USER GUIDE

invitrogen™
by life technologies™

GeneBLAzer® TOPO® Fusion Kits for *In Vivo* or *In Vitro* Detection

Directional TOPO® Cloning of blunt-end PCR products into mammalian expression vectors for fluorescence detection of β-lactamase reporter activity

Catalog numbers 12578-076, 12578-084, 12578-092, 12578-100

Revision date 29 March 2012
Publication Part number 25-0665

MAN0000391

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

life technologies™

Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the directional TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

Step	Action
Design PCR Primers	• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer. • Design the primers such that your gene of interest will be optimally expressed and fused in frame with the β-lactamase reporter gene.
Amplify Your Gene of Interest	1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product. 2. Use agarose gel electrophoresis to check the integrity of your PCR product.
Perform the TOPO® Cloning Reaction	1. Set up the following TOPO® Cloning reaction. **For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector**. **Note:** If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in the TOPO® Cloning reaction. Fresh PCR product 0.5 to 4 µL Salt Solution 1 µL Sterile water add to a final volume of 5 µL TOPO® vector 1 µL Total volume 6 µL 2. Mix gently and incubate for 5 minutes at room temperature. 3. Place on ice and proceed to transform One Shot® Mach1™-T1^R chemically competent *E. coli*, below.
Transform Mach1™-T1^R Chemically Competent *E. coli*	1. Add 2 µL of the TOPO® Cloning reaction into a vial of One Shot® Mach1™-T1^R chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5–30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µL of room temperature S.O.C. medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 50–200 µL of bacterial culture on a **prewarmed** selective plate and incubate at 37°C. Visible colonies should appear within 8 hours for ampicillin selection. Incubate plates overnight, if desired.

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with each kit to perform the control reaction. See the protocol on page 33 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products. All products are also supplied with the GeneBLAzer® Detection Kits manual.

Product	Catalog no.
GeneBLAzer® C-terminal TOPO® Fusion Kit for *In Vitro* Detection	12578-076
GeneBLAzer® C-terminal TOPO® Fusion Kit for *In Vivo* Detection	12578-084
GeneBLAzer® N-terminal TOPO® Fusion Kit for *In Vitro* Detection	12578-092
GeneBLAzer® N-terminal TOPO® Fusion Kit for *In Vivo* Detection	12578-100

Kit Components

The GeneBLAzer® TOPO® Fusion Kits include the following components. For a detailed description of the contents of each component, see pages vii–on page viii.

Component	Catalog no.			
	12578-076	12578-084	12578-092	12578-100
GeneBLAzer® TOPO® Reagents with pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®	√	√		
GeneBLAzer® TOPO® Reagents with pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®			√	√
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	√	√	√	√
GeneBLAzer® *In Vitro* Detection Kit	√		√	
GeneBLAzer® *In Vivo* Detection Kit		√		√

Shipping and Storage

Each GeneBLAzer® TOPO® Fusion Kit is shipped as described in the table below, and contains the following boxes. Note that each kit contains Box 3a **or** 3b, as appropriate. Upon receipt, store the boxes as detailed below.

Box	Item	Shipping	Storage
1	GeneBLAzer® TOPO® Reagents	Dry ice	–20°C
2	One Shot® Mach1™-T1^R Chemically Competent *E. coli*	Dry ice	–80°C
3a	GeneBLAzer® *In Vitro* Detection Kit	Dry ice	**CCF2-FA:** –20°C, desiccated and protected from light
3b	GeneBLAzer® *In Vivo* Detection Kit	Room temperature	**CCF2-AM:** –20°C, desiccated and protected from light **Solutions:** Room temperature, protected from light

Continued on next page

GeneBLAzer® TOPO® Reagents

GeneBLAzer® TOPO® reagents (Box 1) are listed below. **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at –20°C.

Item	Concentration	Amount
GeneBLAzer® vector, linearized and TOPO®-adapted (pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® **or** pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®)	15–20 ng/µL linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/mL BSA 30 µM bromophenol blue	20 µL
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8.0	10 µL
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µL
Sterile Water	–	1 mL
T7 Promoter Primer **(supplied with Catalog nos. 12578-076 and 12578-084 only)**	0.1 µg/µL in TE Buffer, pH 8.0	20 µL
TK polyA Reverse Primer **(supplied with Catalog nos. 12578-092 and 12578-100 only)**	0.1 µg/µL in TE Buffer, pH 8.0	20 µL
Control PCR Primers	0.1 µg/µL each in TE Buffer, pH 8.0	10 µL
Control PCR Template	0.1 µg/µL in TE Buffer, pH 8.0	10 µL
Control Plasmid (pcDNA™6.2/cGeneBLAzer-GW/*lac*Z **or** pcDNA™6.2/nGeneBLAzer-GW/*lac*Z)	0.5 µg/µL in TE, pH 8.0	10 µL

Product Use

For research use only. Not intended for any human or animal therapeutic or diagnostic use.

Continued on next page

One Shot® Mach1™-T1R Reagents

The table below lists the items included in the One Shot® Mach1™-T1R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is 1×10^9 cfu/µg DNA. **Store Box 2 at –80°C.**

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or 4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL
Mach1™-T1R Cells	–	21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL

Genotype of Mach1™-T1R Cells

Use this strain for cloning. Note that this strain cannot be used for single-strand rescue of DNA.

F^- ϕ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R($r_K^- m_K^+$) Δ*rec*A1398 *end*A1 *ton*A

Information for Non-U.S. Customers

The parental strain of Mach1™-T1R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S. A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Sequencing Primers

The table below provides the sequences and amounts supplied of the T7 Promoter and TK polyA Reverse sequencing primers.

Primer	Sequence	pMoles Supplied
T7 Promoter Primer **(Catalog nos. 12578-076 and 12578-084 only)**	5′-TAATACGACTCACTATAGGG-3′	328
TK polyA Reverse Primer **(Catalog nos. 12578-092 and 12578-100 only)**	5′-CTTCCGTGTTTCAGTTAGC-3′	348

GeneBLAzer® Detection Kits

The GeneBLAzer® TOPO® Fusion Kits include either the GeneBLAzer® *In Vitro* Detection Kit or the GeneBLAzer® *In Vivo* Detection Kit for fluorescence detection of β-lactamase reporter activity. Refer to the GeneBLAzer® Detection Kits manual for detailed information pertaining to each kit and a description of the reagents provided in each kit.

Introduction

Product Overview

Description of the System

The GeneBLAzer® TOPO® Fusion Kits provide a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a reporter vector for expression in mammalian cells. The pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vector supplied with each kit facilitates *in vivo* or *in vitro* detection of β-lactamase reporter activity in mammalian cells using the GeneBLAzer® Technology. Use of the GeneBLAzer® Technology provides a highly sensitive and accurate method to quantitate gene expression in mammalian cells.

The pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vectors also allow easy transfer of your gene of interest into multiple vector systems using Gateway® Technology. For more information on the Gateway® System, see page 2.

Features of the pcDNA™6.2/ GeneBLAzer®-GW/ D-TOPO® Vectors

The pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® and pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells
- β-lactamase *bla*(M) reporter gene for C-terminal (pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®) or N-terminal (pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®) fusion to the gene of interest
- *att*B1 and *att*B2 sites for site-specific recombination of the expression clone with a Gateway® donor vector to generate an entry clone
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- The V5 epitope tag for detection using Anti-V5 antibodies (pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® only)
- The Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript
- f1 intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
- SV40 early promoter and origin for expression of the Blasticidin resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Blasticidin resistance gene for selection of stable cell lines
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- The ampicillin resistance gene for selection in *E. coli*

For a map of pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® or pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®, refer to pages 39 and 41, respectively.

Continued on next page

The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest in mammalian cells, simply TOPO® Clone your blunt-end PCR product into a GeneBLAzer® Directional TOPO® vector and transfect your expression clone into the mammalian cell line of choice.

To express your gene of interest in any other expression system:

1. Generate an entry clone by performing a BP recombination reaction between your expression clone and a Gateway® donor vector.
2. Perform an LR recombination reaction between the entry clone and a variety of Gateway® destination vectors to generate an expression construct to express your protein of interest in virtually any expression system.

For more information about the Gateway® Technology, refer to the Gateway® Technology manual. This manual is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 46).

Advantages of the GeneBLAzer® Technology

Using the GeneBLAzer® Technology and the GeneBLAzer® Detection System as a reporter of gene expression in mammalian cells provides the following advantages:

- Suitable for use as a sensitive reporter of gene expression in living mammalian cells using fluorescence microscopy.
- Provides a ratiometric readout to minimize differences due to variability in cell number, substrate concentration, fluorescence intensity, and emission sensitivity.
- Compatible with a wide variety of *in vivo* and *in vitro* applications including microplate-based transcriptional assays and flow cytometry.
- Provides a flexible and simple assay development platform for gene expression in mammalian cells.
- Using a non-toxic substrate allows continued cell culturing after quantitative analysis.

For more information on the GeneBLAzer® Technology, refer to page 4.

One Shot® Mach1™-T1^R *E. coli*

The Mach1™-T1^R *E. coli* strain is modified from the wild-type W strain (ATCC #9637, S. A. Waksman) and has a faster doubling time compared to other standard cloning strains. With Mach1™-T1^R cells, you can visualize colonies 8 hours after plating on ampicillin selective plates. You can also prepare plasmid DNA 4 hours after inoculating a single, overnight-grown colony in the selective media of choice. Note that this feature is not limited to ampicillin selection.

Additional features of the Mach1™-T1^R *E. coli* strain include:

- *lac*ZΔM15 for blue/white color screening of recombinants
- *hsd*R mutation for efficient transformation of unmethylated DNA from PCR applications
- Δ*rec*A1398 mutation for reduced occurrence of homologous recombination in cloned DNA
- *end*A1 mutation for increased plasmid yield and quality
- *ton*A mutation to confer resistance to T1 and T5 phage

How Directional TOPO® Cloning Works

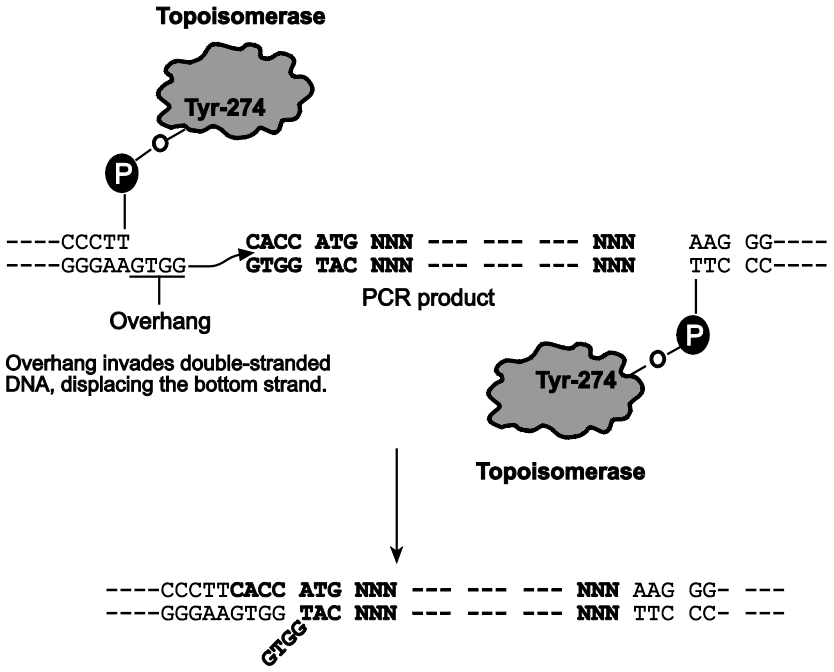
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng & Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. We have modified this idea by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



The GeneBLAzer® Technology

Components of the GeneBLAzer® System

The GeneBLAzer® System facilitates fluorescence detection of β-lactamase reporter activity in mammalian cells, and consists of two major components:

- The β-lactamase reporter gene, *bla*(M), a truncated form of the *E. coli bla* gene. When fused to a gene of interest, the *bla*(M) gene can be used as a reporter of gene expression in mammalian cells. For more information about the *bla*(M) gene, see below.
- A fluorescence resonance energy transfer (FRET)-enabled substrate, CCF2 to facilitate fluorescence detection of β-lactamase activity. In the absence or presence of β-lactamase reporter activity, cells loaded with the CCF2 substrate fluoresce green or blue, respectively. Comparing the ratio of blue to green fluorescence in a population of live cells or in a cell extract of your sample to a negative control provides a means to quantitate gene expression. For more information about the CCF2 substrate and how FRET works, refer to the GeneBLAzer® Detection Kits manual.

β-Lactamase (*bla*) Gene

β-lactamase is the product encoded by the ampicillin resistance gene (*bla*) and is the bacterial enzyme that hydrolyzes penicillins and cephalosporins. The *bla* gene is present in many cloning vectors and allows ampicillin selection in *E. coli*. β-lactamase enzyme activity is not found in mammalian cells.

bla(M) Gene

The GeneBLAzer® Technology uses a modified *bla* gene as a reporter in mammalian cells. This *bla* gene is derived from the *E. coli TEM-1* gene present in many cloning vectors (Zlokarnik *et al.*, 1998), and has been modified in the following ways:

- 72 nucleotides encoding the first 24 amino acids of β-lactamase were deleted from the N-terminal region of the gene. These 24 amino acids comprise the bacterial periplasmic signal sequence, and deleting this region allows cytoplasmic expression of -lactamase in mammalian cells.
- The amino acid at position 24 was mutated from His to Asp to create an optimal Kozak sequence for optimal translation initiation.

This modified reporter gene is named *bla*(M).

Note: The *TEM-1* gene also contains 2 mutations (at nucleotide positions 452 and 753) that distinguish it from the *bla* gene in pBR322 (Sutcliffe, 1978).

Experimental Outline

Experimental Outline

The table below describes the general steps needed to clone and express your gene of interest. For more details, refer to the pages indicated.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the β-lactamase reporter gene. Consult the diagram on page 9 or page 11 to help you design your PCR primers.	6–11
2	Produce your blunt-end PCR product.	12
3	TOPO® Clone your PCR product into a pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vector and transform into One Shot® Mach1™-T1^R *E. coli*. Select for transformants on LB agar plates containing 100 µg/mL ampicillin.	13–17
4	Analyze transformants for the presence and orientation of the insert by restriction digestion, PCR, or sequencing.	18
5	Prepare purified plasmid DNA for transfection.	
6	Transfect your mammalian cell line with the pcDNA™6.2/ GeneBLAzer®-GW/D-TOPO® construct using your method of choice. Select for stable transfectants using Blasticidin, if desired.	20
7	Assay for β-lactamase reporter activity using the appropriate GeneBLAzer® Detection Kit.	24–25

Methods

General Requirements for Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Sequences required to fuse your PCR product in frame with the β-lactamase reporter gene

General Requirements for the Forward Primer

To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vector.

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then the ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

General Requirements for the Reverse Primer

In general, design the reverse PCR primer to allow you to clone your PCR product in frame with any C-terminal fusions, if desired. **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 75%, and may increase the chances of your ORF cloning in the opposite orientation.** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch, but this has not been tested thoroughly.

Continued on next page

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag. The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3′

One possibility is to design the reverse PCR primer to start with the codon just up-stream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another possibility is to design the reverse primer so that it hybridizes just down-stream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine (see below).

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

…**GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TGA-3′**

- To fuse the ORF in frame with a C-terminal tag, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.
- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-TCA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Note

- Remember that the pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vectors accept blunt-end PCR products.
- Do not add 5′ phosphates to your primers for PCR. This will prevent ligation into the pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

Cloning into pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®

Introduction

pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® allows expression of recombinant proteins containing a C-terminal β-lactamase reporter; however, you may use this vector to express native proteins or C-terminal fusion proteins.

Kozak Consensus Sequence

Your sequence of interest should contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. The ATG initiation codon is shown underlined.

(G/A)NN<u>ATG</u>**G**

Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold).

Additional Cloning Considerations

In addition to the guidelines on pages 6–7, consider the following when designing PCR primers to clone your DNA into pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®.

For all cases, design the forward PCR primer such that the ATG initiation codon is in the context of a Kozak consensus sequence (see above) and directly follows the 5´ CACC overhang. To design the reverse PCR primer, consider the following:

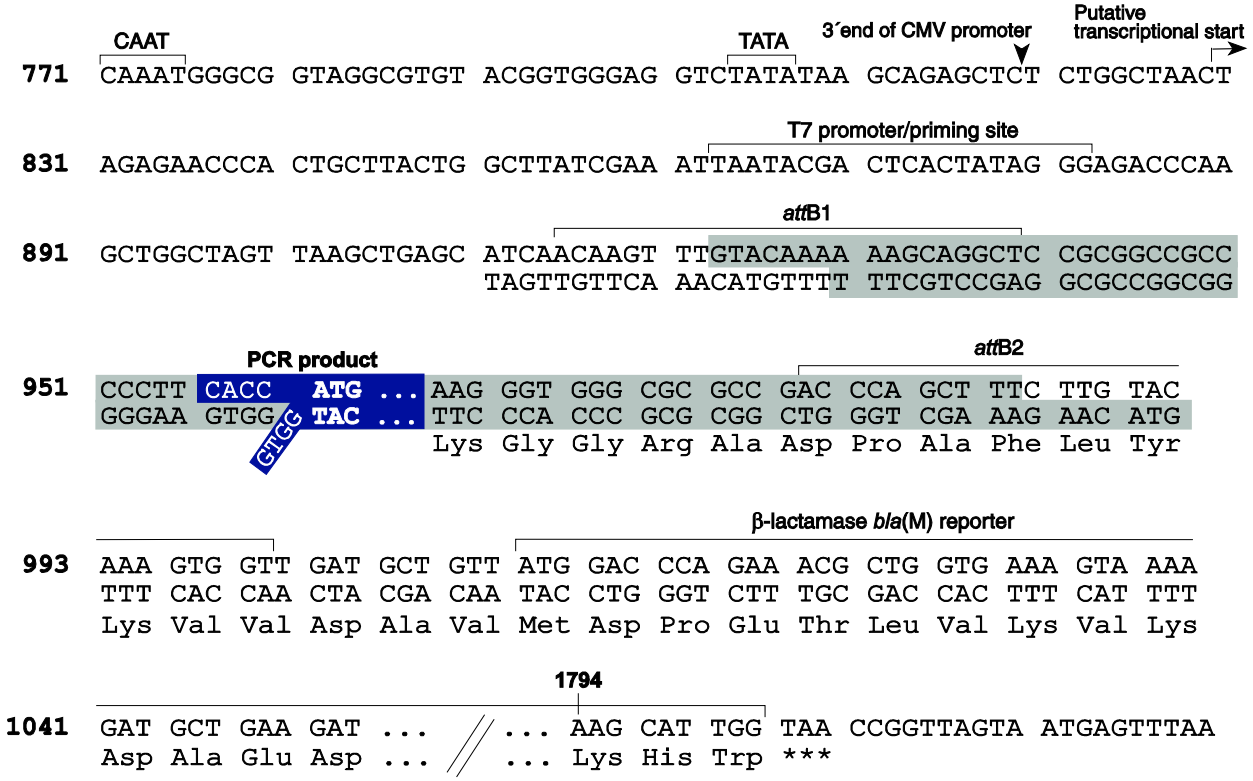
If you wish to...	Then...
include the β-lactamase reporter	design the reverse PCR primer to remove the native stop codon and preserve the reading frame through the *bla*(M) reporter gene
not include the β-lactamase reporter	design the reverse primer to include the native sequence containing the stop codon or make sure the stop codon is upstream from the reverse PCR primer binding site

Continued on next page

Cloning into pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®, Continued

TOPO® Cloning Site of pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®. The shaded region corresponds to sequences that will be transferred from the pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® vector into the entry clone following the BP recombination reaction. The nucleotide sequence of the vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 46).



Cloning into pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®

Introduction

pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® allows expression of recombinant proteins containing an N-terminal β-lactamase reporter and a C-terminal V5 epitope tag, if desired, and contains an ATG initiation codon within the context of a Kozak consensus sequence.

Additional Cloning Considerations

In addition to the guidelines on pages 6–7, consider the following when designing PCR primers to clone your DNA into pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®.

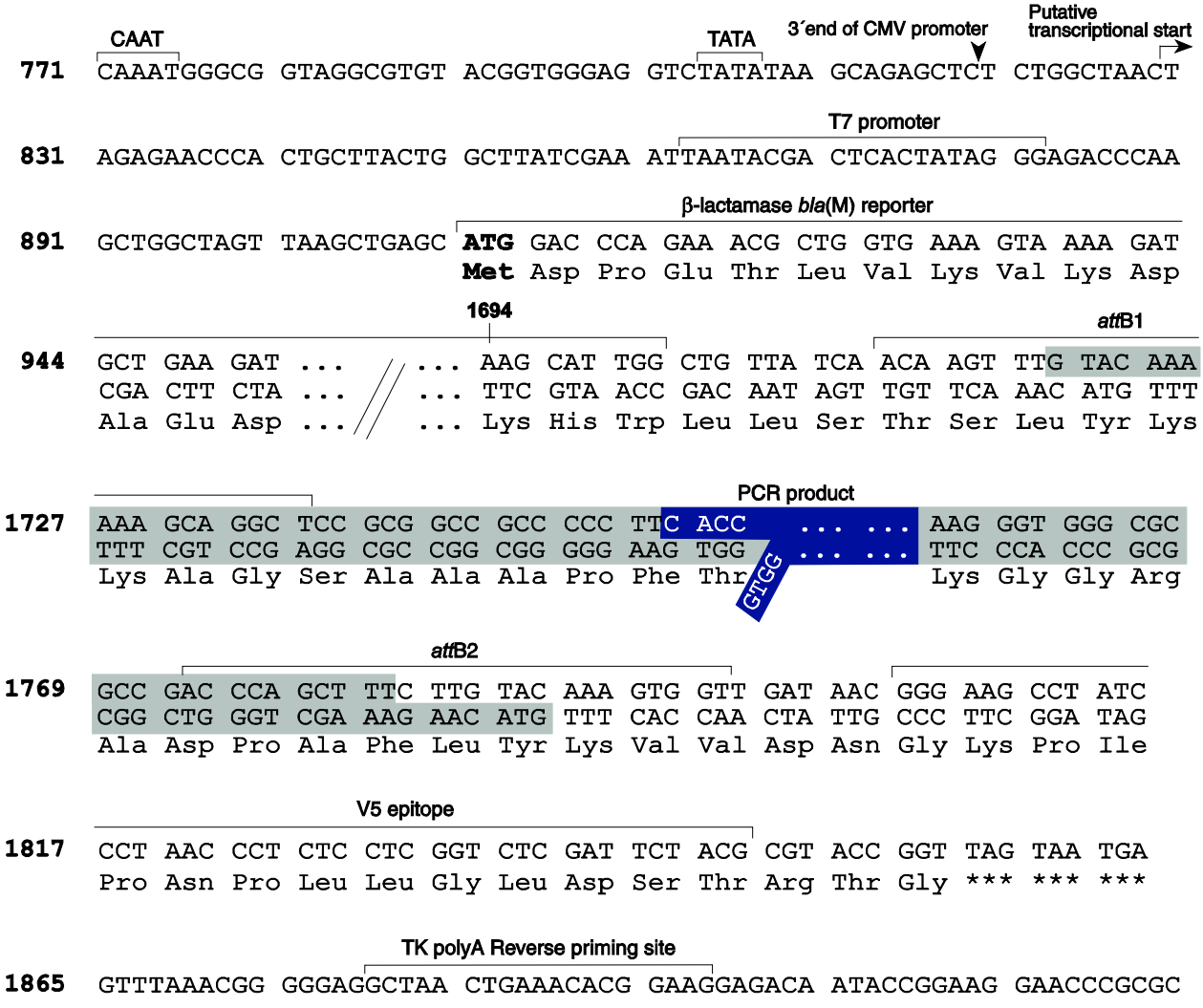
If you wish to...	Then...
include the β-lactamase reporter	design the forward primer to preserve the reading frame with the *bla*(M) reporter gene
include the V5 epitope tag	design the reverse PCR primer to • remove the native stop codon • preserve the reading frame through the V5 epitope tag
not include the V5 epitope tag	design the reverse primer to include the native sequence containing the stop codon or make sure the stop codon is upstream from the reverse PCR primer binding site

Continued on next page

Cloning into pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®, Continued

TOPO® Cloning Site of pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®. The shaded region corresponds to sequences that will be transferred from the pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® vector into the entry clone following the BP recombination reaction. The nucleotide sequence of the vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 46).



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, you are ready to produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Needed

Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers for PCR product

Producing PCR Products

Set up a 25 µL or 50 µL PCR reaction using the guidelines below:

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at –20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations for optimizing your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 35–36).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, page 13 for details).

Performing the TOPO® Cloning Reaction

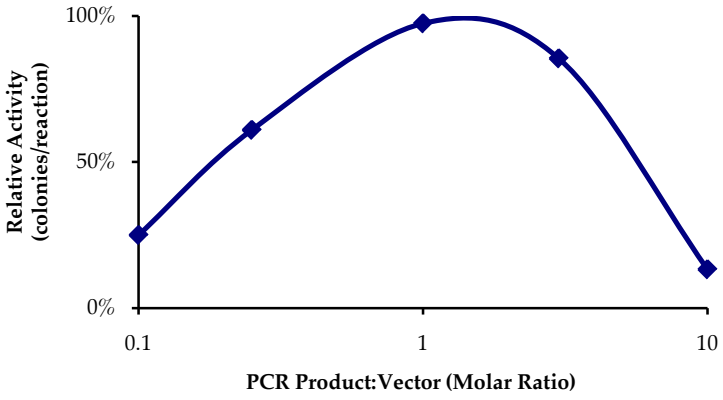
Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into a pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vector and transform the recombinant vector into Mach1™-T1^R cells. To ensure that you obtain the best possible results, we recommend that you read this section and the section entitled **Transforming One Shot® Mach1™-T1^R Competent Cells** (pages 15–17) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 33–34 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For the pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vectors, using 1–5 ng of a 1 kb PCR product or 5–10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



Continued on next page

Performing the TOPO® Cloning Reaction, Continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (i.e. using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page 45 for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR Product	0.5 to 4 µL	0.5 to 4 µL
Salt Solution	1 µL	–
Dilute Salt Solution (1:4)	–	1 µL
Sterile Water	add to a final volume of 5 µL	add to a final volume of 5 µL
pcDNA™6.2/GeneBLAzer®-GW/ D-TOPO® Vector	1 µL	1 µL
Final Volume	**6 µL**	**6 µL**

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or 4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22–23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Mach1™-T1^R Competent Cells**, page 15.

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Transforming One Shot® Mach1™-T1R Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, transform your GeneBLAzer® Directional TOPO® construct into competent *E. coli*. One Shot® Mach1™-T1R Chemically Competent *E. coli* (Box 2) are included to facilitate transformation, however, you may also transform other chemically competent cells (e.g. TOP10) or electrocompetent cells (see page 45 for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Blasticidin Selection

The presence of the EM7 promoter and the Blasticidin resistance gene in the pcDNA™6.2/GeneBLAzer®-GW/-D-TOPO® vectors allows for selection of *E. coli* transformants using Blasticidin. For selection, use Low Salt LB agar plates containing 100 µg/mL Blasticidin (see page 37 for a recipe). For Blasticidin to be active, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.0.

Blasticidin is available separately for purchase (see page 45 for ordering information). Refer to the **Appendix**, page 38 for instructions on how to prepare and store Blasticidin.

Note

The Mach1™-T1R strain allows you to visualize colonies 8 hours after plating on ampicillin selective plates. If you are using Blasticidin selection, you will need to incubate plates overnight in order to visualize colonies.

With the Mach1™-T1R strain, you may also prepare plasmid DNA 4 hours after inoculating a single, overnight-grown colony. Note that you will get sufficient growth of transformed cells within 4 hours with either ampicillin or Blasticidin selection.

Materials Needed

- TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2 (page 14)
- S.O.C. medium (included with the kit)
- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 100 µg/mL ampicillin or Low Salt LB plates containing 100 µg/mL Blasticidin (two for each transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Continued on next page

Transforming One Shot® Mach1™-T1R Competent Cells, Continued

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® Mach1™-T1R cells from Box 2 for each transformation.

Important

If you are using ampicillin selection and wish to visualize colonies within 8 hours of plating, it is essential that you prewarm your LB plates containing 100 µg/mL ampicillin prior to spreading.

One Shot® Mach1™-T1R Chemical Transformation Protocol

1. Add 2 µL of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 14 into a vial of One Shot® Mach1™-T1R Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µL of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50–200 µL from each transformation on a **prewarmed** selective plate. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies.
8. Incubate plates at 37°C. If you are using ampicillin selection, visible colonies should appear within 8 hours. For Blasticidin selection, incubate plates overnight.
9. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 18). Refer to the **Troubleshooting** section on page 27 if you have problems obtaining transformants.

Continued on next page

Transforming One Shot® Mach1™-T1R Competent Cells, Continued

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® Mach1-T1R chemically competent cells for electroporation.

1. Add 2 µL of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 14 into a sterile microcentrifuge tube containing 50 µL of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µL of room temperature S.O.C. medium.
4. Transfer the solution to a 15 mL snap-cap tube (e.g. Falcon) and shake for at least 1 hour at 37°C to allow expression of the ampicillin resistance gene.
5. Spread 20–100 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µL of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 18). Refer to the **Troubleshooting** section on page 27 if you have problems obtaining transformants.

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µL (0.1 cm cuvettes) or 100 to 200 µL (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50–100 µg/mL ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using the PureLink® HQ Mini Plasmid Purification Kit (see page 45 for ordering).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing Primers for pcDNA™6.2/ cGeneBLAzer-GW/D-TOPO®

To confirm that your gene of interest is in frame with the *bla*(M) reporter gene, you may sequence your construct, if desired. Keep the following in mind when designing your sequencing primers:

- Use a forward primer which hybridizes within the 3′ end of your gene of interest to sequence through the 5′ region of the *bla*(M) reporter gene.
- Do not use a reverse primer that hybridizes within the *bla*(M) reporter gene. Any primer that hybridizes within the *bla*(M) reporter gene will also hybridize within the ampicillin resistance gene, contaminating your results.

  Note: Because you will not be using a reverse primer, you will only be able to sequence the sense strand of your construct.
- Use the T7 Promoter primer (supplied with Catalog nos. 12578-076 and 12578-084) to sequence through the 5′ region of your gene of interest. Refer to the diagram on page 9 for the location of the T7 Promoter primer binding site.

Sequencing Primers for pcDNA™6.2/ nGeneBLAzer-GW/D-TOPO®

To confirm that your gene of interest is in frame with the *bla*(M) reporter gene or the V5 epitope tag, you may sequence your construct, if desired. Keep the following in mind when designing your sequencing primers:

- Use a reverse primer which hybridizes within the 5′ end of your gene of interest to sequence through the 3′ region of the *bla*(M) reporter gene.
- Do not use a forward primer that hybridizes within the *bla*(M) reporter gene. Any primer that hybridizes within the β-lactamase reporter gene will also hybridize within the ampicillin resistance gene, contaminating your results.

  Note: Because you will not be using a forward primer, you will only be able to sequence the anti-sense strand of your construct.
- Use the TK polyA Reverse primer (supplied with Catalog nos. 12578-092 and 12578-100) to sequence through the V5 epitope. Refer to the diagram on page 11 for the location of the TK polyA Reverse primer binding site.

Continued on next page

Important

If you download the sequence for pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® or pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® from our website, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. If you are using pcDNA™6.2/ cGeneBLAzer-GW/D-TOPO®, use a combination of the T7 Promoter primer and a primer that hybridizes within your insert. If you are using pcDNA™6.2/ nGeneBLAzer-GW/D-TOPO®, use a combination of the TK polyA Reverse primer and a primer that hybridizes within your insert.

You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (see page 45 for ordering)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µL of PCR SuperMix High Fidelity into a 0.5 mL microcentrifuge tube. Add 1 µL each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µL of the PCR cocktail from Step 1, above.
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Visualize by agarose gel electrophoresis.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at –20°C.

1. Streak the original colony out for single colony on LB plates containing 50–100 µg/mL ampicillin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50–100 µg/mL ampicillin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Transfecting Cells

Introduction

This section provides general information to transfect your expression clone into the mammalian cell line of choice. We recommend that you include a positive control vector (pcDNA™6.2/cGeneBLAzer-GW/*lac*Z or pcDNA™6.2/nGeneBLAzer-GW/*lac*Z) and a mock transfection (negative control) in your experiments to evaluate your results.

If you plan to detect β-lactamase reporter activity *in vivo* using the GeneBLAzer® *In Vivo* Detection Kit (supplied with Catalog nos. 12578-084 and 12578-100 only), note that a number of factors including cell type and cell density can influence the degree of the fluorescence signal detected. We recommend taking these factors into account when designing your transfection experiment. For more information, refer to the section entitled **General Guidelines to Use the GeneBLAzer® *In Vivo* Detection Kit** in the GeneBLAzer® Detection Kits manual.

Plasmid Preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® HQ Mini Plasmid Purification Kit (see page 45 for ordering) or CsCl gradient centrifugation.

Positive Control

pcDNA™6.2/cGeneBLAzer-GW/*lac*Z or pcDNA™6.2/nGeneBLAzer-GW/*lac*Z is provided as a positive control vector for mammalian cell transfection and expression (see pages 43 and 44 for a map) and may be used to optimize recombinant protein expression levels in your cell line. These vectors allow expression of the β-galactosidase gene with either an N-terminal or C-terminal fusion to the β-lactamase reporter.

To propagate and maintain the plasmid:

1. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like Mach1™, TOP10, DH5α™, or equivalent.
2. Select transformants on LB agar plates containing 50–100 µg/mL ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Continued on next page

Methods of Transfection

For established cell lines (e.g. HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen & Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner & Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa & Dower, 1988). For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine® 2000 Reagent (see page 45 for ordering). For more information about Lipofectamine® 2000 and the other transfection reagents available for purchase, refer to www.lifetechnologies.com or contact Technical Support (page 46).

Creating Stable Cell Lines

Introduction

The GeneBLAzer® Directional TOPO® vectors contain the Blasticidin resistance gene to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your construct into the mammalian cell line of choice and select for foci using Blasticidin. General information and guidelines are provided below.

To obtain stable transfectants, we recommend that you linearize your pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in mammalian cells. To linearize your construct, cut at a unique site that is not located within a critical element or within your gene of interest.

Determining Blasticidin Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Blasticidin required to kill your untransfected host cell line by performing a kill curve experiment (see the procedure below). Typically, concentrations ranging from 2.5 to 10 µg/mL Blasticidin are sufficient to kill most untransfected mammalian cell lines. Blasticidin is available separately for purchase (see page 45 for ordering). Refer to the **Appendix**, page 38 for instructions on how to prepare and store Blasticidin.

1. Plate cells at approximately 25% confluence. Prepare a set of 6 plates.
2. On the following day, replace the growth medium with fresh growth medium containing varying concentrations of Blasticidin (e.g. 0, 1, 3, 5, 7.5, and 10 µg/mL Blasticidin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of Blasticidin that prevents growth within 10-14 days after addition of Blasticidin.

Continued on next page

Generating Stable Cell Lines

Once you have determined the appropriate Blasticidin concentration to use for selection, you can generate a stable cell line expressing your pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® construct.

1. Transfect the mammalian cell line of interest with the pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® or pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® construct using your transfection method of choice.
2. 24 hours after transfection, wash the cells and add fresh growth medium.
3. 48 hours after transfection, split the cells into fresh growth medium such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Incubate the cells at 37°C for 2–3 hours until they have attached to the culture dish.
5. Remove the growth medium and replace with fresh growth medium containing Blasticidin at the predetermined concentration required for your cell line.
6. Feed the cells with selective media every 3–4 days until Blasticidin-resistant colonies can be identified.
7. Pick at least 5 Blasticidin-resistant colonies and expand them to assay for recombinant protein expression.

Detecting Recombinant Fusion Proteins

Introduction

Depending on the kit you are using, assay for β-lactamase reporter activity through *in vivo* or *in vitro* detection methods. A brief description of each detection method is provided below. For detailed information, refer to the GeneBLAzer® Detection Kits manual. If you have generated a pcDNA™6.2/ nGeneBLAzer-GW/D-TOPO® construct that contains your gene of interest fused to the V5 epitope tag, you may also detect your recombinant fusion protein by Western blot analysis using one of the Anti-V5 Antibodies available for purchase.

In Vitro Detection

Using the GeneBLAzer® *In Vitro* Detection Kit allows you to quantitate the amount of intracellular β-lactamase in cells based on the β-lactamase activity in lysates.

To detect β-lactamase activity in mammalian cell lysates, you will use the CCF2-FA substrate. CCF2-FA is the non-esterified, free acid form of CCF2, and is recommended for *in vitro* use because it is readily soluble in aqueous solution and may be added directly to pre-made cell lysates. Once added to cell lysates, you may quantitate the CCF2-FA fluorescence signal using a fluorescence plate reader or a fluorometer.

To prepare cell lysates from mammalian cells containing the *bla*(M) reporter gene, you **must** use a method that will preserve the activity of the β-lactamase enzyme. Refer to the GeneBLAzer® Detection Kits manual for detailed guidelines and protocols to prepare CCF2-FA solution, prepare cell lysates and samples, and detect CCF2 signal.

In Vivo Detection

Using the GeneBLAzer® *In Vivo* Detection Kit allows you to measure β-lactamase reporter activity in live mammalian cells. Once β-lactamase reporter activity has been measured, cells may be cultured further for use in additional assays or other downstream applications.

To detect β-lactamase activity in live mammalian cells, you will use the CCF2-AM substrate. CCF2-AM is the membrane-permeable, esterified form of CCF2, and is recommended for *in vivo* use because it is non-toxic, lipophilic, and readily enters the cell. Once cells are "loaded" with CCF2-AM, you may quantitate the CCF2 fluorescence signal using a variety of methods.

Refer to the GeneBLAzer® Detection Kits manual for detailed guidelines and protocols to prepare CCF2-AM solution, load cells with CCF2-AM substrate, and detect CCF2 signal.

Continued on next page

Detecting the V5 Epitope Tag

If you are using pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® vector and you have fused your gene of interest to the V5 epitope tag, you may detect expression of your recombinant fusion protein using the Anti-V5 Antibody, Anti-V5-HRP Antibody, or Anti-V5-AP Antibody (see page 45 for ordering). In addition, the Positope™ Control Protein is available for use as a positive control for detection of fusion proteins containing a V5 epitope (see page 45). The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available for purchase to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to www.lifetechnologies.com or contact Technical Support (page 46).

Note

Expression of your protein fused to the β-lactamase reporter and/or to the V5 epitope tag will increase the size of your recombinant protein. The table below lists the increase in the molecular weight of your recombinant protein that you should expect from a particular fusion. Note that the expected sizes take into account any additional amino acids between the gene of interest and the fusion peptide (see page 9 or page 11 for a diagram).

Vector	Fusion	Expected Size Increase (kDa)
pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®	β-lactamase (C-terminal)	31 kDa
pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®	β-lactamase (N-terminal)	31 kDa
	V5 (C-terminal)	3.5 kDa

Assay for β-Galactosidase

If you use pcDNA™6.2/cGeneBLAzer-GW/*lac*Z or pcDNA™6.2/nGeneBLAzer-GW/*lac*Z) as a positive control vector, you may assay for β-galactosidase expression by Western blot analysis or activity assay (Miller, 1972). We offer the β-Gal Antiserum, the β-Gal Assay Kit, and the β-Gal Staining Kit for fast and easy detection of β-galactosidase expression (see page 45 for ordering information).

Creating an Entry Clone

Introduction

Once you have TOPO® Cloned your gene of interest into a GeneBLAzer® Directional TOPO® vector, you may perform a BP recombination reaction between your expression construct and a Gateway® donor vector to generate an entry clone. Once you generate an entry clone, your gene of interest may then be easily shuttled into a large selection of destination vectors using the LR recombination reaction. To ensure that you obtain the best possible results, we recommend that you read this section and the next section entitled **Performing the BP Recombination Reaction** (pages 28–30) before beginning.

Recombining the Expression Clone with a Donor Vector

Before performing the BP recombination reaction, consider the following points:

- The *bla*(M) reporter gene will not be recombined into the entry clone. If you are using pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®, the V5 epitope tag will also not be recombined into the entry clone. If you wish to fuse your gene of interest to any N-terminal or C-terminal peptides, the peptides will need to be provided by the destination vector in the LR recombination reaction.
- If you cloned the gene of interest to be in frame with an N-terminal or C-terminal peptide in one of the GeneBLAzer® Directional TOPO® vectors, the gene will remain in frame with any N-terminal or C-terminal tags provided by the destination vector following the LR recombination reaction.
- Depending on the design of your forward and reverse primers, your gene in the entry clone may not contain an ATG initiation codon within the context of a Kozak consensus sequence or a stop codon. If either of these are required, they will need to be provided by the destination vector in the LR recombination reaction.

Experimental Outline

To generate an entry clone:

1. Perform a BP recombination reaction between your pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone and an *att*P-containing donor vector (see below)
2. Transform the reaction mixture into a suitable *E. coli* host
3. Select for entry clones

Gateway® Donor Vectors

We offer a variety of Gateway® donor vectors to help you generate an entry clone containing your gene of interest (see page 45 for ordering information). For more information about the vectors available, refer to www.lifetechnologies.com or contact Technical Support (page 46).

Continued on next page

Important

For optimal efficiency, perform the BP recombination reaction using:

- **Linear** pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone (see below for guidelines to linearize expression clones)
- **Supercoiled** *att*P-containing donor vector

Note: Supercoiled or relaxed *att*B expression clones may be used, but will react less efficiently than linear *att*B expression clones.

Linearizing Expression Clones

We recommend that you linearize your pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone using a suitable restriction enzyme (see the guidelines below).

1. Linearize 1 to 2 µg of the expression clone with a unique restriction enzyme that does not digest within the gene of interest and is located outside the *att*B region.
2. Ethanol precipitate the DNA after digestion by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
3. Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.
4. Dissolve the DNA in TE Buffer, pH 8.0 to a final concentration of 50-150 ng/µL.

Performing the BP Recombination Reaction

Introduction

General guidelines and instructions are provided in this section to perform a BP recombination reaction using your pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone and a donor vector, and to transform the reaction mixture into a suitable *E. coli* host to select for entry clones. We recommend that you include a positive control (see the Positive Control below) in your experiment to help you evaluate your results.

Positive Control

pEXP7-tet is provided with the BP Clonase® enzyme mix as a positive control for the BP reaction. pEXP7-tet is an approximately 1.4 kb linear fragment and contains *att*B sites flanking the tetracycline resistance gene and its promoter (Tc^r). Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene. The efficiency of the BP recombination reaction can easily be determined by streaking entry clones onto LB plates containing 20 µg/mL tetracycline.

Determining How Much DNA to Use

For optimal efficiency, we recommend using the following amounts of linearized *att*B expression clone and donor vector in a 20 µL BP recombination reaction:

- An equimolar amount of linearized *att*B expression clone and the donor vector
- 100 femtomoles (fmol) **each** of linearized *att*B expression clone and donor vector is preferred, but the amount of *att*B expression clone used may range from 40–100 fmol

  Note: 100 fmol of donor vector (pDONR™201, pDONR™221, or pDONR™/Zeo) is approximately 300 ng

For a formula to convert fmol of DNA to nanograms (ng), see below. For an example, see page 29.

Important

- Do not use more than 500 ng of donor vector in a 20 µL BP reaction as this will affect the efficiency of the reaction
- Do not exceed more than 1 µg of total DNA (donor vector plus *att*B expression clone) in a 20 µL BP reaction as excess DNA will inhibit the reaction

Converting Femtomoles (fmol) to Nanograms (ng)

Use the following formula to convert femtomoles (fmol) of DNA to nanograms (ng) of DNA:

$$ng = (fmol)(N)\left(\frac{660\ fg}{fmol}\right)\left(\frac{1\ ng}{10^6\ fg}\right)$$

where N is the size of the DNA in bp. For an example, see page 29.

Continued on next page

Example of fmol to ng Conversion

In this example, you need to use 100 fmol of your pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone which is 7.5 kb in size in the BP reaction. Calculate the amount of your pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone required for the reaction (in ng) by using the equation on page 28:

$$(100\text{ fmol})(7500\text{ bp})(\frac{660\text{ fg}}{\text{fmol}})(\frac{1\text{ ng}}{10^6\text{ fg}}) = 495\text{ ng of expression clone required}$$

Materials Needed

- Linearized pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone (see page 28 to determine the amount of DNA to use)
- pDONR™ vector (150 ng/µL)
- BP Clonase® enzyme mix (see page 45 for ordering information; keep at –80ºC until immediately before use)
- 5X BP Clonase® Reaction Buffer (supplied with the BP Clonase® enzyme mix)
- pEXP7-tet positive control, optional (50 ng/µL; supplied with the BP Clonase® enzyme mix)
- TE Buffer, pH 8.0 (10 mm Tris-HCl, pH 8.0; 1 mM EDTA)
- 2 µg/µL Proteinase K solution (supplied with the BP Clonase® enzyme mix; thaw and keep on ice until use)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. medium
- LB agar plates containing the appropriate antibiotic to select for entry clones

Continued on next page

Setting Up the BP Recombination Reaction

1. Add the following components to 1.5 mL microcentrifuge tubes at room temperature and mix.

   Note: To include a negative control, set up a second sample reaction and substitute TE Buffer, pH 8.0 for the BP Clonase® enzyme mix (see Step 4).

Components	Sample	Positive Control
pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® expression clone (40–100 fmol)	1–10 µL	–
pDONR™ vector (150 ng/µL)	2 µL	2 µL
pEXP7-tet positive control (50 ng/µL)	–	2 µL
5X BP Clonase® Reaction Buffer	4 µL	4 µL
TE Buffer, pH 8.0	to 16 µL	8 µL

2. Remove the BP Clonase® enzyme mix from –80°C and thaw on ice (~ 2 minutes).
3. Vortex the BP Clonase® enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 4 µL of BP Clonase® enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return BP Clonase® enzyme mix to –80°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: For most applications, a 1 hour incubation will yield a sufficient number of entry clones. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. An overnight incubation typically yields 5–10 times more colonies than a 1 hour incubation.
6. Add 2 µL of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 µL of the BP recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for entry clones.

   Note: You may store the BP reaction at –20°C for up to 1 week before transformation, if desired.

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1×10^8 cfu/µg, the BP recombination reaction should give >1500 colonies if the entire BP reaction is transformed and plated.

Verifying pEXP7-tet Entry Clones

If you included the pEXP7-tet control in your experiments, you may access the efficiency of the BP reaction by streaking entry clones onto LB plates containing 20 µg/mL tetracycline. True entry clones should be tetracycline-resistant.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions in parallel with your samples (see pages 33–34).

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR product too dilute	• Concentrate the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Long PCR product	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.

Continued on next page

TOPO® Cloning Reaction and Transformation, Continued

Problem	Reason	Solution
Large number of incorrect inserts cloned	PCR reaction contains artifacts (i.e. does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at –80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	One Shot® transformation protocol not followed correctly	Follow the One Shot® transformation protocol provided on page 16.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Selective plates not prewarmed before spreading	Warm selective plates at 37ºC for 30 minutes prior to spreading.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.
No visible colonies 8 hours after plating transformed Mach1™-T1^R cells	Not using ampicillin selection	Colonies will appear 8 hours after plating with ampicillin selection. If you are using Blasticidin selection, incubate plates overnight at 37ºC.
	Selective plates not prewarmed before spreading	Warm selective plates at 37ºC for 30 minutes prior to spreading.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you TOPO® Clone to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50–100 µg/mL ampicillin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µL PCR:

Control DNA Template (100 ng)	1 µL
10X PCR Buffer (appropriate for enzyme)	5 µL
dNTP Mix	0.5 µL
Control PCR Primers (0.1 µg/µL each)	1 µL
Sterile Water	41.5 µL
Thermostable polymerase (1–2.5 units/µL)	1 µL
Total Volume	50 µL

2. Overlay with 70 µL (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR produce used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (i.e. 0.5:1 to 2:1). Proceed to **Control TOPO® Cloning Reactions**, page 34.

Continued on next page

Control TOPO® Cloning Reactions

Using the control PCR product produced on page 33 and a pcDNA™6.2/GeneBLAzer®-GW/D-TOPO® vector, set up two 6 µL TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µL	3 µL
Salt Solution	1 µL	1 µL
Control PCR Product	–	1 µL
pcDNA™6.2/GeneBLAzer®-GW/D-TOPO®	1 µL	1 µL
Final volume	6 µL	6 µL

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µL of each reaction into separate vials of One Shot® Mach1™-T1^R cells using the protocol on page 16.
4. Spread 50–200 µL of each transformation mix onto LB plates containing 50-100 µg/mL ampicillin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® Mach1™-T1^R competent cells. Transform one vial of One Shot® Mach1™-T1^R cells with 10 pg of pUC19 using the protocol on page 16. Plate 10 µL of the transformation mixture plus 20 µL of S.O.C. medium on LB plates containing 100 µg/mL ampicillin. Transformation efficiency should be ~1 × 10^9 cfu/µg DNA.

Analyzing the Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. Refer to the table below for expected digestion patterns.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®	*Ava* I	Correct orientation: 4032, 2617 Reverse orientation: 4603, 2046 Empty vector: 5900
pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®	*Ava* I	Correct orientation: 4829, 1865 Reverse orientation: 5400, 1294 Empty vector: 5945

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation. Relatively few colonies should be produced in the vector-only reaction.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below.

Note

The cloning efficiency may decrease with purification of the PCR product (e.g. PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 8).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 mL at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 × *g* in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µL of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µL of TE or sterile water. Use 4 µL for the TOPO® Cloning reaction and proceed as described on page 14.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1–2 µL of the flow-through in the TOPO® Cloning reaction (page 14). Be sure to make the gel slice as small as possible for best results.

Continued on next page

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µL of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 14.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µL directly into One Shot® Mach1™-T1^R cells using the method on page 16.

Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Recipes

LB (Luria-Bertani) Medium and Plates

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (50–100 µg/mL ampicillin) if needed.
4. Store at room temperature or at 4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50–100 µg/mL of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C.

Low Salt LB Medium with Blasticidin

Low Salt LB Medium:

10 g Tryptone

5 g NaCl

5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust pH to 7.0 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Blasticidin to 100 µg/mL final concentration.
4. Store plates at 4°C in the dark. Plates containing Blasticidin are stable for up to 2 weeks.

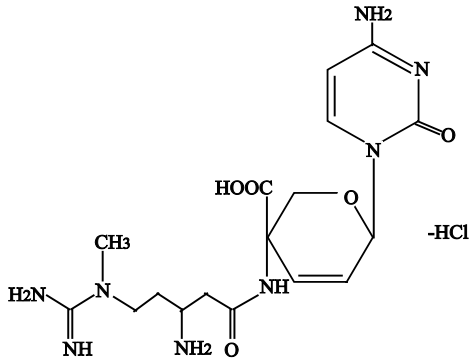
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g. a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

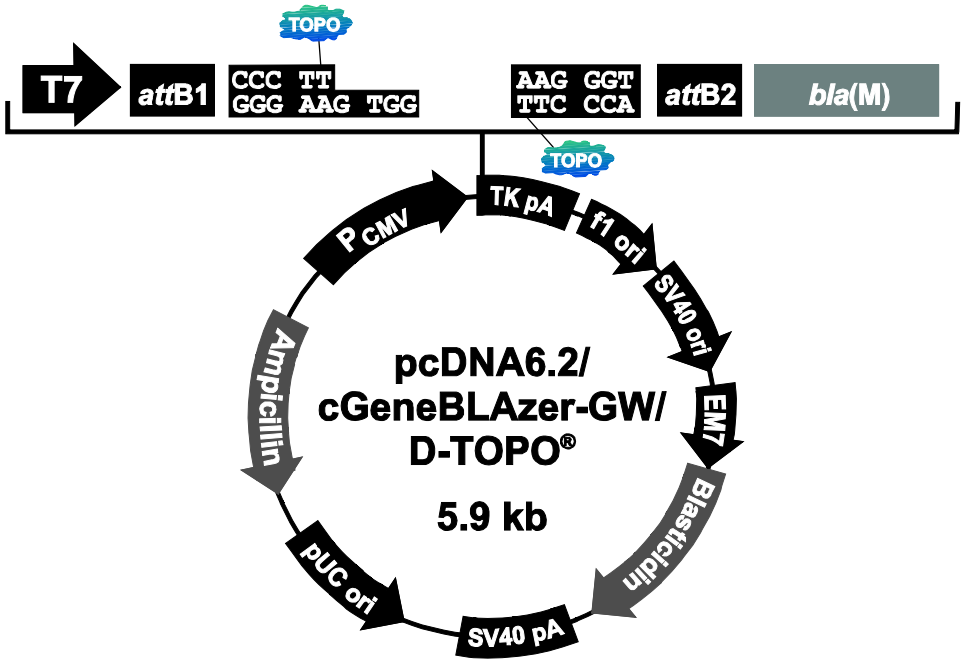
Blasticidin may be obtained separately (see page 45 for ordering) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5–10 mg/mL.

- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at –20°C for long-term storage or store at 4°C for short-term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at -20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at 4°C for up to 2 weeks.
- Medium containing Blasticidin may be stored at 4°C for up to 2 weeks.

Map and Features of pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®

Map

The map below shows the elements of pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®. The nucleotide sequence of the vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 46).



Comments for pcDNA6.2/cGeneBLAzer-GW/D-TOPO®
5900 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
*att*B1 site: bases 915-939
TOPO® recognition site 1: bases 951-955
Overhang sequence (c): bases 956-959
TOPO® recognition site 2: bases 960-964
*att*B2 site: bases 976-1000
β-lactamase *bla*(M) reporter gene: bases 1011-1805
TK polyadenylation signal: bases 1828-2099
f1 origin: bases 2135-2563
SV40 early promoter and origin: bases 2590-2898
EM7 promoter: bases 2953-3019
Blasticidin resistance gene: bases 3020-3418
SV40 early polyadenylation signal: bases 3576-3706
pUC origin (c): bases 4089-4759
Ampicillin resistance gene (c): bases 4904-5764
Ampicillin promoter (c): bases 5765-5863

(c) = complementary strand

Continued on next page

Map and Features of pcDNA™6.2/cGeneBLAzer-GW/D-TOPO®, Continued

Features

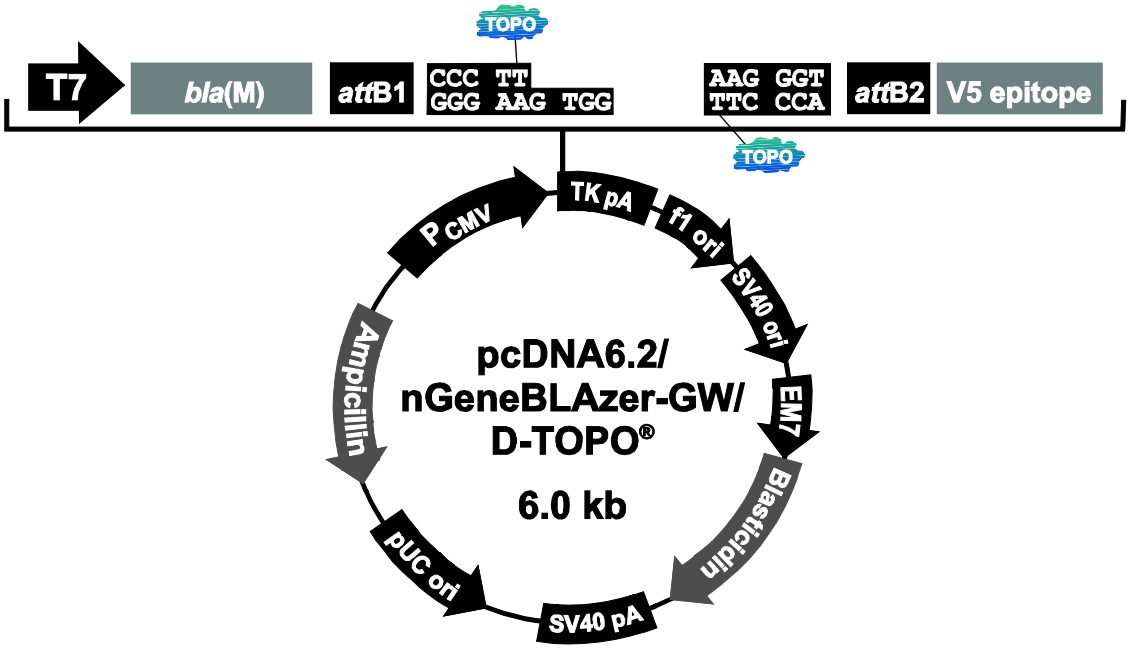
pcDNA™6.2/cGeneBLAzer-GW/D-TOPO® (5900) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter/priming site	Allows *in vitro* transcription in the sense orientation and sequencing through the insert
*att*B1 and *att*B2 sites	Allows recombinational cloning of the gene of interest to generate an entry clone
TOPO® Cloning site (directional)	Allows directional cloning of your PCR product in frame with the C-terminal β-lactamase reporter gene
β-lactamase *bla*(M) reporter gene	Allows fusion of the β-lactamase reporter to the C-terminus of your protein for use as a reporter of gene expression (Zlokarnik *et al.*, 1998)
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole & Stacy, 1985)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Allows expression of the Blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin resistance gene	Allows selection of transformants in *E. coli*

Map and Features of pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®

Map

The map below shows the elements of pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®. The nucleotide sequence of the vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 46).



Comments for pcDNA6.2/nGeneBLAzer-GW/D-TOPO®
5945 nucleotides

CMV promoter: bases 232-819
T7 promoter: bases 863-882
β-lactamase *bla*(M) reporter gene: bases 911-1702
*att*B1 site: bases 1712-1736
TOPO® recognition site 1: bases 1748-1752
Overhang sequence (c): bases 1753-1756
TOPO® recognition site 2: bases 1757-1761
*att*B2 site: bases 1773-1797
V5 epitope: bases 1805-1846
TK polyadenylation signal: bases 1873-2144
TK reverse priming site: bases 1880-1898
f1 origin: bases 2180-2608
SV40 early promoter and origin: bases 2635-2943
EM7 promoter: bases 2998-3064
Blasticidin resistance gene: bases 3065-3463
SV40 early polyadenylation signal: bases 3621-3751
pUC origin (c): bases 4134-4804
Ampicillin resistance gene (c): bases 4949-5809
Ampicillin promoter (c): bases 5810-5908

(c) = complementary strand

Continued on next page

Map and Features of pcDNA™6.2/nGeneBLAzer-GW/D-TOPO®, Continued

Features

pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® (5945) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter	Allows *in vitro* transcription in the sense orientation
β-lactamase *bla*(M) reporter gene	Allows fusion of the β-lactamase reporter to the N-terminus of your protein for use as a reporter of gene expression (Zlokarnik *et al.*, 1998)
*att*B1 and *att*B2 sites	Allows recombinational cloning of the gene of interest to generate an entry clone
TOPO® Cloning site (directional)	Allows directional cloning of your PCR product in frame with the N-terminal β-lactamase reporter gene
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 antibodies (Southern *et al.*, 1991).
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole & Stacy, 1985)
TK polyA reverse priming site	Allow sequencing through the insert
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Allows expression of the Blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin resistance gene	Allows selection of transformants in *E. coli*

Map of pcDNA™6.2/cGeneBLAzer-GW/*lacZ*

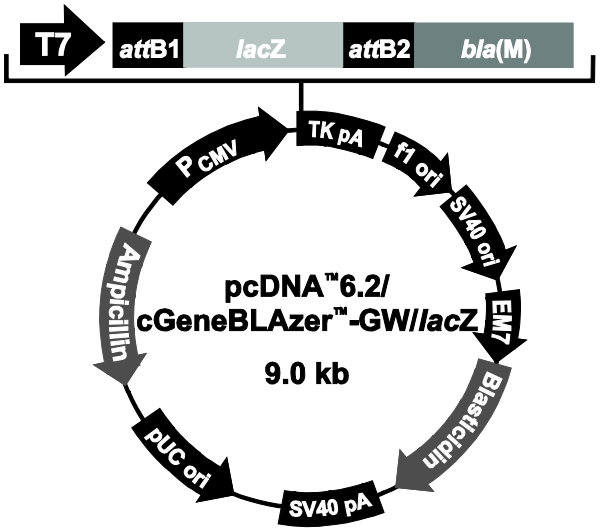
Description

pcDNA™6.2/cGeneBLAzer-GW/*lacZ* (8981 bp) is a control vector containing the *lacZ* gene and was constructed using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pcDNA™6.2/cGeneBLAzer-DEST. *lacZ* is expressed as a fusion to the β-lactamase reporter protein. The molecular weight of the β-galactosidase fusion protein is approximately 147 kDa.

For more information on the Gateway® Technology, refer to the Gateway® Technology manual. For more information on pcDNA™6.2/cGeneBLAzer-DEST, refer to the GeneBLAzer® Gateway® Fusion Kits manual.

Map

The nucleotide sequence of this vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 46).



Comments for pcDNA™6.2/cGeneBLAzer™-GW/*lacZ*
8981 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
attB1 site: bases 915-939
lacZ gene: bases 981-4040
attB2 site: bases 4057-4081
β-lactamase *bla*(M) reporter gene: bases 4092-4886
TK polyadenylation signal: bases 4909-5180
f1 origin: bases 5216-5644
SV40 early promoter and origin: bases 5671-5979
EM7 promoter: bases 6034-6100
Blasticidin resistance gene: bases 6101-6499
SV40 early polyadenylation signal: bases 6657-6787
pUC origin (c): bases 7170-7840
Ampicillin resistance gene (c): bases 7985-8845
Ampicillin promoter (c): bases 8846-8944

(c) = complementary strand

Map of pcDNA™6.2/nGeneBLAzer-GW/*lacZ*

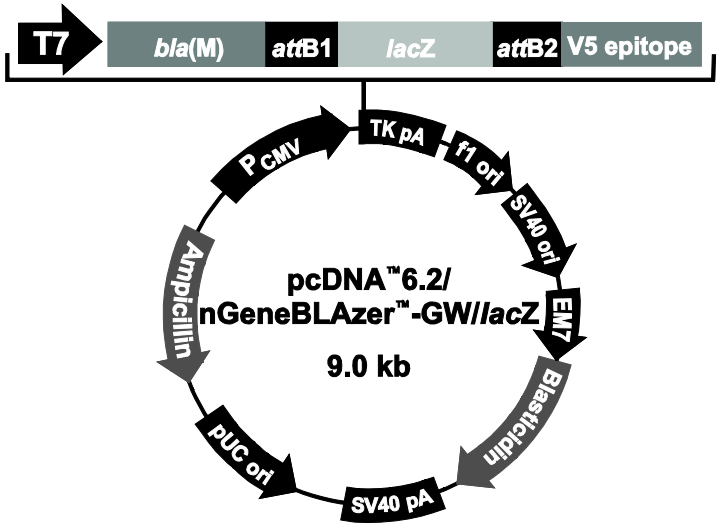
Description

pcDNA™6.2/nGeneBLAzer-GW/*lacZ* (9041 bp) is a control vector containing the *lacZ* gene and was constructed using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pcDNA™6.2/nGeneBLAzer-DEST. *lacZ* is expressed as a fusion to the β-lactamase reporter protein. Note that the *lacZ* gene contains a TAA stop codon and is **not** fused to the V5 tag. The molecular weight of the β-galactosidase fusion protein is approximately 148 kDa.

For more information on the Gateway® Technology, refer to the Gateway® Technology manual. For more information on pcDNA™6.2/nGeneBLAzer-DEST, refer to the GeneBLAzer® Gateway® Fusion Kits manual.

Map

The complete sequence of this vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 46).



Comments for pcDNA™6.2/nGeneBLAzer™-GW/*lacZ*
9041 nucleotides

CMV promoter: bases 232-819
T7 promoter: bases 863-882
β-lactamase *bla*(M) reporter gene: bases 911-1702
*att*B1 site: bases 1712-1736
lacZ gene: bases 1778-4852
*att*B2 site: bases 4869-4893
V5 epitope: bases 4901-4942
TK polyadenylation signal: bases 4969-5240
TK polyA reverse priming site: bases 4976-4994
f1 origin: bases 5276-5704
SV40 early promoter and origin: bases 5731-6039
EM7 promoter: bases 6094-6160
Blasticidin resistance gene: bases 6161-6559
SV40 early polyadenylation signal: bases 6717-6847
pUC origin (c): bases 7230-7900
Ampicillin resistance gene (c): bases 8045-8905
Ampicillin promoter (c): bases 8906-9004

(c) = complementary strand

Accessory Products

Additional Products

Additional products that may be used with the GeneBLAzer® TOPO® Fusion Kits are available for purchase. Ordering information is provided below.

Item	Quantity	Catalog no.
GeneBLAzer® *In Vitro* Detection Kit	100 µg	12578-126
GeneBLAzer® *In Vivo* Detection Kit	50 µg	12578-134
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C4040-03
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions	C4040-50
PCR SuperMix High Fidelity	100 reactions	10790-020
S.O.C. Medium	10 × 10 mL	15544-034
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Gateway® BP Clonase® Enzyme Mix	20 reactions	11789-013
	100 reactions	11789-021
pDONR™221	6 µg	12213-013
pDONR™/Zeo	6 µg	12536-017
Lipofectamine® 2000	0.75 mL	11668-027
	1.5 mL	11668-019
Blasticidin	50 mg	R210-01
β-Gal Assay Kit	100 reactions	K4155-01
β-Gal Staining Kit	1 kit	K1465-01
β-Gal Antiserum*	50 µL	R901-25

*The amount supplied is sufficient to perform 25 Western blots using 10 mL working solution per reaction.

Detecting Fusion Protein

A number of antibodies are available for purchase to detect expression of your fusion protein from the pcDNA™6.2/nGeneBLAzer-GW/D-TOPO® vector. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods. The fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments.

Antibody	Epitope	Catalog no.
Anti-V5	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern et al., 1991). GKPIPNPLLGLDST	R960-25
Anti-V5-HRP		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25
Positope™ Control Protein	--	R900-50

Technical Support

Obtaining support

For the latest services and support information for all locations, go to www.lifetechnologies.com/support.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited warranty

Life Technologies Corporation is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about a Life Technologies product or service, contact our Technical Support Representatives.

All Life Technologies products are warranted to perform according to specifications stated on the certificate of analysis. The Company will replace, free of charge, any product that does not meet those specifications. This warranty limits the Company's liability to only the price of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. The Company reserves the right to select the method(s) used to analyze a product unless the Company agrees to a specified method in writing prior to acceptance of the order.

Life Technologies makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore the Company makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, report it to our Technical Support Representatives.

Life Technologies Corporation shall have no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Limited Use Label License No: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License: ULB ccdB Selection Technology

ccdB selection technology is described in Bernard et al., "Positive Selection Vectors Using the F Plasmid ccdB Killer Gene" Gene 148 (1994) 71-74. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). For licensing information for use in other than research, please contact: outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Life Technologies' policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Life Technologies' commercially available Gateway® Technology.

Gateway® Entry Clones

Life Technologies understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

Gateway® Expression Clones

Life Technologies also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Life Technologies. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Life Technologies.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase® from Life Technologies is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Life Technologies Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to licensing department at outlicensing@lifetech.com.

References

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989) Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, New York

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985) A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530

Chen, C., and Okayama, H. (1987) High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Mol. Cell. Biol. *7*, 2745-2752

Cheng, C., and Shuman, S. (2000) Recombinogenic Flap Ligation Pathway for Intrinsic Repair of Topoisomerase IB-Induced Double-Strand Breaks. Mol. Cell. Biol. *20*, 8059-8068

Chu, G., Hayakawa, H., and Berg, P. (1987) Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326

Cole, C. N., and Stacy, T. P. (1985) Identification of Sequences in the Herpes Simplex Virus Thymidine Kinase Gene Required for Efficient Processing and Polyadenylation. Mol. Cell. Biol. *5*, 2104-2113

Felgner, P. L., Holm, M., and Chan, H. (1989) Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121

Felgner, P. L. a., and Ringold, G. M. (1989) Cationic Liposome-Mediated Transfection. Nature *337*, 387-388

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991) Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exp. Cell Res. *197*, 229-233

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994) Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. ACTA *1219*, 653-659

Kozak, M. (1987) An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148

Kozak, M. (1990) Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305

Kozak, M. (1991) An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903

Landy, A. (1989) Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949

Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987) Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129

Shigekawa, K., and Dower, W. J. (1988) Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751

Shuman, S. (1991) Recombination Mediated by Vaccinia Virus DNA Topoisomerase I in *Escherichia coli* is Sequence Specific. Proc. Natl. Acad. Sci. USA *88*, 10104-10108

Continued on next page

Shuman, S. (1994) Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase. J. Biol. Chem. *269*, 32678-32684

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991) Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557

Sutcliffe, J. G. (1978) Nucleotide Sequence of the Ampicillin Resistance Gene of *Escherichia coli* Plasmid pBR322. Proc. Nat. Acad. Sci. USA *75*, 3737-3741

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958) Blasticidin S, A New Antibiotic. The Journal of Antibiotics, Series A *11*, 1-5

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977) Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965) Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. J. Biochem (Tokyo) *57*, 667-677

Zlokarnik, G., Negulescu, P. A., Knapp, T. E., Mere, L., Burres, N., Feng, L., Whitney, M., Roemer, K., and Tsien, R. Y. (1998) Quantitation of Transcription and Clonal Selection of Single Living Cells with Beta-Lactamase as Reporter. Science *279*, 84-88

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit www.invitrogen.com/support or email techsupport@invitrogen.com

www.lifetechnologies.com

life technologies™