USER GUIDE

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# GeneBLAzer® Gateway® Fusion Kits for *In Vivo* or *In Vitro* Detection

## Gateway®-adapted destination vectors for fluorescence detection of β-lactamase reporter activity in mammalian cells

**Catalog numbers** 12578-035, 12578-043, 12578-050, and 12578-068

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**Publication Part number** 25-0664

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

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# Contents

# 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 Gateway® Fusion Kit for *In Vitro* Detection | 12578-035 |
| GeneBLAzer® C-terminal Gateway® Fusion Kit for *In Vivo* Detection | 12578-043 |
| GeneBLAzer® N-terminal Gateway® Fusion Kit for *In Vitro* Detection | 12578-050 |
| GeneBLAzer® N-terminal Gateway® Fusion Kit for *In Vivo* Detection | 12578-068 |

## Kit Components

The GeneBLAzer® Gateway® Fusion Kits include the following components. For a detailed description of the contents of each component, see page vi.

| Component | Catalog no. | | | |
|---|---|---|---|---|
| | 12578-035 | 12578-043 | 12578-050 | 12578-068 |
| pcDNA™6.2/cGeneBLAzer™-DEST Gateway® Vector | √ | √ | | |
| pcDNA™6.2/nGeneBLAzer™-DEST Gateway® Vector | | | √ | √ |
| GeneBLAzer® *In Vitro* Detection Kit | √ | | √ | |
| GeneBLAzer® *In Vivo* Detection Kit | | √ | | √ |

## Shipping and Storage

The GeneBLAzer® Gateway® Fusion Kits are shipped as detailed below. Upon receipt, store as indicated.

| Box | Shipping | Storage |
|---|---|---|
| GeneBLAzer® Gateway® Vector | Room temperature | **Vectors:** –20ºC |
| GeneBLAzer® *In Vitro* Detection Kit | Dry Ice | **CCF2-FA:** –20°C, desiccated and protected from light |
| GeneBLAzer® *In Vivo* Detection Kit | Room temperature | **CCF2-AM:** –20°C, desiccated and protected from light<br>**Solutions:** Room temperature, protected from light |

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# Kit Contents and Storage, Continued

## GeneBLAzer® Gateway® Vectors

The vectors provided with the GeneBLAzer® Gateway® Fusion Kits are listed below. **Store at –20°C.**

| Item | Concentration | Amount |
|---|---|---|
| Gateway® Destination Vector (pcDNA™6.2/cGeneBLAzer™-DEST **or** pcDNA™6.2/nGeneBLAzer™-DEST) | 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 | 6 µg |
| Control Plasmid (pcDNA™6.2/cGeneBLAzer™-GW/*lac*Z **or** pcDNA™6.2/nGeneBLAzer™-GW/*lac*Z) | 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 | 10 µg |

## GeneBLAzer® Detection Kits

The GeneBLAzer® Gateway® 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.

## Product Use

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

# Introduction

## Product Overview

### Description of the System

The GeneBLAzer® Gateway® Fusion Kits contain Gateway®-adapted destination vectors designed for use with the GeneBLAzer® Technology. The pcDNA™6.2/GeneBLAzer®-DEST vector supplied with each kit facilitates *in vivo* or *in vitro* detection of β-lactamase reporter activity in mammalian cells using a unique fluorescent substrate. Use of the GeneBLAzer® Technology provides a highly sensitive and accurate method to quantitate gene expression in mammalian cells.

### Features

The pcDNA™6.2/cGeneBLAzer™-DEST and pcDNA™6.2/nGeneBLAzer™-DEST 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™-DEST) or N-terminal (pcDNA™6.2/nGeneBLAzer™-DEST) fusion to the gene of interest
- Two recombination sites, *att*R1 and *att*R2, downstream of the CMV promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- The *ccd*B gene located between the two *att*R sites for negative selection
- The V5 epitope tag for detection using Anti-V5 antibodies (pcDNA™6.2/nGeneBLAzer™-DEST 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™-DEST or pcDNA™6.2/nGeneBLAzer™-DEST, refer to pages 21 and 23, respectively.

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### 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 using Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector to create an entry clone.
2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway® destination vector (e.g. pcDNA™6.2/cGeneBLAzer™-DEST or pcDNA™6.2/nGeneBLAzer™-DEST).
3. Transfect your expression clone into the cell line of choice for transient or stable expression of your gene of interest.

For more information on Gateway®, refer to the Gateway® Technology manual. This manual is available for downloading from www.lifetechnologies.com/support or by contacting Technical Support (page 28).

### 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 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 GeneBLAzer® Technology, see page 3.

# 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 β-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).

# Methods

## Generating an Entry Clone

### Introduction

To recombine your gene of interest into pcDNA™6.2/cGeneBLAzer™-DEST or pcDNA™6.2/nGeneBLAzer™-DEST, you will need an entry clone containing the gene of interest. Many entry vectors including pENTR™/D-TOPO® are available to facilitate generation of entry clones (see page 27 for ordering). For more information, refer to www.lifetechnologies.com or contact Technical Support (page 28). Refer to the manual for the specific entry vector you are using for detailed instructions to construct an entry clone.

**Note**

If you wish to express a human or mouse gene of interest, we recommend using an Ultimate™ Human ORF (hORF) or Ultimate™ Mouse ORF (mORF) Clone available for purchase. Each Ultimate™ ORF Clone is a fully-sequenced clone provided in a Gateway® entry vector that is ready-to-use in an LR recombination reaction with a pcDNA™6.2/GeneBLAzer®-DEST vector. In addition, each clone contains a **TAG** stop codon, making it fully compatible for use in the Tag-On-Demand™ System. For more information about the Ultimate™ ORF Clones available, refer to www.lifetechnologies.com or contact Technical Support (page 28).

### Kozak Consensus Sequence

If you will be expressing you protein from pcDNA™6.2/cGeneBLAzer™-DEST, your insert in the entry clone 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)**NNATG**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).

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### Points to Consider for pcDNA™6.2/cGeneBLAzer™-DEST

pcDNA™6.2/cGeneBLAzer™-DEST allows expression of recombinant proteins containing a C-terminal β-lactamase reporter; however, you may use this vector to express a native protein. You may also use this vector in the Tag-On-Demand™ System (see page **Error! Bookmark not defined.**). Consider the following when generating your entry clone.

| If you wish to... | Then your insert... |
|---|---|
| include the β-lactamase reporter | • should contain a Kozak initiation sequence (see page 4)<br>• should **not** contain a stop codon<br>• should be in frame with the *bla*(M) reporter gene after recombination (see page 8 for a diagram) |
| include the β-lactamase reporter for use in the Tag-On-Demand™ System | • should contain a Kozak initiation sequence (see previous page)<br>• should contain a **TAG** stop codon<br>• should be in frame with the *bla*(M) reporter gene after recombination (see page 8 for a diagram) |
| **not** include the β-lactamase reporter | • should contain a Kozak initiation sequence (see page 4)<br>• should contain a stop codon |

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### Points to Consider for pcDNA™6.2/nGeneBLAzer™-DEST

pcDNA™6.2/nGeneBLAzer™-DEST 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 (see page 4). You may use this vector in the Tag-On-Demand™ System (see page **Error! Bookmark not defined.**). Consider the following when generating your entry clone.

| If you wish to... | Then your insert... |
|---|---|
| include the β-lactamase reporter | • should **not** contain a Kozak initiation sequence<br>• should be in frame with the *bla*(M) reporter gene after recombination (see page 9 for a diagram) |
| include the V5 epitope tag | • should **not** contain a stop codon<br>• should be in frame with the V5 epitope after recombination (see page 9 for a diagram) |
| include the V5 epitope for use in the Tag-On-Demand™ System | • should contain a **TAG** stop codon<br>• should be in frame with the V5 epitope after recombination (see page 9 for a diagram) |
| **not** include the V5 epitope tag | • should contain a stop codon |

# Creating an Expression Clone

## Introduction

After you have generated an entry clone, perform the LR recombination reaction to transfer the gene of interest into your pcDNA™6.2/GeneBLAzer®-DEST vector to create your expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the next section entitled **Performing the LR Recombination Reaction** (pages 10–12) before beginning.

## Experimental Outline

To generate an expression clone:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing pcDNA™6.2/GeneBLAzer®-DEST vector.
2. Transform the reaction mixture into a suitable *E. coli* host.
3. Select for expression clones (refer to pages 8–9 for diagrams of the recombination region of the resulting expression clones).

## Propagating the pcDNA™6.2/ GeneBLAzer®-DEST Vectors

If you wish to propagate and maintain the pcDNA™6.2/GeneBLAzer®-DEST vectors, we recommend using Library Efficiency® DB3.1™ Competent Cells for transformation (see page 27 for ordering). The DB3.1™ *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

**Note: DO NOT** use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance of the pcDNA™6.2/GeneBLAzer®-DEST vectors as these strains are sensitive to CcdB effects.

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# Creating an Expression Clone, Continued

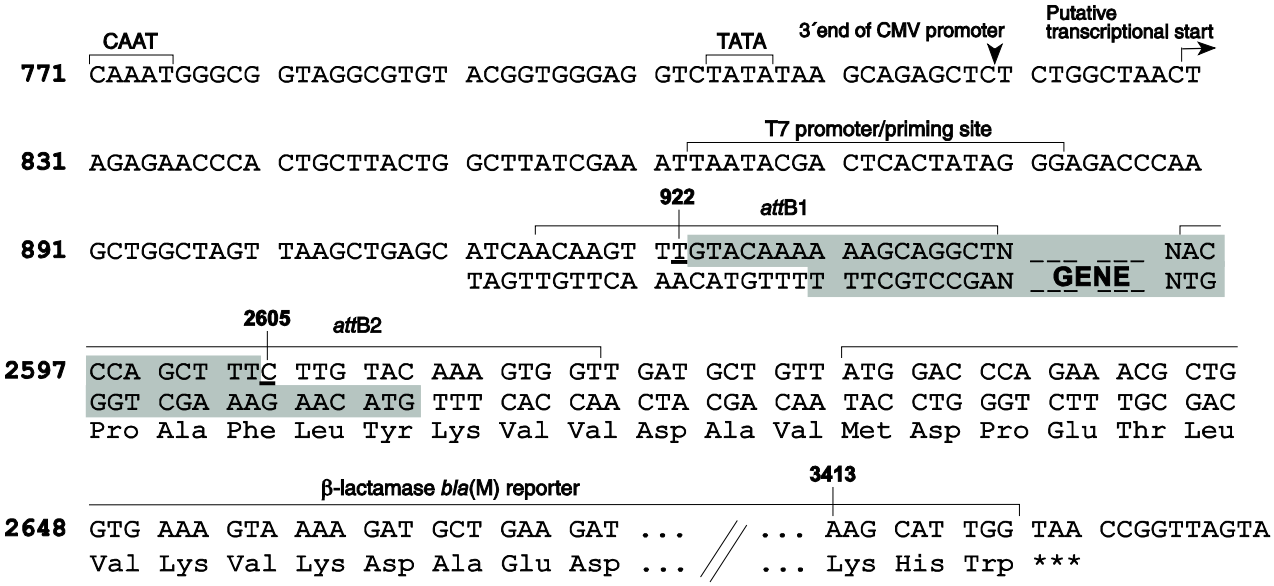
## Recombination Region for pcDNA™6.2/ cGeneBLAzer™-DEST

The recombination region of the expression clone resulting from pcDNA™6.2/ cGeneBLAzer™-DEST × entry clone is shown below.

**Note:** If you are using pcDNA™6.2/cGeneBLAzer™-DEST in the Tag-On-Demand™ System, your gene of interest must contain a TAG stop codon (see page **Error! Bookmark not defined.**).

**Features of the Recombination Region:**

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/cGeneBLAzer™-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/cGeneBLAzer™-DEST vector.
- Bases 922 and 2605 of the pcDNA™6.2/cGeneBLAzer™-DEST vector sequence are marked.

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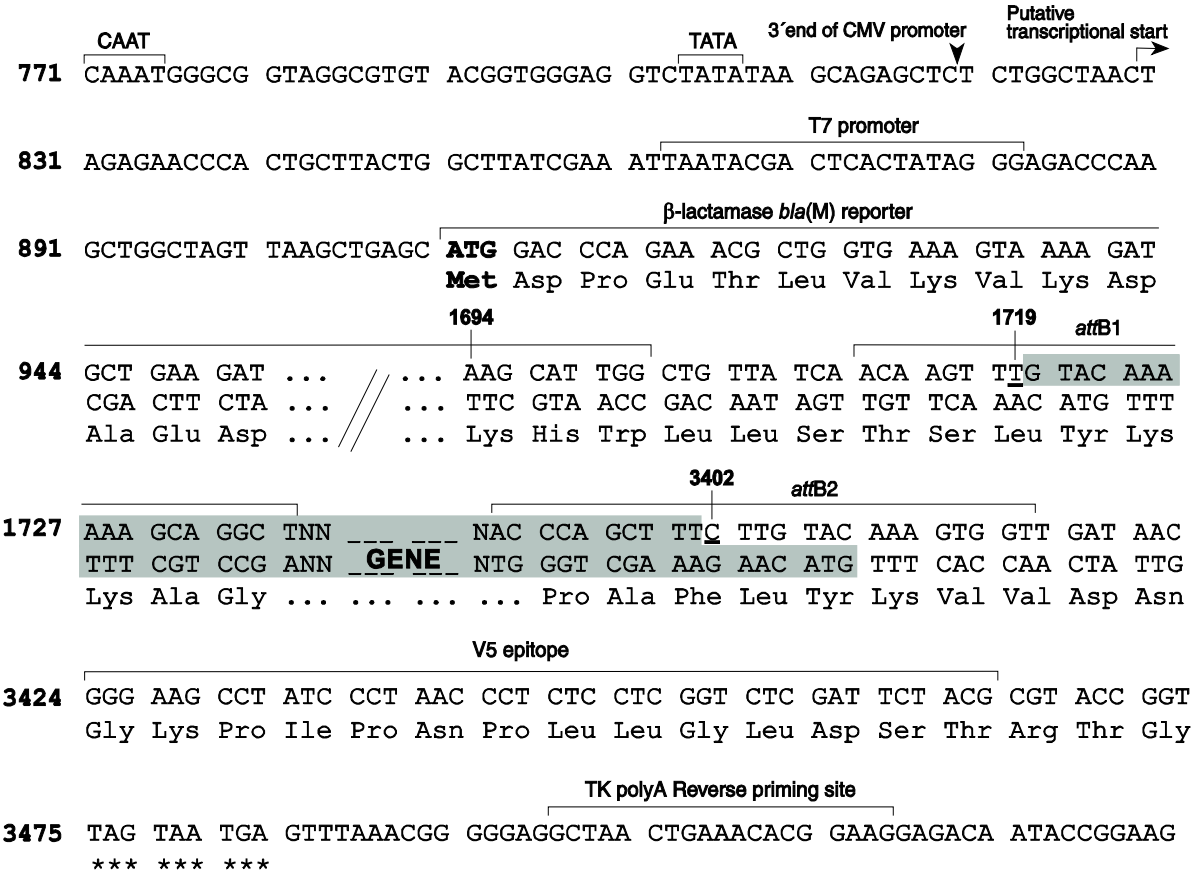
## Recombination Region for pcDNA™6.2/ nGeneBLAzer™-DEST

The recombination region of the expression clone resulting from pcDNA™6.2/ nGeneBLAzer™-DEST × entry clone is shown below.

**Note:** If you are using pcDNA™6.2/nGeneBLAzer™-DEST in the Tag-On-Demand™ System, your gene of interest must contain a TAG stop codon (see page **Error! Bookmark not defined.**).

**Features of the Recombination Region:**

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/nGeneBLAzer™-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/nGeneBLAzer™-DEST vector.
- Bases 1719 and 3402 of the pcDNA™6.2/nGeneBLAzer™-DEST vector sequence are marked.



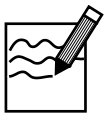
# Performing the LR Recombination Reaction

## Introduction

Once you have obtained an entry clone containing your gene of interest, you may perform an LR recombination reaction between the entry clone and your pcDNA™6.2/GeneBLAzer®-DEST vector, and transform the reaction mixture into a suitable *E. coli* host (see below) to select for an expression clone. We recommend including the pENTR™-gus positive control supplied with the LR Clonase® enzyme mix in your experiments to help you evaluate your results.

## *E. coli* Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, or equivalent for transformation. **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (e.g. TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.



**Note**

The presence of the EM7 promoter and the Blasticidin resistance gene in the pcDNA™6.2/GeneBLAzer®-DEST vectors allows for selection of *E. coli* transformants using Blasticidin. For selection, use Low Salt LB agar plates containing 100 µg/mL Blasticidin (see the **Appendix**, page 19 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 27 for ordering information). See the **Appendix**, page 20 for instructions on how to prepare and store Blasticidin.

## Materials Needed

- Purified plasmid DNA of your entry clone (50–150 ng/ µL in TE, pH 8.0)
- pcDNA™6.2/cGeneBLAzer™-DEST or pcDNA™6.2/nGeneBLAzer™-DEST vector (150 ng/µL in TE, pH 8.0)
- LR Clonase® enzyme mix (keep at –80°C until immediately before use; see page 27 for ordering)
- 5X LR Clonase® Reaction Buffer (supplied with the LR Clonase® enzyme mix)
- pENTR™-gus positive control, optional (50 ng/µL in TE, pH 8.0; supplied with the LR 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 LR 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 expression clones

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# Performing the LR Recombination Reaction, Continued

## Setting Up the LR 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 LR Clonase® enzyme mix (see Step 4).

| Component | Sample | Positive Control |
|---|---|---|
| Entry clone (100–300 ng/reaction) | 1–10 µL | – |
| Destination vector (150 ng/µL) | 2 µL | 2 µL |
| pENTR™-gus (50 ng/µL) | – | 2 µL |
| 5X LR Clonase® Reaction Buffer | 4 µL | 4 µL |
| TE Buffer, pH 8.0 | to 16 µL | 8 µL |

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

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

   **Note:** Extending the incubation time to 18 hours typically yields more colonies.
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 LR recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for expression clones.

   **Note:** You may store the LR 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 $1 \times 10^8$ cfu/µg, the LR reaction should give >5000 colonies if the entire LR reaction is transformed and plated.

## Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/mL chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

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### Sequencing Primers for pcDNA™6.2/cGene BLAzer™-DEST

To confirm that your gene of interest is in frame with the *bla*(M) reporter gene, you may sequence your expression 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 *att*B2 site and the 5′ region of the *bla*(M) reporter gene (see page 8 for a diagram).
- 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 expression construct.
- Use the T7 Promoter primer to sequence through the *att*B1 site and into the 5′ region of your gene of interest. Refer to the diagram on page 8 for the location of the T7 Promoter primer binding site.

### Sequencing Primers for pcDNA™6.2/nGene BLAzer™-DEST

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 expression 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 *att*B1 site and the 3′ region of the *bla*(M) reporter gene (see page 9 for a diagram).
- 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 expression construct.
- Use the TK polyA Reverse primer to sequence through the *att*B2 site and into the V5 epitope. Refer to the diagram on page 9 for the location of the TK polyA Reverse primer binding site.

# Transfecting Cells

## Introduction

This section provides general information for transfecting 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. 12578-043 and 12578-068 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 27 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 25 and 26 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 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.

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### 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 27 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 28).

# Creating Stable Cell Lines

## Introduction

The pcDNA™6.2/GeneBLAzer®-DEST 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®-DEST 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 (see page 27 for ordering). Refer to page 20 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.

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### 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®-DEST construct.

1. Transfect the mammalian cell line of interest with the pcDNA™6.2/cGeneBLAzer™-DEST or pcDNA™6.2/nGeneBLAzer™-DEST expression 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, you will 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™-DEST expression 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 the Anti-V5 Antibodies.

## *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, 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.

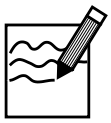
To detect β-lactamase activity in live mammalian cells, use the CCF2-AM substrate. CCF2-AM is the membrane-permeable, esterified form of CCF2, and is recommended for *in vivo* use because it is non-toxic, lipophilic, and readily enters the cell. Once cells are "loaded" with CCF2-AM, you may quantitate 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™-DEST 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 available (see page 27 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 27). The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to www.lifetechnologies.com/support or contact Technical Support (page 28).



**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 pages 8 and 9 for diagrams).

| Vector | Fusion | Expected Size Increase (kDa) |
|---|---|---|
| pcDNA™6.2/cGeneBLAzer™-DEST | β-lactamase (C-terminal) | 30 kDa |
| pcDNA™6.2/nGeneBLAzer™-DEST | β-lactamase (N-terminal) | 30 kDa |
| | V5 (C-terminal) | 3 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-gala*c*tosidase expression (see page 27 for ordering information).

# Appendix

## Recipes

### LB (Luria-Bertani) Medium and Plates

**Composition:**

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 (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 (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.

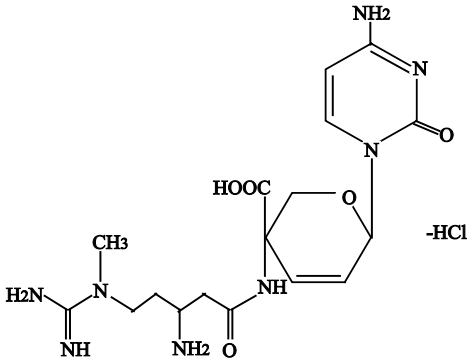
# Blasticidin

## 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

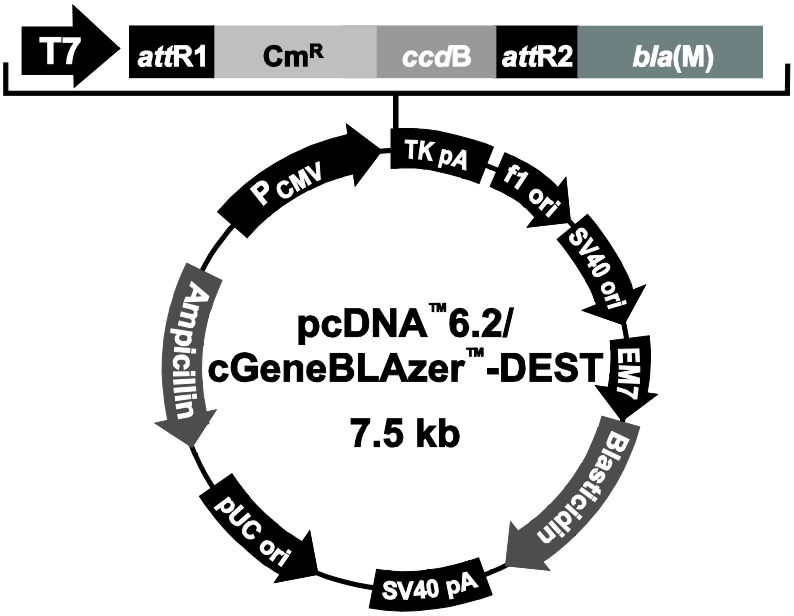
Blasticidin may be obtained separately in 50 mg aliquots (see page 27 for ordering). Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5– 10 mg/mL.

- 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™-DEST

## Map

The map below shows the elements of pcDNA™6.2/cGeneBLAzer™-DEST. DNA from the entry clone replaces the region between bases 918 and 2605. The nucleotide sequence of this vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 28).




**Comments for pcDNA™6.2/cGeneBLAzer™-DEST**
**7519 nucleotides**

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
*att*R1 site: bases 915-1039
Chloramphenicol resistance gene: bases 1148-1807
*ccd*B gene: bases 2149-2454
*att*R2 site: bases 2495-2619
β-lactamase *bla*(M) reporter gene: bases 2630-3424
TK polyadenylation signal: bases 3447-3718
f1 origin: bases 3754-4182
SV40 early promoter and origin: bases 4209-4517
EM7 promoter: bases 4572-4638
Blasticidin resistance gene: bases 4639-5037
SV40 early polyadenylation signal: bases 5195-5325
pUC origin (c): bases 5708-6378
Ampicillin resistance gene (c): bases 6523-7383
Ampicillin promoter (c): bases 7384-7482

(c) = complementary strand

*Continued on next page*

# Map and Features of pcDNA™6.2/cGeneBLAzer™-DEST, Continued

## Features

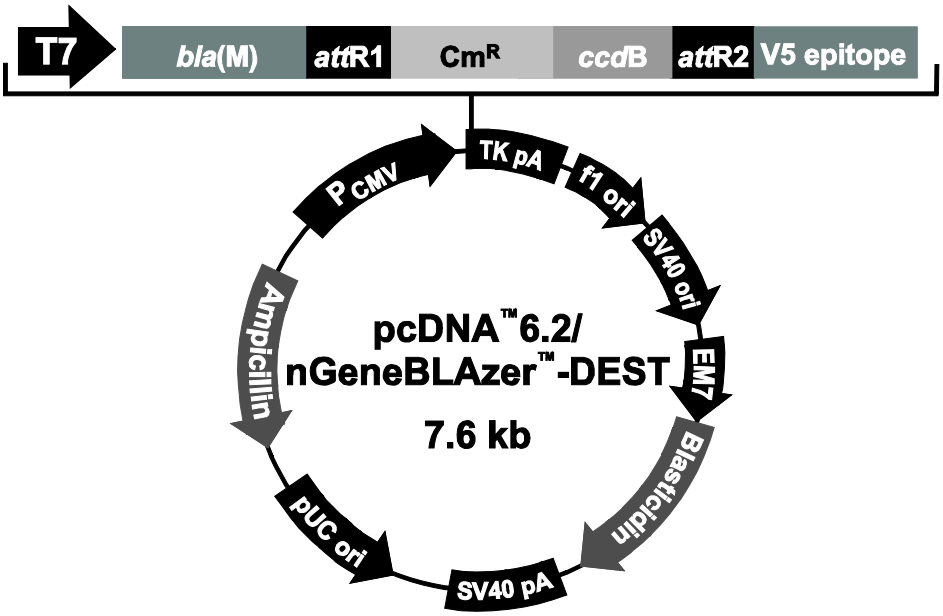
pcDNA™6.2/cGeneBLAzer™-DEST (7519 bp) contains the following elements. All features have been functionally tested.

| 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*R1 and *att*R2 sites | Allows recombinational cloning of the gene of interest from an entry clone |
| Chloramphenicol resistance gene | Allows counterselection of plasmid |
| *ccd*B gene | Allows negative selection of plasmid |
| 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™-DEST

## Map

The map below shows the elements of pcDNA™6.2/nGeneBLAzer™-DEST. DNA from the entry clone replaces the region between bases 1719 and 3402. The nucleotide sequence of this vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 28).




**Comments for pcDNA™6.2/nGeneBLAzer™-DEST**
**7564 nucleotides**

CMV promoter: bases 232-819
T7 promoter: bases 863-882
β-lactamase *bla*(M) reporter gene: bases 911-1702
*att*R1 site: bases 1712-1836
Chloramphenicol resistance gene: bases 1945-2604
*ccd*B gene: bases 2946-3251
*att*R2 site: bases 3292-3416
V5 epitope: bases 3424-3465
TK polyadenylation signal: bases 3492-3763
TK polyA reverse priming site: bases 3499-3517
f1 origin: bases 3799-4227
SV40 early promoter and origin: bases 4254-4562
EM7 promoter: bases 4617-4683
Blasticidin resistance gene: bases 4684-5082
SV40 early polyadenylation signal: bases 5240-5370
pUC origin (c): bases 5753-6423
Ampicillin resistance gene (c): bases 6568-7428
Ampicillin promoter (c): bases 7429-7527

(c) = complementary strand

*Continued on next page*

# Map and Features of pcDNA™6.2/nGeneBLAzer™-DEST, Continued

## Features

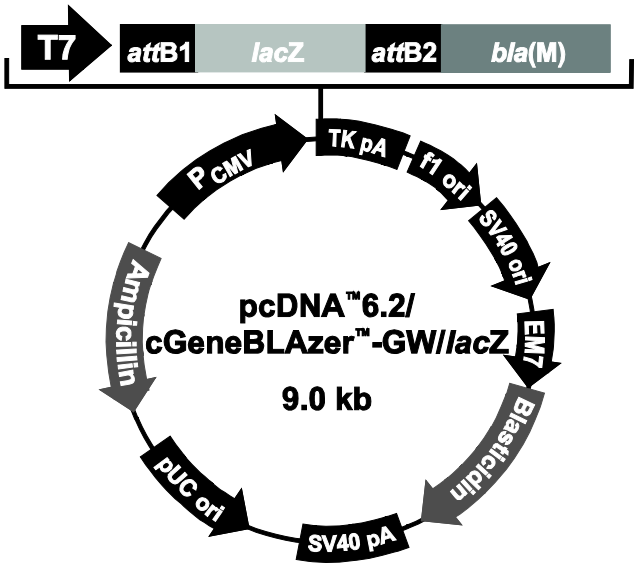
pcDNA™6.2/nGeneBLAzer™-DEST (7564 bp) contain the following elements. All features have been functionally tested.

| 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 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*R1 and *att*R2 sites | Allows recombinational cloning of the gene of interest from an entry clone |
| Chloramphenicol resistance gene | Allows counterselection of plasmid |
| *ccd*B gene | Allows negative selection of plasmid |
| 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 | Allows 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. pcDNA™6.2/cGeneBLAzer™-GW/*lacZ* 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. The nucleotide sequence of this vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 28).




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

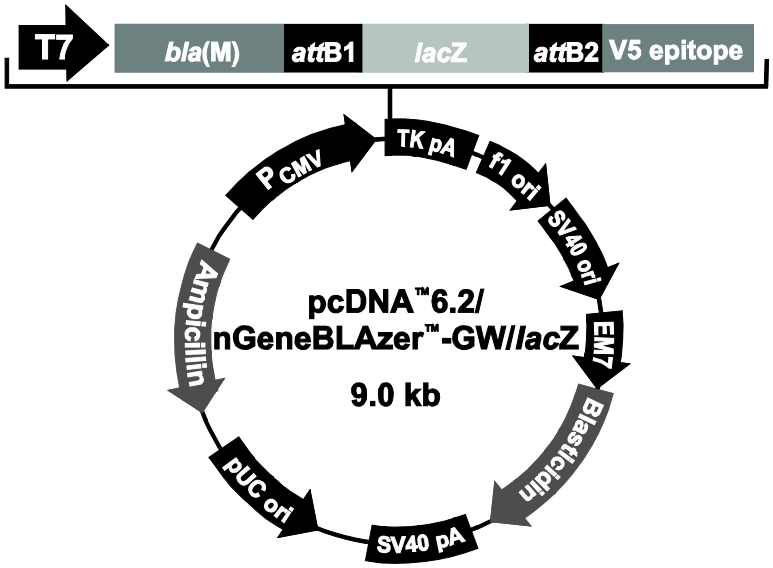
CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
*att*B1 site: bases 915-939
*lacZ* gene: bases 981-4040
*att*B2 site: bases 4057-4081
β-lactamase *bla*(M) reporter gene: bases 4092-4886
TK polyadenylation signal: bases 4909-5180
f1 origin: bases 5216-5644
SV40 early promoter and origin: 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. pcDNA™6.2/nGeneBLAzer™-GW/*lacZ* 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. The nucleotide sequence of this vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (28).




**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™ Gateway® Fusion Kits are available for purchase. Ordering information is provided below.

| Product | Amount | Catalog no. |
|---|---|---|
| pENTR™/D-TOPO® Cloning Kit | 20 reactions | K2400-20 |
| GeneBLAzer® *In Vitro* Detection Kit | 100 µg | 12578-126 |
| GeneBLAzer® *In Vivo* Detection Kit | 50 µg | 12578-134 |
| Gateway® LR Clonase® Enzyme Mix | 20 reactions | 11791-019 |
| | 100 reactions | 11791-043 |
| Library Efficiency® DB3.1™ Competent Cells | 1 mL (5 × 0.2 mL) | 11782-018 |
| One Shot® TOP10 Chemically Competent Cells | 10 reactions | C4040-10 |
| | 20 reactions | C4040-03 |
| One Shot® TOP10 Electrocompetent Cells | 10 reactions | C4040-50 |
| | 20 reactions | C4040-52 |
| PureLink® HQ Mini Plasmid Purification Kit | 100 reactions | K2100-01 |
| Lipofectamine® 2000 | 1.5 mL | 11668-019 |
| | 0.75 mL | 11668-027 |
| 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™-DEST 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).<br>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.

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**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 outlicensing@lifetech.com.

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**Headquarters**
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
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