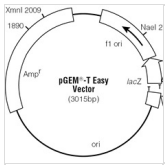

Version 2

Working

Oct 02, 2018

PCR Cloning with Blue/White Selection and Easy Insert Excision

Version 2

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ABSTRACT

Protocol for PCR Cloning with Blue/White Selection and Easy Insert Excision using pGEM®-T Easy Vector Systems.

(For use with A3600, A3610, A1360, or A1380.)

EXTERNAL LINK

https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol.pdf?la=en

pgem-t-and-pgem-t-easy-vector-systems-protocol.pdf

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

1. Introduction

1.A. Vector Features

T-Overhangs for Easy PCR Cloning: The pGEM®-T and pGEM®-T 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 x 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 x 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)

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

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

3.A. Ligation Protocol

See steps for detailed ligation protocol.

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 3015bp.

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

Use high-efficiency competent cells (≥1 x 10^8cfu/µg DNA) for transformations. Ligation of fragments with a singlebase overhang can be inefficient, so it is essential to use cells with a transformation efficiency of1 x 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'L, *tra*D36, *pro*AB, *lac*I^qZΔM15] (4).

4.A. Transformation Protocol

See steps for detaile transformation protocol.

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?

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

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'L-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.

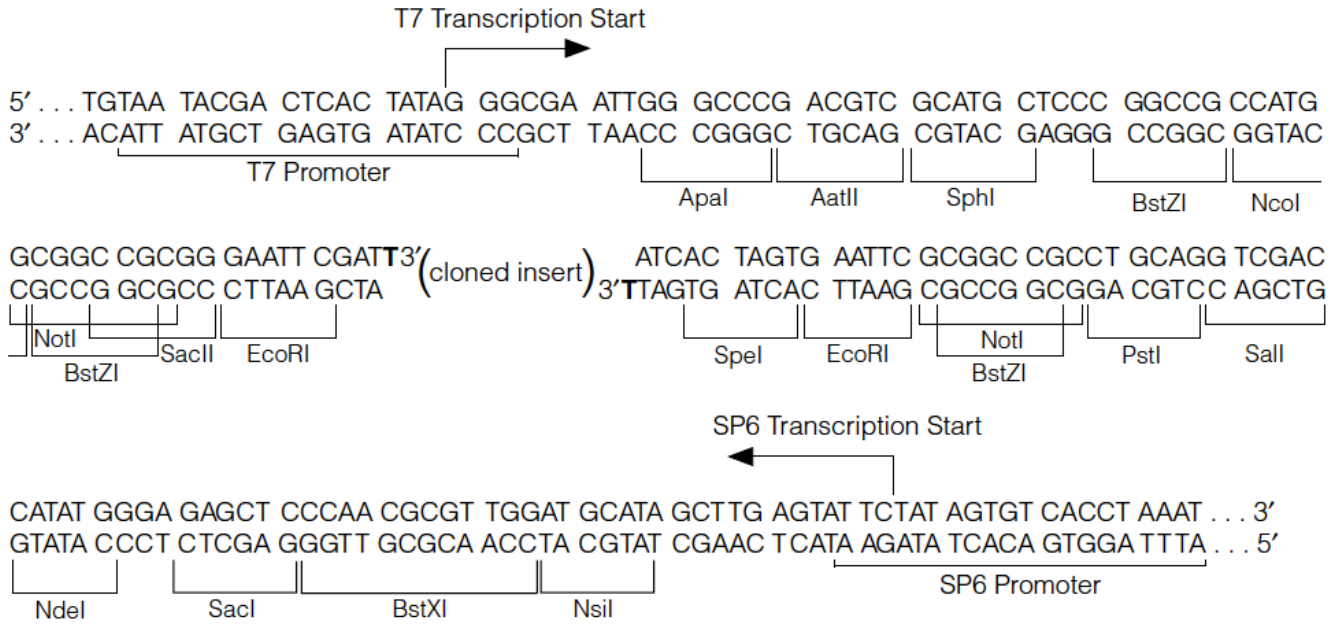
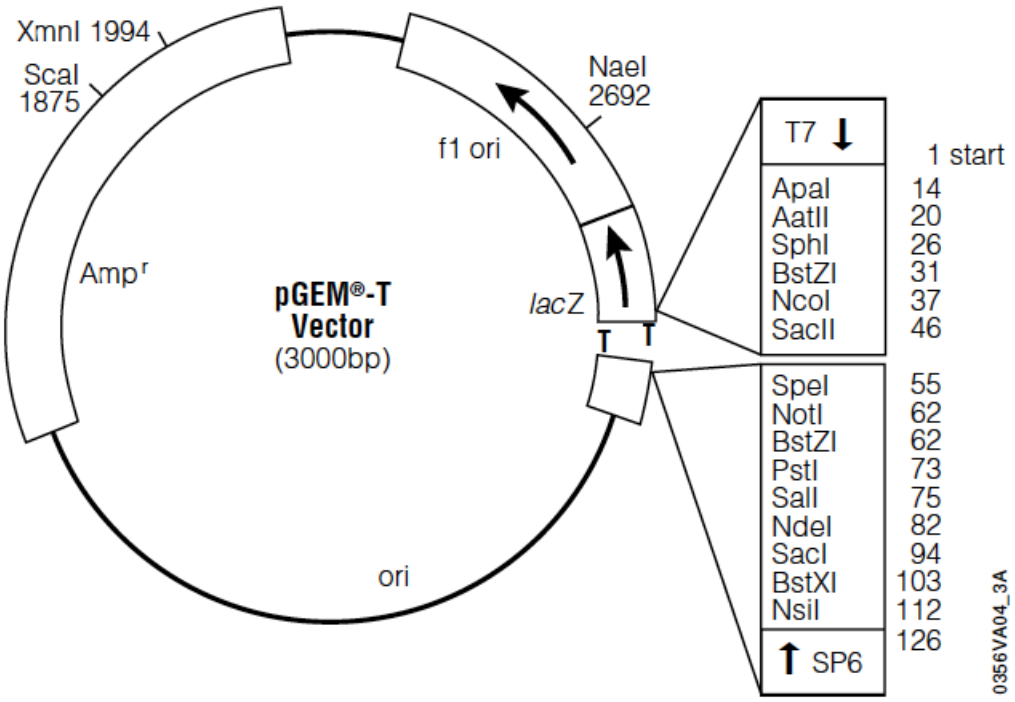


Figure 1. The promoter and multiple cloning sequence of the pGEM®-T Vector. The top strand corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

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 SP6 Promoter Primer (Cat.# Q5011), T7 Promoter Primer (Cat.# Q5021), pUC/M13 Forward Primer (Cat.# Q5601), or pUC/M13 Reverse Primer (Cat.# Q5421).

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/vectors/
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.

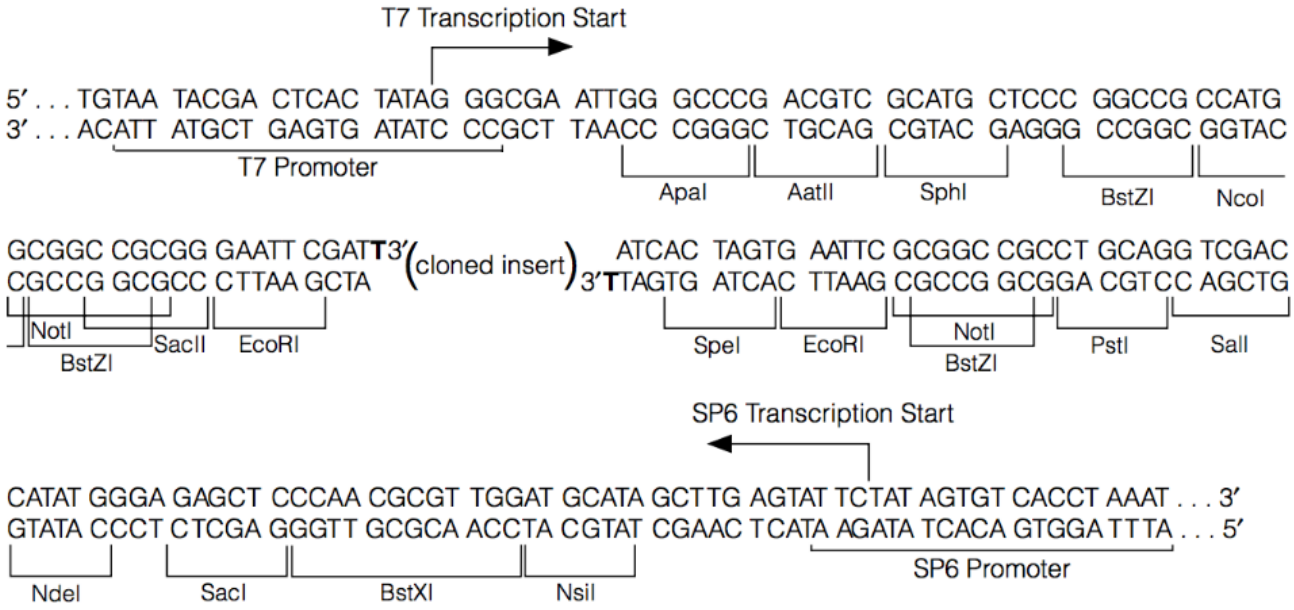
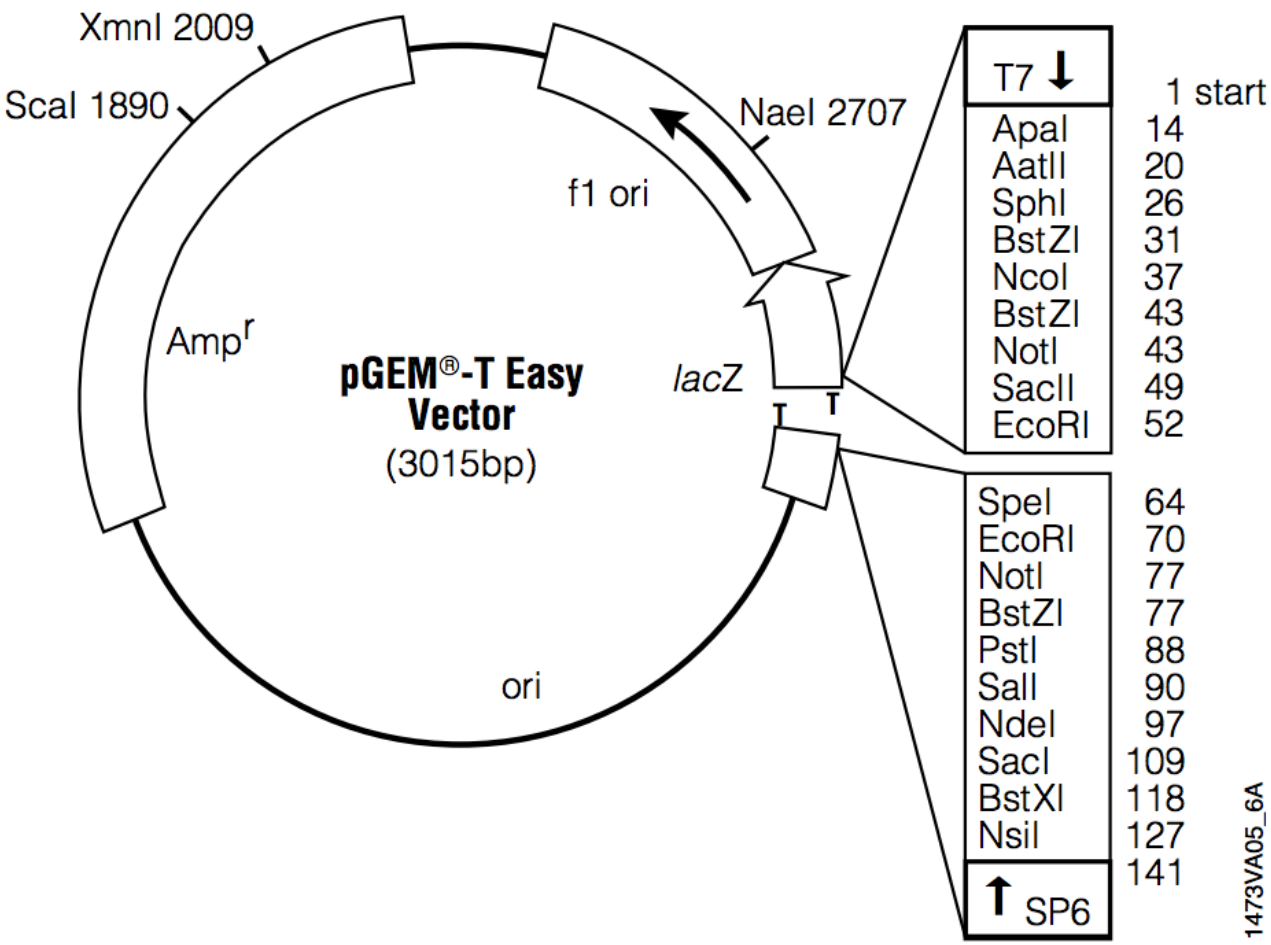


Figure 2. The promoter and multiple cloning sequence of the pGEM®-T Easy Vector. The top strand shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

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 SP6 Promoter Primer (Cat.# Q5011), T7 Promoter Primer (Cat.# Q5021), pUC/M13 Forward Primer (Cat.# Q5601), or pUC/M13 Reverse Primer (Cat.# Q5421).

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.

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.

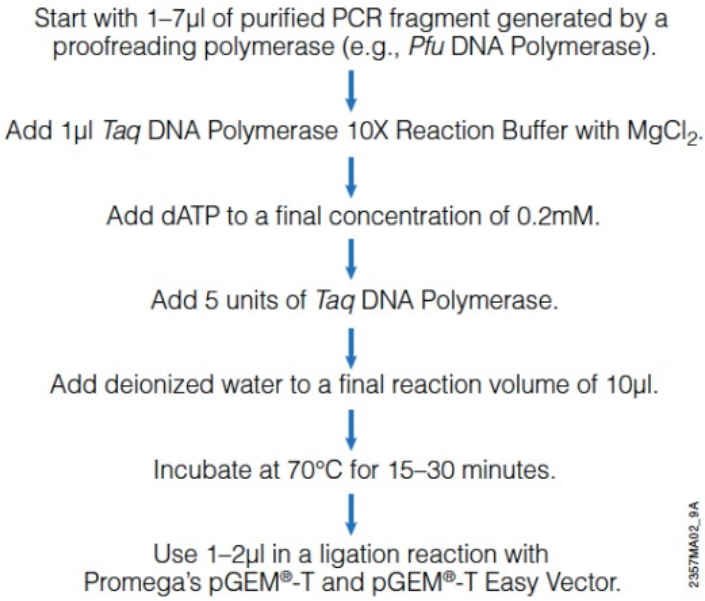


Figure 3. An A-tailing procedure for blunt-ended PCR fragments purified with the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) and used in T-vector cloning.

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 x 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 x 10^8cfu/µg DNA. (Under these conditions, cells that have an efficiency of 1 x 10^7cfu/µg DNA would yield 1–3 blue colonies, and cells with a transformation efficiency of 1 x 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 than1 x 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 x 10^8cfu/µg DNA.

8. Troubleshooting

Please see attached full manuscript for detailed troubleshooting.

9. References

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10. Appendix

Please see attached full manuscript for appendix section.

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for hazard information.

Ligation

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 follows:

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.

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.

NOTE
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.

NOTE
Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the maximum number of transformants.

NOTE
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.

3 Mix the reactions by pipetting.

4 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.
01:00:00 **Incubation**

5 Analyze an aliquot of the PCR reaction 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).

NOTE
Cleanup 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.

Transformation

6 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.

7 Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom. Add 2µl of each ligation reaction to a sterile (17 x100mm) polypropylene tube or a 1.5ml microcentrifuge tube on ice. Set up another tube on ice with 0.1ng uncut plasmid for determination of the transformation efficiency of the competent cells.

> NOTE
> Observations have found that use of larger (17 x 100mm) polypropylene tubes (e.g., Falcon™ Cat.# 2059) increases transformation efficiency. Tubes from some manufacturers bind DNA and should be avoided.

8 Remove tube(s) of frozen JM109 High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes).

00:05:00 **Ice bath**

9 Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.

10 **Carefully** transfer 50µl of cells into each tube prepared in Step 7 (use 100µl of cells for determination of transformation efficiency).

50 µl **Cells**

11 **Gently** flick the tubes to mix and place them on ice for 20 minutes.

00:20:00 **Tubes on ice**

12 Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C (**do not shake**).

00:00:45 **Heat-shock**

42 °C **Water bath**

13 Immediately return the tubes to ice for 2 minutes.

00:02:00 **Tubes on ice**

14 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).

950 µl **SOC medium**

900 µl **SOC medium**

15 Incubate for 1.5 hours at 37°C with shaking (~150rpm).

01:30:00 **Incubation**

37 °C **Incubation**

16 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 x g for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of two plates.

100 µl of each transformation culture

00:10:00 Centrifugation

17 Incubate the plates overnight (16–24 hours) at 37°C.

NOTE

If 100µl is plated, approximately 100 colonies per plate are routinely seen using competent cells that are 1 x 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.

NOTE

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.

NOTE

Blue color will become darker after the plate has been stored overnight at 4°C.

16:00:00 Incubation

37 °C Incubation