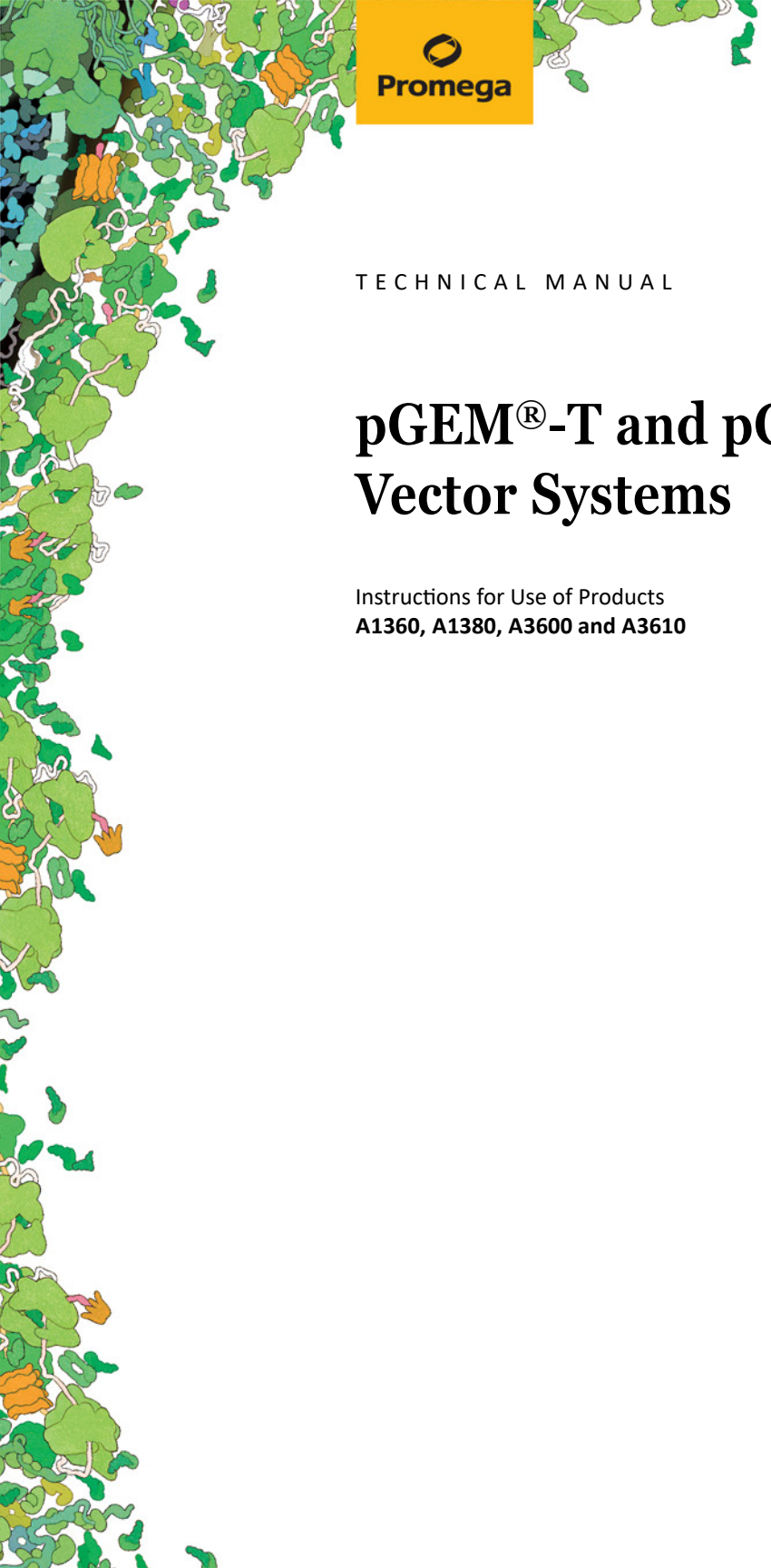

TECHNICAL MANUAL

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

Instructions for Use of Products
A1360, A1380, A3600 and A3610

Revised 10/21
TM042

Promega

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

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Introduction

1.A. Vector Features

T-Overhangs for Easy PCR Cloning: The pGEM®-T and pGEM®-T Easy Vectors are linearized vectors with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (1,2).

Blue/White Selection of Recombinants: The pGEM®-T and pGEM®-T Easy Vectors are high-copy-number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates.

Choice of Restriction Sites for Release of Insert: Both the pGEM®-T and pGEM®-T Easy Vectors contain numerous restriction sites within the multiple cloning region. The pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI, providing three single-enzyme digestions for release of the insert. The pGEM®-T Vector cloning region is flanked by recognition sites for the enzyme BstZI. Alternatively, a double-digestion may be used to release the insert from either vector.

Rapid Ligation: The pGEM®-T and pGEM®-T Easy Vector Systems are supplied with 2X Rapid Ligation Buffer. Ligation reactions using this buffer may be incubated for 1 hour at room temperature. The incubation period may be extended to increase the number of colonies after transformation. Generally, an overnight incubation at 4°C produces the maximum number of transformants.

1.B. Important Considerations for Successful T-Vector Cloning

Avoid introduction of nucleases, which may degrade the T-overhangs on the vector. Use only the T4 DNA Ligase provided with the system, as this has been tested for minimal exonuclease activity. Use sterile, nuclease-free water in your ligation reactions.

Use high-efficiency competent cells (≥1 × 10^8cfu/µg DNA) for transformations. The ligation of fragments with a single-base overhang can be inefficient, so it is essential to use cells with a transformation efficiency of at least 1 × 10^8cfu/µg DNA in order to obtain a reasonable number of colonies. However, use of super high-efficiency competent cells (e.g., XL10 Gold® Cells) may result in a higher background of blue colonies.

Limit exposure of your PCR product to shortwave UV light to avoid formation of pyrimidine dimers. Use a glass plate between the gel and UV source. If possible, only visualize the PCR product with a long-wave UV source.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
pGEM®-T Vector System I	**20 reactions**	**A3600**

Includes:

- 1.2µg pGEM®-T Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase

PRODUCT	SIZE	CAT.#
pGEM®-T Vector System II	**20 reactions**	**A3610**

Includes:

- 1.2µg pGEM®-T Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase
- 1.2ml JM109 Competent Cells, High Efficiency (6 × 200µl)

PRODUCT	SIZE	CAT.#
pGEM®-T Easy Vector System I	**20 reactions**	**A1360**

Includes:

- 1.2µg pGEM®-T Easy Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase

PRODUCT	SIZE	CAT.#
pGEM®-T Easy Vector System II	**20 reactions**	**A1380**

Includes:

- 1.2µg pGEM®-T Easy Vector (50ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl 2X Rapid Ligation Buffer, T4 DNA Ligase
- 1.2ml JM109 Competent Cells, High Efficiency (6 × 200µl)

Storage Conditions: For Cat.# A3610, A1380, store the Competent Cells at –70°C. Store all other components at –20°C.

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3. Protocol for Ligations Using the pGEM®-T and pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer

3.A. Ligation Protocol

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.
2. Set up ligation reactions as described below.

   Note: Use 0.5ml tubes known to have low DNA-binding capacity (e.g., VWR Cat.# 20170-310).

   Vortex the 2X Rapid Ligation Buffer vigorously before each use.
3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature.

   Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.

Reaction Component	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl*	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
nuclease-free water to a final volume of	**10µl**	**10µl**	**10µl**

*Molar ratio of PCR product:vector may require optimization.

Notes:

1. Use only the T4 DNA Ligase supplied with this system to perform pGEM®-T and pGEM®-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may remove the terminal deoxythymidines from the vector.
2. 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.
3. Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the maximum number of transformants.

4. An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction to verify that the reaction produced the desired product. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281). Clean-up of reactions prior to ligation is recommended to remove primer dimers or other undesired reaction products, and to improve ligation efficiency. Exposure of PCR products to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers.

3.B. Optimizing Insert:Vector Molar Ratios

The pGEM®-T and pGEM®-T Easy Vector Systems have been optimized using a 1:1 molar ratio of the Control Insert DNA to the vectors. However, ratios of 8:1 to 1:8 have been used successfully. If initial experiments with your PCR product are suboptimal, ratio optimization may be necessary. Ratios from 3:1 to 1:3 provide good initial parameters. The concentration of PCR product should be estimated by comparison to DNA mass standards on a gel or by using a fluorescent assay (3). The pGEM®-T and pGEM®-T Easy Vectors are approximately 3kb and are supplied at 50ng/µl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Example of insert:vector ratio calculation:

How much 0.5kb PCR product should be added to a ligation in which 50ng of 3.0kb vector will be used if a 3:1 insert:vector molar ratio is desired?

$$\frac{50\text{ng vector} \times 0.5\text{kb insert}}{3.0\text{kb vector}} \times \frac{3}{1} = 25\text{ng insert}$$

Using the same parameters for a 1:1 insert:vector molar ratio, 8.3ng of a 0.5kb insert would be required.

Tip: The Biomath calculator (**www.promega.com/biomath/**) can be used to determine the amount of insert DNA needed. The pGEM®-T Vector size is 3000bp and the pGEM®-T Easy Vector size is 3,015bp.

4. Transformations Using the pGEM®-T and pGEM®-T Easy Vector Ligation Reactions

Use high-efficiency competent cells (≥1 × 10^8cfu/µg DNA) for transformations. Ligation of fragments with a single-base overhang can be inefficient, so it is essential to use cells with a transformation efficiency of 1 × 10^8cfu/µg DNA (or higher) in order to obtain a reasonable number of colonies. We recommend using JM109 High Efficiency Competent Cells (Cat.# L2001); these cells are provided with the pGEM®-T and pGEM®-T Easy Vector Systems II. Other host strains may be used, but they should be compatible with blue/white color screening and standard ampicillin selection.

Note: Use of super high-efficiency competent cells (e.g., XL10 Gold® Ultracompetent Cells) may result in a higher background of blue colonies.

If you are using competent cells other than JM109 High Efficiency Competent Cells purchased from Promega, it is important that the appropriate transformation protocol be followed. Selection for transformants should be on LB/ampicillin/IPTG/X-Gal plates (See recipe in Section10.C). For best results, do not use plates that are more than 1 month old.

The genotype of JM109 is *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (rK–,mK+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´, *tra*D36, *pro*AB, *lac*I^qZΔM15] (4).

4.A. Transformation Protocol

Materials to Be Supplied by the User
(Solution Compositions are provided in Section 10.C.)

- LB plates with ampicillin/IPTG/X-Gal
- SOC medium

1. Prepare two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency. Equilibrate the plates to room temperature.
2. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom. Add 2µl of each ligation reaction to a sterile (17 × 100mm) polypropylene tube or a 1.5ml microcentrifuge tube on ice (see Note 1). Set up another tube on ice with 0.1ng uncut plasmid for determination of the transformation efficiency of the competent cells.
3. Remove tube(s) of frozen JM109 High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.
4. **Carefully** transfer 50µl of cells into each tube prepared in Step 2 (use 100µl of cells for determination of transformation efficiency).
5. **Gently** flick the tubes to mix and place them on ice for 20 minutes.
6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C (**do not shake**).

7. Immediately return the tubes to ice for 2 minutes.
8. Add 950µl room-temperature SOC medium to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid (LB broth may be substituted, but colony number may be lower).
9. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
10. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × *g* for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of two plates.
11. Incubate the plates overnight (16–24 hours) at 37°C. If 100µl is plated, approximately 100 colonies per plate are routinely seen using competent cells that are 1×10^8cfu/µg DNA. Use of ultra-high- efficiency competent cells may result in a higher number of background colonies. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to facilitate blue color development. White colonies generally contain inserts; however, inserts may also be present in blue colonies.

Notes:

1. We have found that use of larger (17 × 100mm) polypropylene tubes (e.g., Falcon™ Cat.# 2059) increases transformation efficiency. Tubes from some manufacturers bind DNA and should be avoided.
2. Colonies containing β-galactosidase activity may grow poorly relative to cells lacking this activity. After overnight growth, the blue colonies may be smaller than the white colonies, which are approximately one millimeter in diameter.
3. Blue color will become darker after the plate has been stored overnight at 4ºC.

4.B. Example of Transformation Efficiency Calculation

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

$$\frac{200\text{cfu}}{0.001\text{ng}} = 2 \times 10^5\text{cfu/ng} = 2 \times 10^8\text{cfu/µg DNA}$$

4.C. Screening Transformants for Inserts

Successful cloning of an insert into the pGEM®-T or pGEM®-T Easy Vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates. However, the characteristics of the PCR products cloned into the vectors can significantly affect the ratio of blue:white colonies obtained. Usually clones containing PCR products produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lacZ* gene. Such fragments are usually a multiple of 3 base pairs long (including the 3´-A overhangs) and do not contain in-frame stop codons. There have been reports of DNA fragments up to 2kb that have been cloned in-frame and have produced blue colonies. Even if your PCR product is not a multiple of 3 bases long, the amplification process can introduce mutations (deletions or point mutations) that may result in blue colonies.

The Control Insert DNA supplied with the pGEM®-T and pGEM®-T Easy Systems is a 542bp fragment from pGEM®-*luc* Vector DNA (Cat.# E1541). This sequence has been mutated to contain multiple stop codons in all six reading frames, which ensures a low background of blue colonies for the control reaction. Results obtained with the Control Insert DNA may not be representative of those achieved with your PCR product.

5. pGEM®-T and pGEM®-T Easy Vector Sequences, Multi-Cloning Sites and Circle Maps

5.A. Sequence and Multi-Cloning Site of the pGEM®-T Vector

The pGEM®-T Vector is derived from the pGEM®-5Zf(+) Vector (GenBank® Accession No. **X65308**). The pGEM®-T Vector was created by linearizing the pGEM®-5Zf(+) Vector with EcoRV at base 51 and adding a T to both 3´-ends. The EcoRV site will not be recovered upon ligation of the vector and insert.

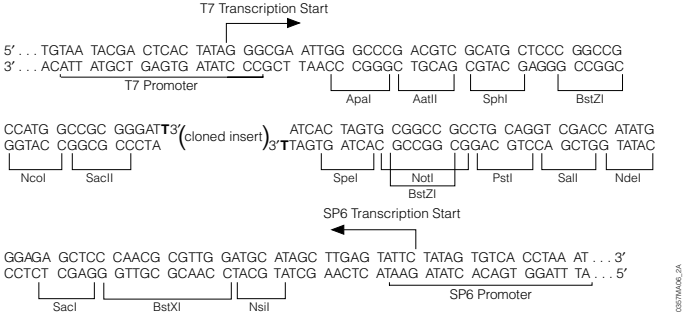
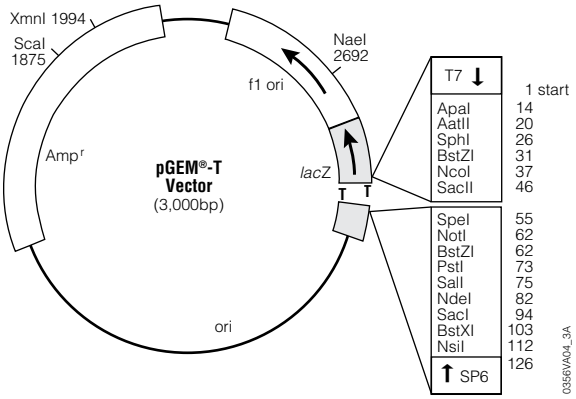


Figure 1. The promoter and multiple cloning sequence of the pGEM®-T Vector. The top strand corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

5.B. pGEM®-T Vector Map and Sequence Reference Points



pGEM®-T Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
lacZ start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

Note: Inserts can be sequenced using the pUC/M13 Forward Primer (Cat.# Q5601) or pUC/M13 Reverse Primer (Cat.# Q5401).

Note: A single digest with BstZI will release inserts cloned into the pGEM®-T Vector. Double digests can also be used to release inserts. Isoschizomers of BstZI include EagI and Eco52I.

5.C. Sequence and Multi-Cloning Site of the pGEM®-T Easy Vector

The sequence of the pGEM®-T Easy Vector is available at: **www.promega.com/resources/vector-sequences/** The pGEM®-T Easy Vector has been linearized at base 60 with EcoRV and a T added to both 3´-ends. The EcoRV site will not be recovered upon ligation of the vector and insert.

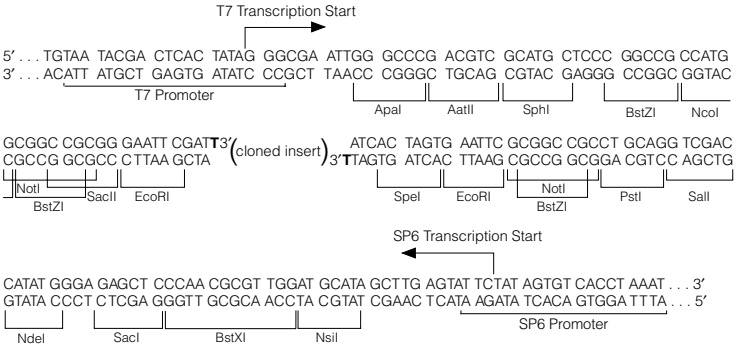
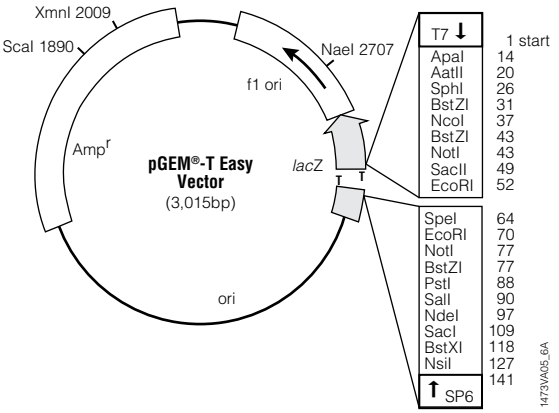


Figure 2. The promoter and multiple cloning sequence of the pGEM®-T Easy Vector. The top strand shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

More PCR Cloning Resources are available at: **www.promega.com/resources/guides/**

5.D. pGEM®-T Easy Vector Map and Sequence Reference Points



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Note: Inserts can be sequenced using the pUC/M13 Forward Primer (Cat.# Q5601) or pUC/M13 Reverse Primer (Cat.# Q5401).

Note: A single digest with BstZI, EcoRI (Cat.# R6011) or NotI (Cat.# R6431) will release inserts cloned into the pGEM®-T Easy Vector. Double digests can also be used to release inserts. Isoschizomers of BstZI include EagI and Eco52I.

6. General Considerations for PCR Cloning

6.A. PCR Product Purity

An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction. The PCR product can be gel-purified or purified directly from the PCR amplification using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281). Exposure to shortwave ultraviolet light should be minimized to avoid the formation of pyrimidine dimers. Even if distinct bands of the expected size are observed, primer-dimers should be removed by gel purification or by using the Wizard® SV Gel and PCR Clean-Up System. Use of crude PCR product may produce successful ligation in some cases; however, the number of white colonies containing the relevant insert may be reduced due to preferential incorporation of primer-dimers or other extraneous reaction products. Therefore, it may be necessary to screen numerous colonies in order to identify clones that contain the PCR product of interest.

6.B. Properties of Various Thermostable Polymerases

Not all thermostable polymerases generate fragments with 3´A-tailed fragments. Table 1 lists the properties of several commonly used polymerase enzymes.

Table 1. Comparison of PCR Product Properties for Thermostable DNA Polymerases.

	Thermostable DNA Polymerase						
Characteristic	GoTaq®/ *Taq*/ AmpliTaq®	*Tfl*	*Tth*	Vent® (*Tli*)	Deep Vent®	*Pfu*	*Pwo*
Resulting DNA ends	3´A	3´A	3´A	Blunt	Blunt	Blunt	Blunt
5´→ 3´exonuclease activity	Yes	Yes	Yes	No	No	No	No
3´→ 5´exonuclease activity	No	No	No	Yes	Yes	Yes	Yes

6.C. Cloning Blunt-Ended PCR Products

Thermostable DNA polymerases with proofreading activity, such as *Pfu* DNA Polymerase (Cat.# M7741), *Pwo* DNA polymerase and *Tli* DNA Polymerase, generate blunt-ended fragments. Nevertheless, PCR products generated using these polymerases can be modified using the A-tailing procedure outlined in Figure 3 and ligated into the pGEM®-T and pGEM®-T Easy Vectors (5). Using this method, only one insert will be ligated into the vector (as opposed to multiple insertions that can occur with blunt-ended cloning). In addition, with T-vector cloning there is no need to dephosphorylate the vector, and there is a low background of religated vector.

Using this procedure with optimized insert:vector ratios, 55–95% recombinants were obtained when *Pfu* and *Tli* DNA Polymerases were used to generate the insert DNA (Table 2). It is critical that the PCR fragments are purified using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) or by direct isolation from a gel by other means. In the absence of purification, the proofreading activity of the *Pfu*, *Pwo* and *Tli* DNA Polymerases will degrade the PCR fragments, or remove the 3´-terminal deoxyadenosine added during tailing or the 3´-terminal deoxythymidine from the vector during the A-tailing reaction or ligation.

To optimize cloning efficiency, the amount of DNA in the A-tailing reaction and the 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 and/or good amplification, small volumes of the PCR fragment are needed for the A-tailing and ligation reactions. However, when molar concentration is low due to large fragment size and/or poor amplification, large volumes of the PCR fragment are needed for the A-tailing and ligation reactions. We have successfully used 1–7µl of purified PCR fragment in A-tailing reactions to optimize the insert:vector ratio. (See Section 3.B for further discussion of optimizing the insert:vector ratio.) Recombinants were identified by blue/white screening, and 70–100% were shown to have the correct size insert by PCR. Few recombinants were observed in control reactions in which the PCR fragment was not tailed. These control results confirm that the majority of the pGEM®-T Easy Vector used contained 3´-terminal deoxythymidine and that, during the A-tailing, *Taq* DNA Polymerase added a 3´-terminal deoxyadenosine to a significant proportion of the PCR fragments.

6.C. Cloning Blunt-Ended PCR Products (continued)

Table 2. Comparison of A-Tailing Procedures.

	% Recombinants[1]			
	1-Hour Ligation at 24°C (Standard)		16-Hour Ligation at 4°C (Alternative)	
Polymerase	**542bp**	**1.8kb**	**542bp**	**1.8kb**
Pfu DNA Polymerase	65–84%[2]	31–55%[3]	81–95%[2]	50–75%[3]
Tli DNA Polymerase	68–77%[4]	37–65%[5]	85–93%[4]	60–81%[5]

PCR fragments generated by *Pfu* and *Tli* DNA Polymerase were A-tailed and ligated into pGEM®-T Easy Vector for 1 hour at 24°C or for 16 hours at 4°C. Two microliters of ligation mix was transformed into JM109 Competent Cells and plated on LB/amp/IPTG/X-gal plates.

[1]% Recombinants = % white and/or pale blue colonies. PCR fragments were purified with the Wizard® PCR Preps DNA Purification System prior to A-tailing.

[2]Insert:vector ratios tested: 5:1, 3:1, 1:1. Volume of PCR amplification product used in A-tailing: 1–2µl.

[3]Insert:vector ratios tested: 3:1, 2:1, 1:1. Volume of PCR amplification product used in A-tailing: 3–7µl.

[4]Insert:vector ratios tested: 3:1, 2:1, 1:1. Volume of PCR amplification product used in A-tailing: 1–2µl.

[5]Insert:vector ratios tested: 2:1, 1:1. Volume of PCR amplification product used in A-tailing: 4–7µl.

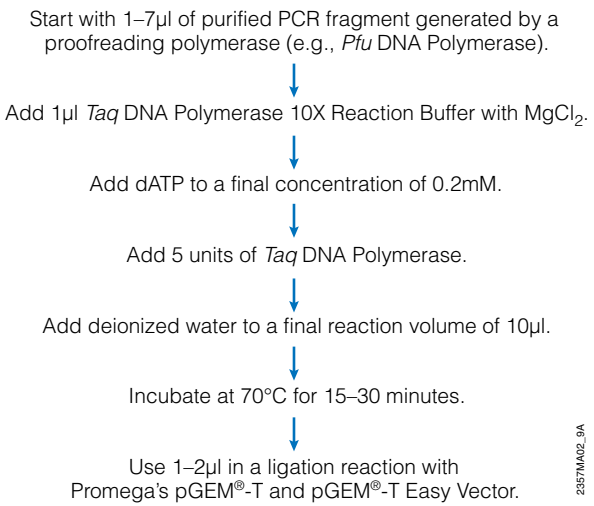


Figure 3. An A-tailing procedure for blunt-ended PCR fragments purified with the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) and used in T-vector cloning.

7. Experimental Controls

Positive Control: Set up a ligation reaction with the Control Insert DNA as described in Section 3 and use it for transformations. This control will allow you to determine whether the ligation is proceeding efficiently. Typically, approximately 100 colonies should be observed, 10–40% of which are blue, when competent cells that have a transformation efficiency of 1×10^8cfu/µg DNA are transformed. Greater than 60% of the colonies should be white. The Control Insert DNA is designed to produce white colonies; however, other insert DNA may not yield white colonies (see Section 4.C). Background blue colonies from the positive control ligation reaction arise from non-T-tailed or undigested pGEM®-T or pGEM®-T Easy Vector. These blue colonies are a useful internal transformation control; if no colonies are obtained, the transformation has failed. If small numbers of blue colonies are obtained, but no whites, the ligation reaction may have failed. If <50% white colonies are seen in the positive control reaction, then the ligation conditions were probably suboptimal or nuclease contamination of the ligation reaction may have occurred.

The concentration of the Control Insert DNA is such that 2µl (4ng/µl) can be used in a 10µl ligation reaction to achieve a 1:1 molar ratio with 50ng of the pGEM®-T or pGEM®-T Easy Vectors.

Background Control: Set up a ligation reaction with 50ng of pGEM®-T or pGEM®-T Easy Vector and no insert as described in Section 3, and use it for transformations. This control allows determination of the number of background blue colonies resulting from non-T-tailed or undigested pGEM®-T or pGEM®-T Easy Vector alone. If the recommendations in Section 4 are followed, 10–30 blue colonies will typically be observed if the transformation efficiency of the competent cells is 1×10^8cfu/µg DNA. (Under these conditions, cells that have an efficiency of 1×10^7cfu/µg DNA would yield 1–3 blue colonies, and cells with a transformation efficiency of 1×10^9cfu/µg DNA would yield 100–300 blue colonies). Compare the number of blue colonies obtained with this background control to the number of blue colonies obtained in the standard reaction using the PCR product. If ligation of the PCR product yields dramatically more blue colonies than the background control reaction, then recombinants are probably among these blue colonies (see Section 4.C).

Transformation Control: Check the transformation efficiency of the competent cells by transforming them with an uncut plasmid (not pGEM®-T or pGEM®-T Easy, since these vectors are linearized) and calculating cfu/µg DNA. If the transformation efficiency is lower than 1×10^8cfu/µg DNA, prepare fresh cells. If you are not using JM109 High Efficiency Competent Cells (provided with pGEM®-T and pGEM®-T Easy Vector Systems II; Cat.# A3610 and A1380, respectively), be sure the cells are compatible with blue/white screening and standard ampicillin selection and have a transformation efficiency of at least 1×10^8cfu/µg DNA.

8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
No colonies	A problem has occurred with the transformation reaction or the cells have lost competence. Background undigested vector and religated non-T-tailed vector should yield 10–30 blue colonies independent of the presence of insert DNA. Check the background control (Section 7).
	Use high-efficiency competent cells (≥1 × 10^8cfu/µg DNA). Test the efficiency by transforming the cells with an uncut plasmid that allows for antibiotic selection, such as the pGEM®-5Zf(+) Vector. If the guidelines in Section 4 are followed, cells at 1 × 10^8cfu/µg DNA typically yield 100 colonies. Therefore, you would not see any colonies from cells that are <1 × 10^7cfu/µg DNA (Section 7).
Less than 10% white colonies with Control Insert DNA	Improper dilution of the 2X Rapid Ligation. The T4 DNA ligase buffer is provided at a concentration of 2X. Use 5µl in a 10µl reaction.
	If the total number of colonies is high, but there are few/no white colonies, competent cells may be high efficiency (≥1 × 10^9cfu/µg), but there may be a ligation problem. Approximately 1,000 colonies can be obtained from the positive control ligation using cells that are 10^9cfu/µg DNA, with 70–90% white colonies. If ligation is suboptimal or fails, the total number of colonies will be high (up to 300 cells at 1 × 10^9cfu/µg), but the amount of white colonies will be low or zero.

Symptoms	Causes and Comments
Less than 10% white colonies with Control Insert DNA (continued)	Ligation reaction has failed. Ligase buffer may DNA have low activity. The 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles by making single-use aliquots of the buffer. Use a fresh vial of buffer. To test the activity of the ligase and buffer, set up a ligation with ~20ng of DNA markers (e.g., Lambda DNA/HindIII Markers, Cat.# G1711). Compare ligated and nonligated DNA on a gel and check that the fragments have been religated into high-molecular-weight material.
	T-overhangs have been removed, allowing blunt-ended ligation of vector and giving rise to more blue than white colonies. Avoid introduction of nucleases, which may degrade the T-overhangs. Use only the T4 DNA Ligase provided with the system, which has been tested for minimal exonuclease activity. Also, use sterile, nuclease-free water.
Less than 60% white colonies with Control Insert DNA	Improper dilution of the Rapid Ligation Buffer. The Rapid Ligation Buffer is provided at a 2X concentration. Use 5µl in a 10µl reaction.
	T-overhangs have been removed, allowing blunt-ended ligation of vector and giving rise to more blue than white colonies. Avoid introduction of nucleases, which may degrade the T-overhangs. Use only the T4 DNA Ligase provided with the system, which has been tested for minimal exonuclease activity.
	Ligation temperature is too high. Higher temperatures (>28°C) give rise to increased background and fewer recombinants.
Low number or no white colonies containing PCR product	Improper dilution of the Rapid Ligation Buffer. The Rapid Ligation Buffer is provided at a 2X concentration. Use 5µl in a 10µl reaction.
	Ligation incubation is not long enough. Optimal results are seen with an overnight ligation.
	Failed ligation due to an inhibitory component in the PCR product. Mix some of the PCR product with the positive control ligation to determine whether an inhibitor is present. If an inhibitor is indicated, repurify the PCR fragment.

8. Troubleshooting (continued)

Symptoms	Causes and Comments
Low number or no white colonies containing PCR product (continued)	PCR product is not ligating because there are no 3´-A overhangs. As summarized in Table 1, not all thermostable DNA polymerases create a 3´-A overhang (6,7). Blunt-ended fragments may be subsequently A-tailed by treatment with an appropriate polymerase and dATP (8–10).
	PCR product cannot be ligated due to pyrimidine dimers formed from UV over-exposure. This is a common problem with gel-purified DNA. There is no way to fix this; the DNA must be remade. Exposure to shortwave UV should be limited as much as possible. Use a glass plate between the gel and UV source to decrease UV overexposure. If possible, only visualize the PCR product using a longwave UV source.
	The PCR fragment is inserted, but it is not disrupting the *lacZ* gene. If there are a higher number of blue colonies resulting from the PCR fragment ligation than with the background control, some of these blue colonies may contain insert. Screen blue and pale blue colonies (see Section 4.C).
	Insert:vector ratio is not optimal. Check the integrity and quantity of your PCR fragment by gel analysis. Optimize the insert:vector ratio (see Section 3.B).
	There may be primer-dimers present in PCR fragment preparation. Primer-dimers will ligate into the pGEM®-T or pGEM®-T Easy Vector but may not be seen after restriction digestion and gel analysis because of their small size. The vector will appear to contain no insert. More blue colonies may be seen with the ligation than on the background control plates. The PCR fragment should be gel-purified.
	Multiple PCR products may have been generated and cloned into the pGEM®-T or pGEM®-T Easy Vector. Gel-purify the PCR fragment of interest.

Symptoms	Causes and Comments
Low number or no white colonies containing PCR product (continued)	DNA has rearranged. Check a number of clones to see whether the rearrangement is random. If so, the clone of interest should be present and can be identified by screening several clones. If the same rearrangement is found in all of the clones, use of a repair-deficient bacterial strain (e.g., SURE® cells) may reduce recombination events.
PCR product ligation reaction produces white colonies only (no blue colonies)	Ampicillin is inactive, allowing ampicillin- sensitive cells to grow. Check that ampicillin plates are made properly and used within one month. Test ampicillin activity by streaking plates, with and without ampicillin, using an ampicillin-sensitive clone.
	The bacterial strain (e.g., JM109) has lost its F´ episome, or the bacterial strain used is not compatible with blue/white screening. Check the background control. If these colonies are not blue, the cells may have lost the F´ episome (assuming *lac*I^{q}ZΔM15 is located on the F´ in the transformed strain and appropriate plates were used). Be sure that the cells are prepared properly for use with this system (see Section 4).
	Plates are incompatible with blue/white screening. Check the background control. If these colonies are not blue, check that the plates have ampicillin/IPTG/X-Gal and are fresh. If there is any question about the quality of the plates, repeat plating with fresh plates.
Not enough clones contain the PCR product of interest	Insufficient A-tailing of the PCR fragment. After the PCR product of interest urification of the PCR fragment, set up an A-tailing reaction (8–10). Clean up the sample and proceed with the protocol.
	Insert:vector ratio is not optimal. Check the integrity and quality of your PCR fragment by gel analysis. Optimize the insert:vector ratio (see Section 3.B).
	Multiple PCR products are generated and cloned into the pGEM®-T or pGEM®-T Easy Vector. Gel-purify the PCR fragment of interest.

9. References

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10. Appendix

10.A. pGEM®-T Vector Restriction Enzyme Sites

The pGEM®-T Vector is derived from the circular pGEM®-5Zf(+) Vector (GenBank® Accession No. **X65308**). The pGEM®-5Zf(+) Vector sequence is available at: **www.promega.com/resources/vector-sequences/**

The following restriction enzyme tables are based on those of the circular pGEM®-5Zf(+) Vector. The pGEM®-T Vector has been created by linearizing the pGEM®-5Zf(+) Vector with EcoRV at base 51 and adding a T to both 3´ ends. This site will not be recovered upon ligation of the vector and insert. The following tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3´ end of the cut DNA (the base to the left of the cut site). Please contact your local Promega Branch Office or Distributor if you identify a discrepancy. In the U.S., contact Technical Services at 1-800-356-9526.

Table 3. Restriction Enzymes That Cut the pGEM®-T Vector 1–5 Times.

Enzyme	# of Sites	Location
AatII	1	20
AccI	1	76
AcyI	2	17, 1932
AflIII	2	99, 502
Alw26I	2	1456, 2232
Alw44I	2	816, 2062
AlwNI	1	918
ApaI	1	14
AspHI	4	94, 820, 1981, 2066
AvaII	2	1533, 1755
BanI	3	246, 1343, 2626
BanII	3	14, 94, 2664
BbuI	1	26
BglI	3	39, 1515, 2833
BsaI	1	1456
BsaAI	1	2589
BsaHI	2	17, 1932
BsaJI	5	37, 43, 241, 662, 2936
Bsp120I	1	10
BspHI	2	1222, 2230
BspMI	1	62
BssSI	2	675, 2059
BstOI	5	242, 530, 651, 664, 2937
BstXI	1	103
BstZI	2	31, 62
Cfr10I	2	1475, 2690
DdeI	4	777, 1186, 1352, 1892
DraI	3	1261, 1280, 1972
DraIII	1	2589
DrdI	2	610, 2544
DsaI	2	37, 43
EagI	2	31, 62
EarI	3	386, 2190, 2878
EclHKI	1	1395
Eco52I	2	31, 62
EcoICRI	1	92
EcoRV	1	51*

Enzyme	# of Sites	Location
FokI	5	119, 1361, 1542, 1829, 2919
FspI	2	1617, 2840
HaeII	4	380, 750, 2740, 2748
HgaI	4	613, 1191, 1921, 2806
HincII	1	77
HindII	1	77
Hsp92I	2	17, 1932
MaeI	5	56, 997, 1250, 1585, 2740
MluI	1	99
NaeI	1	2692
NciI	4	30, 882, 1578, 1929
NcoI	1	37
NdeI	1	82
NgoMIV	1	2690
NotI	1	62
NsiI	1	112
NspI	2	26, 506
Ppu10I	1	108
PstI	1	73
PvuI	2	1765, 2861
PvuII	2	326, 2890
RsaI	1	1875
SacI	1	94
SacII	1	46
SalI	1	75
ScaI	1	1875
SfiI	1	39
SinI	2	1533, 1755
SpeI	1	55
SphI	1	26
Sse8387I	1	73
SspI	2	2199, 2381
StyI	1	37
TaqI	4	76, 602, 2046, 2622
TfiI	2	337, 477
VspI	3	273, 332, 1567
XmnI	1	1994

*The pGEM®-T Vector has been created by linearizing the pGEM®-5Zf(+) Vector with EcoRV at base 51 and adding a T to both 3´ ends. This site will not be recovered upon ligation of the vector and insert.

Note: The enzymes listed in boldface type are available from Promega.

10.A.pGEM®-T Vector Restriction Enzyme Sites (continued)

Table 4. Restriction Enzymes That Do Not Cut the pGEM®-T Vector.

AccB7I	BbsI	BstEII	FseI	PinAI	SplI
AccIII	**BclI**	Bsu36I	**HindIII**	PmeI	SrfI
Acc65I	**BglII**	**ClaI**	HpaI	PmlI	StuI
AflII	BlpI	CspI	**I-PpoI**	PpuMI	SwaI
AgeI	Bpu1102I	Csp45I	KasI	PshAI	Tth111I
AscI	BsaBI	DraII	**KpnI**	Psp5II	**XbaI**
AvaI	BsaMI	Eco47III	NarI	PspAI	XcmI
AvrII	BsmI	Eco72I	**NheI**	RsrII	**XhoI**
BalI	BsrGI	Eco81I	NruI	**SgfI**	XmaI
BamHI	BssHII	EcoNI	PacI	SgrAI	
BbeI	Bst1107I	**EcoRI**	PaeR7I	**SmaI**	
BbrPI	Bst98I	EheI	PflMI	SnaBI	

Note: The enzymes listed in boldface type are available from Promega.

10.B.pGEM®-T Easy Vector Restriction Enzyme Sites

The sequence of the pGEM®-T Easy Vector is available on the Internet at:
www.promega.com/resources/vector-sequences/
The pGEM®-T Easy Vector has been linearized at base 60 with EcoRV and a T added to both 3´ends. This site will not be recovered upon ligation of the vector and insert. The following tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3´end of the cut DNA (the base to the left of the cut site). Please contact your local Promega Branch Office or Distributor if you identify a discrepancy. In the U.S., contact Technical Services at 1-800-356-9526.

Table 5. Restriction Enzymes that Cut the pGEM®-T Easy Vector 1–5 Times.

Enzyme	# of Sites	Location
AatII	1	20
AccI	1	91
AcyI	2	17, 1947
AflIII	2	114, 517
Alw26I	2	1471, 2247
Alw44I	2	831, 2077
AlwNI	1	933
ApaI	1	14
AspHI	4	109, 835, 1996, 2081
AvaII	2	1548, 1770
BanI	3	261, 1358, 2641
BanII	3	14, 109, 2679
BbuI	1	26
BglI	4	39, 42, 1530, 2848
BsaI	1	1471
BsaAI	1	2604
BsaHI	2	17, 1947
BsaJI	5	37, 46, 256, 677, 2951
Bsp120I	1	10
BspHI	2	1237, 2245
BspMI	1	77
BssSI	2	690, 2074
BstOI	5	257, 545, 666, 679, 2952
BstXI	1	118
BstZI	3	31, 43, 77
Cfr10I	2	1490, 2705
DdeI	4	792, 1201, 1367, 1907
DraI	3	1276, 1295, 1987
DraIII	1	2604
DrdI	2	625, 2559
DsaI	2	37, 46
EagI	3	31, 43, 77
EarI	3	401, 2205, 2893
EclHKI	1	1410
Eco52I	3	31, 43, 77
EcoICRI	1	107
EcoRI	2	52, 70

Enzyme	# of Sites	Location
EcoRV	1	60*
FokI	5	134, 1376, 1557, 1844, 2931
FspI	2	1632, 2855
HaeII	4	395, 765, 2755, 2763
HgaI	4	628, 1206, 1936, 2821
HincII	1	92
HindII	1	92
Hsp92I	2	17, 1947
MaeI	5	65, 1012, 1265, 1600, 2755
MluI	1	114
NaeI	1	2707
NciI	4	30, 897, 1593, 1944
NcoI	1	37
NdeI	1	97
NgoMIV	1	2705
NotI	2	43, 77
NsiI	1	127
NspI	2	26, 521
Ppu10I	1	123
PstI	1	88
PvuI	2	1780, 2876
PvuII	2	341, 2905
RsaI	1	1890
SacI	1	109
SacII	1	49
SalI	1	90
ScaI	1	1890
SinI	2	1548, 1770
SpeI	1	64
SphI	1	26
Sse8387I	1	88
SspI	2	2214, 2396
StyI	1	37
TaqI	5	56, 91, 617, 2061, 2637
TfiI	2	352, 492
VspI	3	288, 347, 1582
XmnI	1	2009

*The pGEM®-T Easy Vector has been linearized at base 60 with EcoRV and a T added to both 3´ends. This site will not be recovered upon ligation of the vector and insert.

Note: The enzymes listed in boldface type are available from Promega.

10.B.pGEM®-T Easy Vector Restriction Enzyme Sites (continued)

Table 6. Restriction Enzymes That Do Not Cut the pGEM®-T Easy Vector.

AccB7I	BbsI	BstEII	**HindIII**	PmeI	SplI
AccIII	**BclI**	Bsu36I	HpaI	PmlI	SrfI
Acc65I	**BglII**	**ClaI**	**I-PpoI**	PpuMI	StuI
AflII	BlpI	CspI	KasI	PshAI	SwaI
AgeI	Bpu1102I	Csp45I	**KpnI**	Psp5II	Tth111I
AscI	BsaBI	DraII	NarI	PspAI	**XbaI**
AvaI	BsaMI	Eco47III	**NheI**	RsrII	XcmI
AvrII	BsmI	Eco72I	NruI	SfiI	**XhoI**
BalI	BsrGI	Eco81I	PacI	**SgfI**	XmaI
BamHI	BssHII	EcoNI	PaeR7I	SgrAI	
BbeI	Bst1107I	EheI	PflMI	**SmaI**	
BbrPI	Bst98I	FseI	PinAI	SnaBI	

Note: The enzymes listed in boldface type are available from Promega.

10.C.Composition of Buffers and Solutions

IPTG stock solution (0.1M)

1.2g IPTG

Add water to 50ml final volume. Filter-sterilize and store at 4°C.

X-Gal (2ml)

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Dissolve in 2ml N,N´-dimethyl-formamide. Cover with aluminum foil and store at –20°C.

LB medium (per liter)

10g Bacto®-tryptone
5g Bacto®-yeast extract
5g NaCl

Adjust pH to 7.0 with NaOH.

LB plates with ampicillin

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 100µg/ml. 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.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5mM IPTG and 80µg/ml X-Gal and pour the plates. Alternatively, 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal may be spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

SOC medium (100ml)

2.0g	Bacto®-tryptone
0.5g	Bacto®-yeast extract
1ml	1M NaCl
0.25ml	1M KCl
1ml	2M Mg^{2+} stock, filter-sterilized
1ml	2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring to 100ml with sterile, distilled water. The final pH should be 7.0.

2M Mg^{2+} stock

20.33g	$MgCl_2 \cdot 6H_2O$
24.65g	$MgSO_4 \cdot 7H_2O$

Add distilled water to 100ml. Filter sterilize.

2X Rapid Ligation Buffer, T4 DNA Ligase (provided)

60mM	Tris-HCl (pH 7.8)
20mM	$MgCl_2$
20mM	DTT
2mM	ATP
10%	polyethylene glycol (MW8000, ACS Grade)

Store in single-use aliquots at –20°C. Avoid multiple freeze-thaw cycles.

TYP broth (per liter)

16g	Bacto®-tryptone
16g	Bacto®-yeast extract
5g	NaCl
2.5g	K_2HPO_4

10.D.Related Products

PCR Cloning Systems

Product	Size	Cat.#
pTargeT™ Mammalian Expression Vector System	20 reactions	A1410

Direct mammalian expression from a T-Vector.

Amplification Products

A partial list of our amplification products is given on the next page. Please visit our Web site at: **www.promega.com/products/pcr/** to see a complete list.

10.D. Related Products (continued)

Thermostable DNA Polymerases

Product	Size	Cat.#
GoTaq® Hot Start Polymerase	100u	M5001
GoTaq® DNA Polymerase	100u	M3171[1], M3001[2]
GoTaq® Flexi DNA Polymerase (allows optimization of Mg^{2+} concentration in reaction)	100u	M8301[1], M8291[2]

Additional sizes available.

[1]Cat.# M3171 & M8301 are available in Europe or through Distributors supported by Promega European Branch Offices.

[2]Cat.# M3001 & M8291 are available in all other countries, including the United States.

PCR Master Mixes

Product	Size	Cat.#
GoTaq® Hot Start Green Master Mix	100 reactions	M5122
	1,000 reactions	M5123
GoTaq® Hot Start Colorless Master Mix	100 reactions	M5132
	1,000 reactions	M5133
GoTaq® Green Master Mix	100 reactions	M7112[1], M7122[2]
	1,000 reactions	M7113[1], M7123[2]
GoTaq® Colorless Master Mix	100 reactions	M7142[1], M7132[2]
	1,000 reactions	M7143[1], M7133[2]

GoTaq® Master Mixes are premixed solutions containing GoTaq® DNA Polymerase, GoTaq® Reaction Buffer (Green or Colorless), dNTPs and Mg^{2+}.

[1]Cat.# M7112, M7113, M7142 & M7143 are available in Europe or through Distributors supported by Promega European Branch Offices.

[2]Cat.# M7122, M7123, M7132 & M7133 are available in all other countries, including the United States.

PCR Purification Systems

Product	Size	Cat.#
Wizard® SV Gel and PCR Clean-Up System	50 preps	A9281
	250 preps	A9282
Wizard® SV 96 PCR Clean-Up System	1 × 96 preps	A9340

Additional sizes available.

Competent Cells

Product	Size	Cat.#
JM109 Competent Cells, >10^8cfu/µg	5 × 200µl	L2001
Single Step (KRX) Competent Cells	20 × 50µl	L3002

RT-PCR Systems

Product	Size	Cat.#
Access RT-PCR System	100 reactions	A1250
AccessQuick™ RT-PCR System	100 reactions	A1702
	500 reactions	A1703
ImProm-II™ Reverse Transcription System	100 reactions	A3800

Available in additional sizes.

dNTPs

Product	Size	Cat.#
PCR Nucleotide Mix (10mM each)	200µl	C1141
	1,000µl	C1145
dATP, dCTP, dGTP, dTTP, each at 100mM	10µmol of each	U1330
dATP, dCTP, dGTP, dTTP, each at 100mM	40µmol of each	U1240
dATP, dCTP, dGTP, dTTP, each at 100mM	200µmol of each	U1410

Accessory Products

Product	Size	Cat.#
X-Gal	100mg (50mg/ml)	V3941
IPTG, Dioxane-Free	1g	V3955
	5g	V3951

10.E.Summary of Changes

The following changes were made to the 10/21 revision of this document:

1. Updated Sections 5.B, 5.C, 5.D, 10.A and 10.B.
2. Updated cover page.

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Promega Corporation · 2800 Woods Hollow Road · Madison, WI 53711-5399 USA · Toll Free in USA 800-356-9526 · 608-274-4330 · Fax 608-277-2516
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