Rapid Ligation for the pGEM®-T and pGEM®-T Easy Vector Systems

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The pGEM®-T and pGEM®-T Easy Systems are now provided with a new 2X Rapid Ligation Buffer that allows the user to perform ligation reactions in as little as one hour. In this study the performance of the 2X Rapid Ligation Buffer is compared with that of the previously supplied T4 DNA Ligase 10X Buffer in both one-hour and 16-hour ligation reactions. The effects of incubation time, ligation reaction temperature, and insert:vector ratio on cloning efficiencies are also evaluated using the 2X Rapid Ligation Buffer.

Introduction

The pGEM®-T and pGEM®-T Easy Vector Systems[a,b] facilitate the cloning of PCR[c] products by providing linear vectors that have a single thymidine extension at the 3´-ends. These terminal thymidines are complementary to the nontemplate-derived 3´ adenosine residues that are added to double-stranded DNA products by many nonproofreading DNA polymerases (1). The single complementary base pair that forms between the vector and PCR product provides the conditions for an efficient ligation reaction. The pGEM®-T and pGEM®-T Easy Vector Systems now contain a new buffer that shortens the incubation time required for ligation reactions. This new 2X Rapid Ligation Buffer for T4 DNA Ligase can be used to perform one-hour ligation reactions, a great savings in time compared to the overnight ligation recommended for the buffer previously supplied with these systems. In this study we have examined some of the parameters of ligation reactions using the new Rapid Ligation Buffer.

Cloning Efficiency

The cloning efficiency of the 2X Rapid Ligation Buffer was compared to the cloning efficiency of the T4 DNA Ligase 10X Buffer previously provided with the pGEM®-T and pGEM®-T Easy Vector Systems. The results in Table 1 clearly show that cloning efficiencies are greatly improved with the new buffer when the ligation reaction is incubated either at 24°C for one hour or at 4°C for 16 hours.

Table 1. Comparison of Cloning Efficiencies Using the 2X Rapid Ligation Buffer and the T4 DNA Ligase 10X Buffer.

Reaction Conditions	2X Rapid Ligation Buffer	T4 DNA Ligase 10X Buffer
1 hour at 24°C	259	29
16 hours at 4°C	580	164

Cloning efficiency was calculated as the number of white colonies/ng vector DNA from a ligation containing 8ng Control Insert DNA (542bp) and 50ng pGEM®-T Easy Vector.

Each value is the average of three independent ligation reactions, followed by transformation of 2µl into High Efficiency JM109 Competent Cells.

Time and Temperature of Ligation Reaction

The recommended time and temperature for incubation of ligation reactions using the 2X Rapid Ligation Buffer with either the pGEM®-T or the pGEM®-T Easy Vector Systems is one hour at room temperature (24°C) or, for greater numbers of recombinants, overnight at 4°C. Table 2 shows the results obtained from ligation reactions incubated for various times at 24°C and 4°C. In this experiment the Control Insert DNA supplied with the vector systems was ligated to the pGEM®-T Easy Vector at a 1:1 insert:vector molar ratio (2). Some recombinants were produced after only 15 minutes at either temperature; however, for incubation times less than two hours more recombinants were generated at 24°C than at 4°C. For incubation times greater than two hours the cloning efficiency was better at 4°C than at 24°C. A 16-hour incubation at 4°C generally produced the maximum number of recombinants. An incubation temperature >28°C is not recommended because it results in decreased numbers of recombinants and an increase in the number of clones containing recircularized vector (data not shown).

Table 2. The Relationship Between Incubation Time, Ligation Reaction Temperature and Cloning Efficiency using the 2X Rapid Ligation Buffer.

Time (hours)	Ligation Reaction Temperature			
	24°C		4°C	
	Cloning Efficiency	Percent Recombinants	Cloning Efficiency	Percent Recombinants
0.25	72	89	50	91
0.5	181	94	119	91
1	259	93	172	92
2	275	93	270	92
3	269	93	363	92
6	368	91	341	93
16	422	93	1344	95

Cloning efficiency and reaction conditions are as defined in Table 1. Percent recombinants were calculated as the number of white colonies divided by the total number of colonies.

INSERT:VECTOR RATIO

The pGEM®-T and pGEM®-T Easy Vector Systems containing the 2X Rapid Ligation Buffer may be used to clone different sized PCR products generated from various templates. As in all cloning experiments, it may be necessary to optimize the insert:vector molar ratio used in each ligation reaction. In general we recommend 1:1 to 10:1 insert:vector ratios; however, in some cases ratios outside this range will be optimal. Table 3 shows the cloning efficiency of different sized PCR products used in ligation reactions at various insert:vector ratios. The results indicate that a wide range of insert:vector ratios may provide acceptable results. Each of the PCR products used for cloning had different optimal insert:vector ratios. It is likely that the optimal ratio is determined by a number of factors that influence cloning efficiency, one of the most important being the method of purification following amplification. All of the PCR fragments used in this study were amplified from either human genomic DNA or *Pseudomonas aeruginosa* genomic DNA using *Taq* DNA Polymerase. The Wizard® PCR Preps DNA Purification System[(d)] (Cat.# A7170) was used to purify the PCR products prior to cloning. In this experiment the larger (2.4kb) product had a significantly lower cloning efficiency than smaller (<1.8kb) PCR products. The sequence of a given DNA fragment may also have a significant effect on cloning efficiency.

Table 3. Cloning Efficiencies of Various PCR Products into the pGEM®-T Easy Vector.

Insert Size (bp)	Cloning Efficiency[1] (Insert:Vector Molar Ratio)					
	1:1	2:1	3:1	5:1	8:1	10:1
200	56	331	205	369	632	964
400	80	370	292	396	341	459
1,000	163	169	272	650	746	720
1,800	916	1052	608	478	356	346
2,400	128	160	72	50	25	13
Control Insert						
(542)	287					

Each value represents the result obtained from transformation of 2µl of a ligation reaction containing 50ng pGEM®-T Easy Vector into High Efficiency JM109 Competent Cells (12×10^8 transformants/µg DNA). The presence of the DNA fragment in the vector was confirmed by PCR from a representative number of recombinant colonies.

[1]Cloning efficiency was calculated as the number of colonies/ng vector DNA (200 and 1,000bp inserts); as

the number of white colonies/ng vector DNA (400 and 2,400bp inserts and the Control Insert); or as the number of white plus pale blue colonies/ng vector DNA (1,800bp insert).

It is interesting to note that the PCR products used in this study gave different patterns of beta-galactosidase expression when inserted into the pGEM®-T Easy Vector. The pGEM®-T Vectors carry a segment of the *lacZ* gene that encodes the amino terminal fragment of beta-galactosidase. Expression of this fragment will complement certain *E. coli lacZ* mutations. The multiple cloning site of the pGEM®-T Vectors is within the *lacZ* coding region on the plasmid and inserted fragments will interrupt the expression of the *lacZ* sequences. In the presence of an inducer of the *lac* operon (IPTG) and an indicator substrate (X-Gal) colonies expressing *lacZ* sequences from the plasmid are blue, while those containing plasmids with inserts are white. This is a common method for detecting recombinant plasmids. However, this method does not always effectively distinguish between recombinant and nonrecombinant plasmids since it is possible to get expression of *lacZ* from the vector even when a DNA fragment has been inserted into the coding region. In this study only two of the five PCR fragments cloned into the pGEM®-T Easy Vector yielded recombinants that resulted in only white colonies. These were a 400bp fragment from the human *aromatase* gene and a 2.4kb fragment from the *P. aeruginosa trp* A and B genes. The colonies containing plasmids with a 200bp region of the human Y chromosome or a 1.0kb segment of the *P. aeruginosa gro*EL gene inserted into the vector were blue and pale blue (no white colonies). There were pale blue and white colonies carrying plasmids with a 1.8kb segment of the human *adenomatous polyposis coli* (*APC*) gene. In each case the two colony phenotypes were present in equal numbers, suggesting that insertion into the plasmid in different orientations results in different expression levels of the *lacZ* gene. This has been verified for the 1.8kb fragment of human *APC* gene by sequence analysis (see the article on page 10).

When cloning PCR products into the pGEM®-T Vectors it is important to realize that blue/white screening for beta-galactosidase expression may not reveal all recombinants. In some cases the insertion of a DNA fragment in one or both orientations may not completely interrupt expression of the plasmid *lacZ* sequences. The presence of a large number of blue colonies after transformation with a ligation mix may be indicative that some of the blue colonies contain recombinant plasmids. In such situations it is helpful to perform a negative control (no insert DNA) ligation to determine the expected background of blue colonies. In some cases colonies containing recombinant plasmids will be a paler shade of blue than those without the insert, providing a means to distinguish blue colonies carrying recombinant plasmids from those containing only vector DNA.

Summary

The new 2X Rapid Ligation Buffer for use with T4 DNA Ligase provided with the pGEM®-T and pGEM®-T Easy Vector Systems allows investigators flexibility in determining the time and temperature of ligation reaction incubation. The recommended one-hour incubation at room temperature will produce >100 white colonies/ng vector DNA when using the Control Insert DNA supplied with these systems. Shorter or longer incubation times can be used; for incubation times greater than one hour a temperature of 4°C is recommended. The 2X Rapid Ligation Buffer can be used to clone various sized PCR products from different templates into the pGEM®-T Vectors. We used an insert:vector ratio of 1:1 to 10:1 for the PCR products; however, the optimal insert:vector ratio is different for each PCR product.

Acknowledgement

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References

1. Clark, J.M. (1988) *Nucl. Acids Res.* **16**, 9677.
2. *pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual* #TM042.

Ordering Information

Product	Size	Cat.#
pGEM®-T Vector System I	20 reactions	A3600
pGEM®-T Vector System II	20 reactions	A3610
pGEM®-T Easy Vector System I	20 reactions	A1360
pGEM®-T Easy Vector System II	20 reactions	A1380
Wizard® PCR Preps DNA Purification System	50 preps	A7170
JM109 Competent Cells, High Efficiency >10^8cfu/µg (5 x 200µl)	1ml	L2001

The pGEM®-T and pGEM®-T Easy Vector Systems II include JM109 Competent Cells, High Efficiency.

[a]U.S. Pat. No. 4,766,072.

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

[c]The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

[d]Licensed under U.S. Pat. No. 5,075,430.
