Technical Manual

pTARGET™ Mammalian Expression Vector System

INSTRUCTIONS FOR USE OF PRODUCT A1410.

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pTARGET™ Mammalian Expression Vector System

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I. Description

The pTARGET™ Mammalian Expression Vector System[a,b,c] is a convenient system for cloning PCR[d] products and for expression of cloned PCR products in mammalian cells. The vector is prepared by cutting the pTARGET™ Vector with *Eco*R V and adding a 3´ terminal thymidine to both ends. These single 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid in two ways. First the overhangs prevent recircularization of the vector; second they provide a compatible overhang for PCR products generated by certain thermostable polymerases (1,2). As

summarized in Table 1, these polymerases often add a single deoxyadenosine in a template-independent fashion to the 3′-ends of amplified fragments (3,4). The pTARGET™ Vector also contains a modified version of the coding sequence of the α-peptide of β-galactosidase, allowing blue/white recombinant screening.

The pTARGET™ Vector carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter[c] region to promote constitutive expression of cloned DNA inserts in mammalian cells. This vector also contains the neomycin phosphotransferase gene, a selectable marker for mammalian cells. The pTARGET™ Vector can be used for transient expression or for stable expression by selecting transfected cells with the antibiotic G-418.

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

	Thermostable DNA Polymerase						
Characteristic	***Taq*/ AmpliTaq®**	***Tfl***	***Tth***	**Vent$_R$®/ (*Tli*)**	**Deep Vent$_R$®**	***Pfu***	***Pwo***
Resulting DNA ends	3′ A	3′ A	3′ A	>95% Blunt	>95% 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

II. Vector Maps

II.A. pTARGET™ Vector Multiple Cloning Region

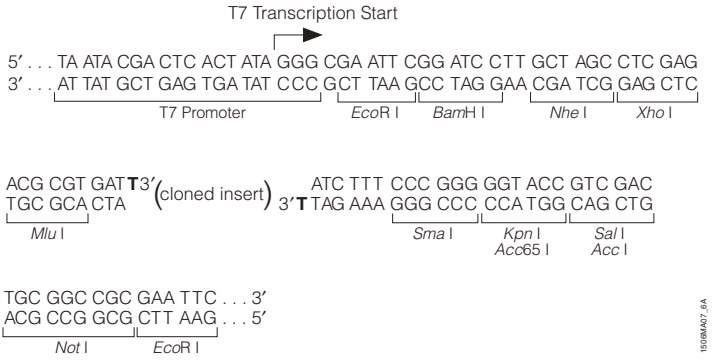

Figure 1. The promoter and multiple cloning region of the pTARGET™ Vector. The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase. The top strand shown is the same as the ssDNA produced by this vector. The codons of the *lac*Zα region within the multiple cloning region are indicated by the spacing of the nucleotides.

II.B. pTARGET™ Vector Map and Sequence Reference Points

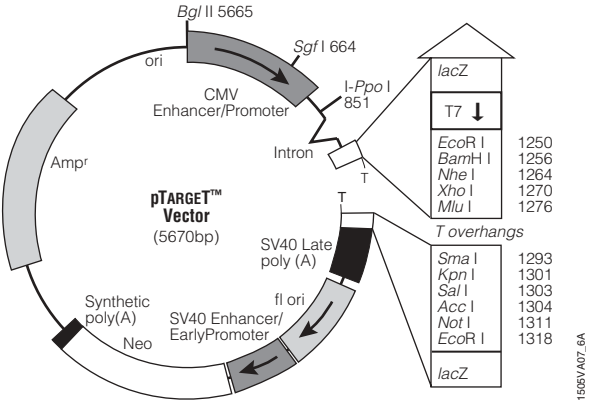

Figure 2. Vector circle map and sequence reference points.

pTARGET™ Vector sequence reference points:

Cytomegalovirus immediate early enhancer	1–659
Cytomegalovirus immediate early promoter	669–750
Chimeric intron	890–1022
pTARGET™ Sequencing Primer	1367–1344
*lac*Zα start codon	1377
*lac*Zα stop codon	1053
lac operon sequences	1066–1226, 1363–1499
lac operator	1397–1413
T7 promoter (–17 to +3)	1229–1248
Multiple cloning region	1250–1323
SV40 late polyadenylation signal	1535–1755
Phage f1 region	1798–2252
Neomycin selectable marker	
SV40 enhancer and early promoter	2260–2630
SV40 minimum origin of replication	2528–2593
coding region of neomycin phosphotransferase	2675–3469
synthetic polyadenylation signal	3533–3581
β-lactamase (Amp^r) coding region	3978–4838

III. Product Components and Storage Conditions

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

Includes:

- 1.2µg pTARGET™ Vector (60ng/µl)
- 12µl Control Insert DNA (4ng/µl)
- 100u T4 DNA Ligase
- 200µl T4 DNA Ligase 10X Buffer
- 1.2ml JM109 Competent Cells, High Efficiency (6 × 200µl)
- 1 Protocol

Storage Conditions: Store Competent Cells at –70°C. See the expiration date on the tube label. All other components can be stored at –20°C or –70°C. T4 DNA Ligase 10X Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaws by making smaller aliquots of the buffer.

IV. Ligation and Transformation Protocols

IV.A. Ligations Using pTARGET™ Vector

1. Briefly centrifuge the pTARGET™ Vector and Control Insert DNA tubes to collect the contents at the bottom of the tube.
2. Set up ligation reactions as described below.

   Note: Use 0.5ml tubes known to have low DNA-binding properties (e.g., Sarstedt Cat.# 72687005).

	Standard Reaction	Positive Control	Background Control
T4 DNA Ligase 10X Buffer	1µl	1µl	1µl
pTARGET™ Vector (60ng)	1µl	1µl	1µl
PCR product	Xµl*	–	–
Control Insert DNA (8ng)	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
deionized water to a final volume of	10µl	10µl	10µl

*Molar ratio of PCR product to pTARGET™ Vector may require optimization (see Section V.C).

3. Incubate overnight at 4°C. Shorter incubation times (3-hour minimum) may be used but may result in fewer colonies.

Notes:

1. Use only the T4 DNA Ligase supplied with this system in performing pTARGET™ Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activity, which may remove the terminal thymidines from the vector.
2. T4 DNA Ligase 10X Buffer contains ATP, which can degrade with temperature fluctuations. Avoid multiple freeze-thaw cycles by making smaller aliquots of the buffer.
3. Vortex the thawed T4 DNA Ligase 10X Buffer before use.
4. When using T4 DNA Ligase 10X Buffer, low temperature ligations are necessary for annealing single-base overhangs. Ligation temperatures higher than 15°C may significantly reduce the number of recombinants.

IV.B. Transformations Using pTARGET™ Vector

Use only high-efficiency competent cells (≥1 × 10^8cfu/µg DNA) for transformations. The ligation efficiency of fragments with a single-base overhang can be inefficient; use competent cells that have transformation efficiencies of 1 × 10^8cfu/µg DNA (or higher) in order to obtain a reasonable number of colonies (see Section V.E).

Note: The JM109 competent cells supplied with the pTARGET™ System are ready for blue/white selection. No further selection on M9 plates is necessary.

We highly recommend using JM109 High Efficiency Competent Cells (Cat.# L2001). In our experience, other strains such as DH5α™ and XL1-Blue have a much higher frequency of background white colonies than JM109. If you make your own competent cells, grow and maintain JM109 cells on M9 minimal medium plates supplemented with thiamine hydrochloride prior to the preparation of competent cells. This will ensure that the cells retain the F′ episome. The episome contains the genetic information for proline biosynthesis, a nutritional requirement for growth on minimal medium. The F′ episome also includes *lac*I^{q}ZΔM15, which is required in the blue/white color screening process. Selection for transformants should be on LB/ampicillin/IPTG/X-Gal plates (see Section VII.C). For best results, do not use plates more than 30 days old.

The genotype of JM109 is *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_K–,m_K+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15] (5).

IV.B. Transformations Using pTARGET™ Vector (continued)

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

- LB plates with ampicillin/IPTG/X-Gal
- SOC medium
- 0.1–1.0ng uncut plasmid for determination of transformation efficiency

1. Prepare two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency (see Section V.E). Equilibrate the plates to room temperature prior to plating (Step 10).
2. Remove frozen JM109 High Efficiency Competent Cells from –70°C storage and place on ice until just thawed (about 5 minutes). Mix the cells by **gently** flicking the tube. Prechill tubes on ice for each transformation reaction.
3. For each ligation reaction and control, carefully transfer 50µl of cells into each tube prepared in Step 2 (100µl of cells for control to determine transformation efficiency). Avoid excessive pipetting because the cells are very fragile.
4. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom of the tube. Add 2µl of each ligation reaction to a sterile 1.5ml microcentrifuge tube on ice. Add 0.1–1.0ng uncut plasmid to the tube containing the 100µl cells for determining the transformation efficiency of the competent cells (see Section V.E).

   Note: In our experience, using larger 17 × 100mm polypropylene tubes (e.g., Falcon Cat.# 2059) can increase transformation efficiency. Tubes from some manufacturers bind DNA. These tubes should be avoided.
5. **Gently** flick the tubes to mix and place them on ice for 20 minutes.
6. Heat shock the cells for 45–50 seconds at exactly 42°C in a water bath. **Do Not Shake.**
7. Immediately return the tubes to ice for 2 minutes.
8. Add **950**µl of room temperature SOC medium to each sample tube and 900µl SOC medium to the transformation control tubes (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 reaction onto duplicate antibiotic plates. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × *g* for 10 minutes, resuspended in 100µl of SOC medium and plated on one plate. For the transformation control, we recommend plating 100µl a 1:10 dilution with SOC medium.
11. Incubate the plates overnight (16–24 hours) at 37°C. We routinely see approximately 100 colonies per plate when using competent cells that are 1×10^8cfu/µg DNA if a 100µl aliquot is plated. Longer incubations or

storing plates at 4°C (after 37°C overnight incubation) may facilitate blue/white screening. White colonies generally contain inserts, but inserts may also be present in blue colonies. Please see Section V.D for more information.

IV.C. Isolating Recombinant Plasmid DNA

A convenient and reliable method for isolating plasmid DNA is the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330). Alternatively, a standard plasmid miniprep procedure, such as the one described in the *Protocols and Applications Guide* (6), may be used.

The Wizard® *Plus* SV Minipreps DNA System, a silica membrane-based spin or vacuum-driven system, isolates plasmid DNA that is ready for direct use in molecular biology applications, including restriction enzyme digestion and automated fluorescent sequencing.

IV.D. Transfecting Mammalian Cells with the pTARGET™ Vector

The pTARGET™ Vector can be used for both transient and stable expression of genes. Transfection of DNA into mammalian cells may be mediated by cationic lipids (7,8), calcium phosphate (9,10), DEAE-dextran (11–13), polybrene-DMSO (14,15) or electroporation (16,17). Transfection systems based on the cationic lipids (TransFast™ Transfection Reagent, Transfectam® Reagent and Tfx™ Reagents), calcium phosphate and DEAE-dextran (ProFection® Mammalian Transfection Systems) are available from Promega. For more information on using TransFast™ Transfection Reagent, please request the *TransFast™ Transfection Reagent Technical Bulletin* (#TB260). For more information and a protocol for the Transfectam® Reagent, please request the *Transfectam® Reagent for the Transfection of Eukaryotic Cells Technical Bulletin* (#TB116). For more information on transfection using the Tfx™ Reagents, please request the *Tfx™-20 and Tfx™-50 Reagents for the Transfection of Eukaryotic Cells Technical Bulletin* (#TB216). For transfection procedures using calcium phosphate or DEAE-dextran, please request the *ProFection® Mammalian Transfection Systems Technical Manual* (#TM012).

Note: Visit the Transfection Assistant for help in selecting a transfection reagent: **www.promega.com/transfectionasst/**

For stable expression, the transfected cells must be selected with the antibiotic G-418 (Cat.# V7981). Following transfection, seed the cells at a low density and apply the G-418 antibiotic to the medium at a concentration between 100µg/ml and 1mg/ml. The concentration of G-418 required to select and maintain drug resistance depends on the cell type and growth rate. In general, mammalian cells require a concentration of 400–600µg/ml of active G-418 for selection and 200–400µg/ml of active G-418 for maintenance of stable transformants (18). A stock solution of 100mg/ml of G-418 can be made up in 40mM HEPES (pH 7.3), PBS or water. (Store the stock solution of G-418 at –20°C.) For effective selection, the cells should be subconfluent, since confluent, nongrowing cells are very resistant to the effects of G-418. Change

the medium every 3 days until drug-resistant clones appear (2–5 weeks, depending on the cell type). Death of cells not expressing neomycin phosphotransferase may require 3–9 days following addition of G-418. Cell strains vary in the level of resistance to antibiotics, so the level of resistance of a particular cell strain must be tested before attempting stable selection. A "kill curve" will determine the minimum concentration of antibiotic needed to kill nontransfected cells.

Note: Prepare a control plate for all selection experiments by treating untransfected cells with G-418 in medium under the experimental conditions. This control plate will indicate whether the conditions for antibiotic selection were sufficiently stringent to eliminate cells not expressing neomycin phosphotransferase.

V. General Considerations for Cloning PCR Products

V.A. Generating PCR Product

For efficient expression of the gene, the first ATG in the 5´-region of the mRNA transcript should be the translational start site, since upstream, out-of-frame ATGs can significantly reduce the rate of translation of genes in eukaryotes (19). Another consideration in designing PCR products is the inclusion of the consensus sequence (–9GCCGCC($^{A}/_{G}$)CCAUGG+4) surrounding the initiation of translation. Kozak reported that efficient translation is obtained as long as the –3 position relative to the ATG contains a purine base (19). However, if the –3 position does not contain a purine, efficient translation can still be obtained if a guanine is at the +4 position.

The fidelity of the PCR depends upon the cycle number, the polymerase and the reaction conditions (20). Because successful expression of the inserted gene product depends upon integrity of the sequence, optimizing PCR fidelity is important (20). The DNA sequence cloned into the pTARGET™ Vector should also be verified. To sequence upstream of the multiple cloning region toward the insert, use the T7 Promoter Primer (Cat.# Q5021). To sequence downstream of the multiple cloning region toward the insert, use the pTARGET™ Sequencing Primer (Cat.# Q4461).

V.B. PCR Product Purity

Analyze an aliquot of the PCR amplification product on an agarose gel before using it in the ligation reaction. Several convenient methods exist for purifying the PCR products from an agarose gel. The Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) is designed to extract and purify DNA fragments of 100bp–10kb from standard or low-melt agarose gels. During gel purification, use longwave ultraviolet light (366nm) to visualize the PCR product so that the formation of pyrimidine dimers is minimized. If gel separation is not required, the Wizard® SV Gel and PCR Clean-Up System can be used to purify the bands of interest directly from the reaction mix.

Even if distinct bands of the expected size are observed, primer-dimers should be removed. Using crude PCR product may also produce successful ligations; however, the number of white colonies containing the relevant insert may be reduced because of preferential incorporation of primer-dimers or other extraneous reaction products. Therefore, you may need to screen numerous colonies in order to identify clones that contain the insert of interest when you use crude product.

V.C. Optimizing Insert:Vector Molar Ratios

The pTARGET™ Vector System uses a 1.4:1 molar ratio of the Control Insert DNA to the pTARGET™ Vector. Ratios of 8:1 to 1:8 have been used successfully. If initial experiments with your PCR product are suboptimal, you may need to optimize the ratio of insert to vector. Ratios from 3:1 to 1:3 provide good initial parameters. Estimate the concentration of PCR product by comparing it to DNA mass standards on a gel or by using a fluorescent assay (21). The pTARGET™ Vector is 5.67kb in size and is supplied at a concentration of 60ng/µl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation:

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

Sufficient pTARGET™ Vector is provided to vary insert:vector ratios as recommended and to perform control reactions.

Example of insert:vector ratio calculation:

How much 0.5kb PCR product should be added to a ligation in which 60ng of 5.67kb vector will be used if a 3:1 insert:vector molar ratio is desired?

$$\frac{60\text{ng vector} \times 0.5\text{kb insert}}{5.67\text{kb vector}} \times 3/1 = 15.9\text{ng insert}$$

Note: Using the same parameters for a 1:1 insert:vector molar ratio, 5.3ng of a 0.5kb insert would be required.

V.D. Screening Transformants for Inserts

Successful cloning of an insert in the pTARGET™ Vector interrupts the coding sequence of the α-peptide of β-galactosidase. Recombinant clones can usually be identified by color screening on indicator plates. However, the characteristics of PCR products cloned into the pTARGET™ Vector can significantly affect the ratio of blue:white colonies obtained following transformation of competent cells. Clones that contain PCR products produce white colonies in most cases, 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.

V.D. Screening Transformants for Inserts (continued)

Even if your expected PCR product is not a multiple of 3 bases long, the amplification process can introduce mutations (e.g., deletions or point mutations) that may result in blue colonies when the fragment is inserted into the pTARGET™ Vector.

The Control Insert DNA supplied with the pTARGET™ Vector System is a 542bp sequence containing 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.

V.E. Experimental Controls

We strongly recommend performing the controls detailed below. These are necessary to accurately assess the performance of the pTARGET™ Vector System.

Positive Control

Set up a ligation reaction with the Control Insert DNA as described in the protocol (Section IV.A) and use it for transformations as described in Section IV.B. This control will allow you to determine the ligation efficiency. You will typically observe approximately 100 colonies, of which 10–30% are blue, if you use competent cells that have a transformation efficiency of 1×10^8cfu/µg DNA. Greater than 60% of the colonies should be white and, therefore, recombinant, since the Control Insert DNA is designed to reduce the number of background blue colonies (discussed in Section V.D). Background blue colonies arise from non-T-tailed or undigested pTARGET™ Vector. These blue colonies are a useful internal transformation control; if no colonies are obtained, the transformation has failed. If blue colonies are obtained, but no whites, the result suggests that the ligation reaction failed. If <50% white colonies are seen in this positive control reaction, then the ligation conditions were probably suboptimal. Also, fewer total colonies can indicate inefficient ligation.

Note: The Control Insert DNA should only be used as a ligation control and should not be used as a control for expression in mammalian cells.

Background Control

Set up a ligation reaction with 60ng of pTARGET™ Vector and no insert as described in the protocol (Section IV.A) and use it for transformations as described in Section IV.B. This ligation will allow you to determine the number of background blue colonies resulting from non-T-tailed or undigested pTARGET™ Vector.

If you follow the recommendations in Section IV.B closely, you should observe 20–60 colonies from competent cells with a transformation efficiency of 1×10^8cfu/µg DNA. Of these, approximately 4–15 colonies will be white. The background white colonies seen with the pTARGET™ Vector result from

deletions generated in the *lacZ*α region during transformation. Deletions of plasmid DNA during the transformation of *E. coli* with linear plasmid molecules are a well-documented phenomenon (22–25).

Transformation Control

Check the transformation efficiency of the competent cells by transforming them with an uncut plasmid and calculating cfu/µg DNA. If the transformation efficiency is lower than 1×10^8cfu/µg DNA, prepare fresh cells.

Example of transformation efficiency calculation:

After 100µl competent cells are transformed with 0.1ng 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 per plate (average of two plates), the transformation efficiency is calculated as shown below:

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

VI. 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	Problem with the transformation reaction or cells not competent. Background undigested vector and religated non-T-tailed vector should yield 20–60 blue colonies independent of the presence of insert DNA. Check the background control (Section V.E). Test the efficiency of the cells by transforming them with an uncut plasmid that allows antibiotic selection, such as the pGEM®-5Zf(+) Vector. If the recommendations in Section IV.B are followed, cells that are 1×10^8cfu/µg DNA typically yield 100 colonies. Therefore, you would not see any colonies from cells that are 1×10^6cfu/µg DNA.
Less than 10% white colonies in the positive control ligation	Failed ligation. Ligase buffer may have low activity. T4 DNA Ligase 10X Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles by making smaller aliquots of the buffer. Use a fresh vial of buffer.

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
Less than 10% white colonies in the positive control ligation (continued)	Failed ligation. Ligase activity may be low. To test the activity of the ligase and buffer, set up a ligation with approximately 200ng of DNA markers (e.g., Lambda DNA/*Hin*d III Markers, Cat.# G1711). Compare ligated and nonligated DNA on a gel and check that the fragments have been religated into high molecular weight material.
	Loss of T-overhangs allowing blunt-ended ligation of vector. Avoid introducing nucleases that may degrade the T-overhangs. Use only the T4 DNA Ligase provided with the system that has been tested for minimal exonuclease activity.
Less than 60% white colonies in the positive control ligation	Ligation temperature too high. Low temperature ligations (i.e., 4°C) favor annealing of the A-overhang of the insert with the T-overhang of the vector. Higher temperatures (i.e., room temperature) favor blunt-ended ligations.
	Ligation temperature too high. There is a background of non-T-tailed vector that will favor blunt-ligated at higher ligation temperatures.
	Loss of T-overhangs allowing blunt-ended ligation of vector. Avoid introduction of nucleases that may degrade the T-overhangs. Use only the T4 DNA Ligase provided with the system that has been tested for minimal exonuclease activity.
	Ligation incubation too short. Overnight ligation yields optimal results.
High colony number but low percentage of white colonies in the positive control ligation	Failed ligation. If the ligation is suboptimal or fails, and the competent cells have a high transformation frequency (1×10^9cfu/µg), the total number of colonies will be high, but the amount of white colonies will be low.
Low number or no white colonies with PCR product	Failed ligation. See above comments.
	Ligation incubation too short. Overnight ligation yields optimal results.

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
Low number or no white colonies with PCR product (continued)	Failed ligation due to inhibitory component in the PCR product. Mix some of the PCR product with the control ligation to see if it is inhibiting the reaction. If an inhibitor is suspected, repurify the PCR fragment.
	No 3´-A overhangs to allow PCR product ligation. As summarized in Table 1, not all thermostable DNA polymerases create a 3´A overhang (3,4). Blunt-ended fragments may be subsequently A-tailed by treatment with an appropriate polymerase and dATP (26,27).
	PCR fragment does not disrupt the *lac*Z gene. If there are a higher number of blue colonies resulting from the PCR fragment ligation than from the background control, some of these blue colonies may contain insert (especially with smaller insert fragments). Screen blue and pale blue colonies.
	Nonoptimal insert:vector ratio. Check the integrity and quantity of your PCR fragment by gel analysis. Optimize the insert:vector ratio (see Section V.C).
PCR product ligation reaction produces white colonies that do not contain insert (blue colonies are present)	Primer-dimers present in PCR fragment preparation. Primer-dimers will ligate into the pTARGET™ Vector but may not be seen after gel analysis of recombinant plasmid DNA because of their small size. Gel purify the PCR fragment.
	Multiple PCR products generated and cloned. Gel purify the PCR fragment of interest.
	DNA rearranged. Check a number of clones to see whether rearrangement is random. If so, the clone of interest should be present and can be identified by screening several clones. Toxic, unstable or homologous elements may also be a factor. Grow cells at a lower temperature or use a recombination-deficient cell line.
PCR product ligation reaction produces white colonies only (no blue colonies are present)	Inactive ampicillin. Check that ampicillin plates are made properly and used within two weeks. Test ampicillin by streaking plates with and without ampicillin with an ampicillin-sensitive strain of *E. coli*.

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
PCR product ligation reaction produces white colonies only (no blue colonies are present; continued)	No F′ episome in bacterial strain. Check the background control. If these colonies are not blue, and fresh plates that contain ampicillin/IPTG/X-Gal were used, the cells may have lost the F′ episome. Be sure that the cells are prepared properly (see Section IV.B).
	Plates 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 that they 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 of the PCR fragment. After purifying the PCR fragment, set up an A-tailing reaction (28,29). Clean up the sample and proceed with the protocol.
Expression of recombinant protein not detected in the transfected cells	No ATG sequence present in insert. Insert DNA must contain an ATG for initiation of translation
	Failed transfection. Check efficiency of transfection by cotransfecting with a reporter vector together with the pTARGET™ Vector recombinant clone and measuring expression of reporter gene.
	PCR-induced mutation in insert introducing a stop codon. Sequence insert to ensure no PCR-induced mutations are present.

VII. Appendix

VII.A. Vector Components

Enhancer/Promoter Regions

The CMV immediate-early enhancer/promoter region present in the pTARGET™ Vector allows strong, constitutive expression in a variety of cell types. The promiscuous nature of the CMV enhancer/promoter has been demonstrated in transgenic mice, where expression of the chloramphenicol acetyltransferase (CAT) gene regulated by the CMV enhancer/promoter was observed in 24 of the 28 tissues examined (28). The pTARGET™ Vector also 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 (29).

Chimeric Intron

Downstream of the CMV enhancer/promoter region is a chimeric intron composed of the 5´-donor site from the first intron of the human β-globin gene and the branch and 3´-acceptor site from the intron of an immunoglobulin gene heavy chain variable region (30). The sequences of the donor and acceptor sites, along with the branchpoint site, have been changed to match the consensus sequences for splicing (31). The intron is located upstream of the cDNA insert in order to prevent utilization of possible cryptic 5´-donor splice sites within the cDNA sequence (32).

Transfection studies have demonstrated that an intron flanking the cDNA insert frequently increases the level of gene expression (33–36). The increase in expression level due to the intron depends on the particular cDNA insert. For example, in transient transfections of 293 cells, the presence of this chimeric intron results in an approximate 20-fold increase in expression of the CAT gene (37). In contrast, the chimeric intron increases the gene expression level from luciferase cDNA by only threefold (37). In transgenic experiments, an intron is necessary to promote a high level of expression for virtually all cDNA inserts (38–40).

T7 RNA Polymerase Promoter

In the pTARGET™ Vector, a T7 RNA polymerase promoter is located downstream of the intron. This promoter can be used to synthesize RNA transcripts in vitro using T7 RNA polymerase.

Multiple Cloning Region, Other Convenient Restriction Enzyme Sites and *lac*Zα Region

The multiple cloning region in the pTARGET™ Vector is immediately downstream from the T7 promoter. Recognition sites for the restriction enzyme *Eco*R I flank the cloning site. This provides a convenient, single-enzyme digestion for release of the insert. Alternatively, a double-digestion may be used to release the insert.

Large hairpin structures in the 5´-end of untranslated mRNAs can reduce the level of in vitro and in vivo translation in higher eukaryotes (41–45). RNA transcribed from the multiple cloning region of the pTARGET™ Vector upstream of the cloning site does not contain hairpin structures that would interfere with translation (44).

Because there are no ATG sequences in either the multiple cloning region or between the transcriptional start site and the multiple cloning region, **an ATG for the initiation of translation must be present in the inserted DNA.**

Note: Additional requirements for efficient translation, including the Kozak consensus sequence (19), are described in Section V.A of this technical manual.

Unique restriction sites flank the CMV enhancer (*Bgl* II and *Sgf* I) and the CMV promoter (*Sgf* I and I-*Ppo* I), allowing easy replacement of these regulatory regions with other regulatory regions of interest.

VII.A. Vector Components (continued)

Multiple Cloning Region, Other Convenient Restriction Enzyme Sites and *lac*Zα Region (continued)

The multiple cloning region is within a *lac*Zα region, allowing identification of recombinant clones by color screening on indicator plates. The *lac*Zα region was modified to eliminate potential splice donor and acceptor sites and to eliminate an ATG sequence upstream of the cloning site. These modifications were made in an attempt to reduce any effect the *lac*Zα region may have on the expression level of the insert DNA. Recombinant clones may be identified by blue/white colony screening.

SV40 Late Polyadenylation Signal

Polyadenylation signals terminate transcription by RNA polymerase II and cause the addition of approximately 200 to 250 adenosine residues to the 3′-end of the RNA transcript (46). Polyadenylation enhances RNA stability and translation efficiency (47,48). To facilitate efficient processing of cloned DNA inserts not containing polyadenylation signals, the SV40 late polyadenylation signal has been positioned downstream from the multiple cloning region. The SV40 late polyadenylation signal is extremely efficient and can increase the steady-state level of RNA approximately fivefold more than the SV40 early polyadenylation signal (49).

Neomycin Phosphotransferase Selectable Marker

The neomycin phosphotransferase gene from Tn5 is present in the pTARGET™ Vector and is under the regulation SV40 enhancer and early promoter region. A synthetic polyadenylation signal based on the highly efficient polyadenylation signal of the rabbit β-globin gene (50) is located downstream of the neomycin phosphotransferase gene. To increase the translational efficiency of the neomycin phosphotransferase gene, the upstream, out-of-frame ATG sequences present in the wildtype neomycin phosphotransferase gene have been eliminated. Additionally, the sequence upstream of the initiator ATG has been changed to match a sequence shown to improve the context for initiating translation (46).

Expression of the neomycin phosphotransferase gene in mammalian cells confers resistance to the antibiotic G-418 (18). G-418 is an aminoglycoside antibiotic produced by *Streptomyces*, which induces cytotoxicity by blocking translation. Neomycin phosphotransferase inactivates G-418 through phosphorylation, thereby blocking its toxic effects (51).

f1 Origin of Replication and Plasmid Replicon

The backbone for the pTARGET™ Vector is derived from the pGEM®-3Zf(+) Vector. As a result, this vector is a high-copy-number plasmid and contains the origin of replication of the filamentous phage f1. To generate single-stranded DNA (ssDNA) from the f1 origin, bacteria transformed with the pTARGET™ Vector carrying the cloned DNA of interest are infected with an appropriate

helper phage. The plasmid then enters the f1 replication mode, and the resulting ssDNA is exported from the cell as an encapsidated virus particle. The ssDNA molecule exported corresponds to the top strand shown for the multiple cloning region (Figure 1). The ssDNA is purified from the supernatant by simple precipitation and extraction procedures, which are described in detail in the *Protocols and Applications Guide* (6).

Note: For further information about ssDNA purification, please contact your local Promega Branch Office or Distributor. In the U.S., contact Technical Services at 1-800-356-9526.

The "poison" sequence present in pBR322 that has been shown to inhibit replication of SV40 origin-containing vectors in COS cells has been deleted in the pTARGET™ Vector (52). This results in more efficient expression of the cloned cDNAs in COS cells and other cells transformed with the SV40 large T antigen.

VII.B. pTARGET™ Vector Restriction Enzyme Tables

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software with the pTARGET™ Vector prior to cutting with *Eco*R V and T-tailing. 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). For more information about the cut sites for these enzymes, or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. The pTARGET™ Vector is supplied linearized with a "T" added to both 3´ ends. The *Eco*R V site will not be recovered upon ligation of vector and insert. Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number **AY540613**) and on the Internet at: **www.promega.com/vectors/**

Table 2. Restriction Enzymes That Cut the pTARGET™ Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
***Aat* II**	5	278, 331, 414, 600, 3846	***Bal* I**	3	10, 64, 2885
***Acc* I**	1	1304	***Bam*H I**	1	1256
***Acc* III**	1	1775	***Ban* II**	3	729, 1956, 3168
***Acc*65 I**	1	1297	*Bbe* I	1	2806
Afl II	3	828, 847, 2657	*Bbr*P I	1	1849
Afl III	1	1276	*Bbs* I	2	961, 1540
***Alw*44 I**	3	3596, 4093, 5339	***Bbu* I**	3	2357, 2429, 3208
*Alw*N I	1	5244	***Bgl* II**	1	5665
***Ava* I**	2	1270, 1291	*Bsa* I	2	915, 4699
***Ava* II**	3	3319, 4401, 4623	*Bsa*A I	4	493, 1849, 2027, 3107
Avr II	1	2609	*Bsa*B I	1	1766

VII.B. pTARGET™ Vector Restriction Enzyme Tables (continued)

Table 2. Restriction Enzymes That Cut the pTARGET™ Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location
***Bsa*M I**	2	1585, 1678
Bsm I	2	1585, 1678
*Bsp*H I	3	3820, 3925, 4933
*Bsp*M I	4	877, 2690, 3071, 3521
*Bsr*G I	1	96
***Bss*H II**	1	3200
*Bss*S I	4	3395, 3789, 4096, 5480
***Bst*98 I**	3	828, 847, 2657
***Bst*X I**	1	3524
***Bst*Z I**	2	1311, 2709
*Cfr*10 I	4	1922, 3122, 3303, 4680
***Cla* I**	1	1770
***Csp* I**	1	3319
***Csp*45 I**	1	3485
***Dra* I**	4	1725, 4187, 4879, 4898
Dra II	1	3785
Dra III	1	2030
Dsa I	3	513, 2516, 3235
Eag I	2	1311, 2709
Ear I	4	1123, 3147, 3357, 3966
***Ecl*HK I**	1	4765
*Eco*52 I	2	1311, 2709
*Eco*72 I	1	1849
***Eco*ICR I**	1	727
***Eco*R I**	2	1250, 1318
Ehe I	1	2804
Fsp I	3	1085, 2905, 4542
***Hae* II**	4	1872, 1880, 2806, 5413
***Hinc* II**	3	677, 1305, 1664
*Hin*d II	3	677, 1305, 1664
***Hind* III**	3	756, 1325, 2625
***Hpa* I**	1	1664
I-*Ppo* I	1	851
Kas I	1	2802
***Kpn* I**	1	1301
***Mlu* I**	1	1276
***Nar* I**	1	2803
***Nco* I**	3	513, 2516, 3235
***Nde* I**	1	387
***Ngo*M IV**	2	1922, 3303
***Nhe* I**	1	1264
***Not* I**	1	1311
***Nsi* I**	2	2359, 2431
Nsp I	4	2357, 2429, 3208, 3740
*Pae*R7 I	1	1270
Pml I	1	1849
*Ppu*10 I	2	2355, 2427
*Psp*A I	1	1291
***Pst* I**	2	838, 2856
***Pvu* I**	2	664, 4396
***Pvu* II**	3	1135, 2285, 2909
Rsr II	1	3319
***Sac* I**	1	729
***Sal* I**	1	1303
***Sca* I**	1	4284
***Sfi* I**	1	2562
***Sgf* I**	1	664
***Sin* I**	3	3319, 4401, 4623
***Sma* I**	1	1293
***Sna*B I**	1	493
***Spe* I**	1	152
***Sph* I**	3	2357, 2429, 3208
***Ssp* I**	4	5, 52, 2235, 3960
***Stu* I**	1	2608
***Sty* I**	4	513, 2516, 2609, 3235
Tfi I	4	1772, 2631, 3288, 3422
***Tth*111 I**	1	2921
***Vsp* I**	3	160, 1485, 4590
***Xho* I**	1	1270
***Xma* I**	1	1291
***Xmn* I**	1	4165

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

VII.B. pTARGET™ Vector Restriction Enzyme Tables (continued)

Table 3. Restriction Enzymes That Do Not Cut the pTARGET™ Vector.

***Acc*B7 I**	*Bsi*W I	*Eco*N I	*Pme* I	*Srf* I
***Age* I**	*Bsp*120 I	*Fse* I	*Ppu*M I	*Sse*8387 I
***Apa* I**	*Bst*1107 I	*Ksp* I	*Psh*A I	*Swa* I
***Asc* I**	***Bst*E II**	***Nru* I**	*Psp*5 II	***Xba* I**
***Bcl* I**	***Bsu*36 I**	*Pac* I	***Sac* II**	*Xcm* I
Blp I	***Eco*47 III**	*Pfl*M I	*Sgr*A I	
*Bpu*1102 I	*Eco*81 I	*Pin*A I	*Spl* I	

Table 4. Restriction Enzymes That Cut the pTARGET™ Vector 6 or More Times.

Aci I	***Bsp*1286 I**	*Eae* I	***Hsp*92 II**	*Nla* III
Acy I	*Bsr* I	*Fnu*4H I	*Mae* I	*Nla* IV
***Alu* I**	***Bsr*S I**	***Fok* I**	*Mae* II	*Ple* I
***Alw*26 I**	*Bst*71 I	***Hae* III**	*Mae* III	***Rsa* I**
*Asp*H I	***Bst*O I**	*Hga* I	***Mbo* I**	***Sau*3A I**
Ban I	*Bst*U I	***Hha* I**	***Mbo* II**	*Sau*96 I
Bbv I	***Cfo* I**	*Hin*P I	*Mnl* I	*Scr*F I
***Bgl* I**	***Dde* I**	***Hinf* I**	*Mse* I	*Sfa*N I
*Bsa*H I	***Dpn* I**	***Hpa* II**	***Msp* I**	***Taq* I**
*Bsa*J I	*Dpn* II	*Hph* I	***Msp*A1 I**	***Tru*9 I**
*Bsa*O I	*Drd* I	***Hsp*92 I**	***Nci* I**	***Xho* II**

VII.C. Composition of Buffers and Solutions

IPTG stock solution (0.1M)

1.2g IPTG (Cat.# V3951)

Add deionized 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 1L of LB medium. 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 one month or at room temperature for up to one 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.

VII.C. Composition of Buffers and Solutions (continued)

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 (as prepared below)
1ml	2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml deionized 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 deionized water. Filter the complete medium through a 0.2µm filter unit. 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.

T4 DNA Ligase 10X Buffer (provided)

300mM	Tris-HCl (pH 7.8)
100mM	$MgCl_2$
100mM	DTT
10mM	ATP

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

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VII.E. Related Products

Basic PCR Cloning Systems

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

For Laboratory Use.

Product	Size	Cat.#
JM109 Competent Cells* (>1 × 10^8cfu/µg DNA efficiency)	1ml (5 × 200µl)	L2001
Select96™ Competent Cells	1 × 96 reactions	L3300

*For Laboratory Use.

Gel and PCR Purification Products

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

For Laboratory Use.

Amplification Products

Product	Size	Cat.#
PCR Master Mix	10 reactions*	M7501
GoTaq® DNA Polymerase	100 units*	M3001

For Laboratory Use. *Available in additional sizes.

VII.E. Related Products (continued)

Reverse Transcription

Product	Size	Cat. #
Access RT-PCR System	100 reactions*	A1250
ImProm-II™ Reverse Transcription System	100 reactions	A3800
AccessQuick™ RT PCR System	20 reactions*	A1701

For Laboratory Use. *Available in additional sizes.

Transfection Products

Product	Size	Cat.#
TransFast™ Transfection Reagent	1.2mg	E2431
Tfx™-20 Reagent	4.8mg	E2391
Tfx™-50 Reagent	2.1mg	E1811
Tfx™ Reagents Transfection Trio	5.4mg	E2400
Transfectam® Reagent for the Transfection of Eukaryotic Cells	0.5mg	E1232
	1.0mg	E1231
ProFection® Mammalian Transfection System—Calcium Phosphate	1 system	E1200
ProFection® Mammalian Transfection System—DEAE-Dextran	1 system	E1210

Plasmid DNA Purification Systems

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
Wizard® *Plus* SV Minipreps DNA Purification System (ready for spin protocols using a microcentrifuge)	50 preps*	A1330
Wizard® *Plus* SV Minipreps DNA Puification System (with vacuum adapters; ready for spin or vacuum protocols**)	50 preps*	A1340

For Laboratory Use. *Available in additional sizes.
**Vacuum protocol requires Vac-Man® Laboratory Vacuum Manifold.

Product	Capacity	Cat.#
Vac-Man® Laboratory Vacuum Manifold	20 samples	A7231

Sequencing Primers

Product	Size	Cat.#
T7 Promoter Primer	2µg	Q5021
pTARGET™ Sequencing Primer	2µg	Q4461

Reagents

Product	Size	Cat.#
X-Gal	100mg	V3941
IPTG, Dioxane Free	1g	V3955
	5g	V3951
	50g	V3953
Antibiotic G-418 Sulfate	100mg	V7981
	1g	V7982
	5g	V7983
Antibiotic G-418 Sulfate Solution	20ml	V8091

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