

Ligation has failed. Avoid multiple freeze/thaws of the ligation buffer. Ligase buffer contains ATP and could be damaged by freeze-thaws. You may need to aliquot the ligase buffer into useful portions for your experimental needs.

Few/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 $>1 \times 10^8\text{cfu}/\mu\text{g}$ to insure >100 colonies from the Control Insert ligation.

Experimental insert gives more blue colonies than the Control Insert or negative control and less white colonies than the Control Insert.

In-frame insertion with no interruption of the α -fragment. Although the pGEM-T Vector Control Insert will produce recombinants that generate white colonies, the insertion of other DNA fragments into the *lacZ* coding sequence may not result in white colonies unless the fragments disrupt the *lacZ* reading frame. Although this tends to occur most frequently with PCR products of 500bp or less, inserts of up to 2kb have been reported to result in blue colonies. Moreover, some insert DNAs can also give pale blue colonies or "bull's eye" colonies that have a blue center and a white perimeter. In one case, we found that a 1.8kb insert produced white colonies when oriented in one direction and bull's eye colonies when oriented in the opposite direction (1).

1. 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.

Cloning PCR DNA

Basic Subcloning

Product	Size	Cat.#
pGEM®-T Vector System I ^(h,i)	20 reactions	A3600
pGEM®-T Vector System II ^(h,i)	20 reactions	A3610
pGEM®-T Easy Vector System I ^(h,i)	20 reactions	A1360
pGEM®-T Easy Vector System II ^(h,i)	20 reactions	A1380

For Laboratory Use. pGEM®-T and pGEM®-T Easy Vector Systems I do not include competent cells. With System II, competent cells are provided.

Direct Mammalian Expression

Product	Size	Cat.#
pTARGET™ Mammalian Expression Vector System ^(i,j)	20 reactions	A1410

Competent Cells provided.

Primers

Product	Size	Cat.#
T7 Promoter Primer (10µg/ml) [5'-d(TAATAGGACTCACTATAGGG)-3']	2µg	Q5021
SP6 Promoter Primer (10µg/ml) [5'-d(TATTTAGGTGACACTATAG)-3']	2µg	Q5011
pUC/M13 Forward Primer (10µg/ml) [5'-d(GGCCAGGGTTTTCCAGTCACGAC)-3']	2µg	Q5601
pUC/M13 Reverse Primer (10µg/ml) [5'-d(TCACACAGGAAACAGCTATGAC)-3']	2µg	Q5421
pTARGET™ Sequencing Primer (10µg/ml) [5'-d(TTACGCCAAGTTATTTAGGTGACA)-3']	2µg	Q4461
PinPoint™ Vector Sequencing Primer [5'-d(CGTGACGCGGTGCAGGGCG)-3']	2µg	V4211

DNA Purification Systems

Product	Size	Cat.#
Wizard® Plus SV Minipreps DNA Purification System ^{(q,r)*}	50 preps	A1330
	250 preps	A1460
Wizard MagneSil Tfx™ System ^(s)	4 × 96 preps	A2380
	8 × 96 preps	A2381

(Automated transfection-grade plasmid purification.)

*For Laboratory Use.

Cloning PCR DNA

Accessory Products

Product	Size	Cat.#
Select96™ Competent Cells (Single aliquot competent cells, use 1 to 96 at a time, $>1 \times 10^8$ cfu/ μ g DNA)	1 \times 96 preps	L3300
JM109 High Efficiency Competent Cells (10 ⁸ cfu/ μ g)* (Packaged 5 \times 200 μ l; use 50 μ l per transformation $>1 \times 10^8$ cfu/ μ g DNA)	1ml	L2001
IPTG, Dioxane-Free	5g	V3951
	50g	V3953
X-Gal (50mg/ml)	100mg	V3941
Antibiotic G-418 Sulfate (potency $>500\mu$ g/mg)	100mg	V7981
	1g	V7982
	5g	V7983
Antibiotic G-418 Sulfate Solution (potency 40–60mg/ml)	20ml	V8091

*For Laboratory Use.

Transfection Reagents

Product	Size	Cat.#
TransFast™ Transfection Reagent ^(U)	1.2mg	E2431
Tfx™-10 Reagent ^(U)	9.3mg	E2381
Tfx™-20 Reagent ^(U)	4.8mg	E2391
Tfx™-50 Reagent ^(U)	2.1mg	E1811