USER GUIDE

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pcDNA™6 BioEase™ Gateway® Biotinylation System

Gateway®-adapted destination vector for cloning and expression of biotinylated fusion proteins in mammalian cells

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

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Contents

Kit Contents and Storage

Shipping and Storage

The pcDNA™6 BioEase™ Gateway® Biotinylation System is shipped at 4°C and contains the following items. Upon receipt, store as detailed in the following table.

Item	Storage
Vectors	–30°C to –10°C
Streptavidin-HRP Conjugate	2°C to 8°C

Note: Avoid freezing the streptavidin-HRP conjugate as this may result in loss of activity.

Contents

The following reagents are supplied in the pcDNA™6 BioEase™ Gateway® Biotinylation System. The amount of streptavidin-HRP conjugate supplied is sufficient for 25 western blots using a 10 mL working solution. **Store the vectors at –30°C to –10°C and the streptavidin-HRP conjugate at 2°C to 8°C.**

Vector	Composition	Amount
pcDNA™6/BioEase™-DEST vector	40 µL of vector at 150 ng/µL in TE, pH 8.0	6 µg
pcDNA™6/BioEase™-GW/lacZ control vector	20 µL of vector at 0.5 µg/µL in TE, pH 8.0	10 µg
Streptavidin-HRP Conjugate	Supplied in MOPS Buffer; refer to the label on the tube for concentration	50 µL

Introduction

About the kit

System Overview

The pcDNA™6 BioEase™ Gateway® Biotinylation System is designed to allow high-level expression of biotinylated recombinant fusion proteins in mammalian cells. Biotinylated recombinant protein may then be easily detected or immobilized to a solid support for other downstream applications. The main component of the System is the pcDNA™6/BioEase™-DEST vector into which the gene of interest is inserted through Gateway® cloning. For more information about the pcDNA™6/BioEase™-DEST vector, see below. For more information about the Gateway® Technology, see page 6.

Features of the Vector

The pcDNA™6/BioEase™-DEST vector contains the following elements:

- The human cytomegalovirus (CMV) immediate early enhancer/promoter for high-level constitutive expression of the gene of interest in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
- BioEase™ tag to allow biotinylation of the recombinant protein of interest for easy detection or use in other applications.
- 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 (Cm^R) located between the two *att*R sites for counterselection.
- The *ccd*B gene located between the *att*R sites for negative selection.
- Blasticidin (*bsd*) resistance gene for selection of stable cell lines using blasticidin (Izumi *et al.*, 1991; Kimura *et al.*, 1994; Takeuchi *et al.*, 1958).
- Ampicillin resistance gene for selection in *E. coli.*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli.*

The control plasmid, pcDNA™6/BioEase™-GW/lacZ, is included for use as a positive control for transfection and expression in the mammalian cell line of choice.

BioEase™ Tag

The BioEase™ tag is a 72 amino acid peptide derived from the C-terminus (amino acids 524–595) of the *Klebsiella pneumoniae* oxalacetate decarboxylase α subunit (Schwarz *et al.*, 1988). Biotin is covalently attached to the oxalacetate decarboxylase α subunit and peptide sequencing has identified a single biotin binding site at lysine 561 of the protein (Schwarz *et al.*, 1988). When fused to a heterologous protein, the BioEase™ tag is both necessary and sufficient to facilitate *in vivo* biotinylation of the recombinant protein of interest (see the diagram on page 9 for the location of the biotin binding site). The entire 72 amino acid domain is required for recognition by the cellular biotinylation enzymes. For more information about the cellular biotinylation enzymes and the mechanism of biotinylation, refer to the review by Chapman-Smith and Cronan, 1999.

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The Gateway® Technology

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 the Gateway® Technology:

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone.
2. Perform an LR recombination reaction between the entry clone and a Gateway® destination vector (*e.g.* pcDNA™6/BioEase™-DEST). Transform *E. coli* and select for an expression clone.
3. Purify plasmid and transfect your expression clone into the mammalian cell line of interest.

For more detailed information about the Gateway® Technology, generating an entry clone, and performing the LR recombination reaction, refer to the Gateway® Technology manual. This manual is available from **www.lifetechnologies.com/manuals** or by contacting Technical Support (see page 22).

Methods

Using pcDNA™6/BioEase™-DEST

IMPORTANT!

The pcDNA™6/BioEase™-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual has previously recommended using a linearized destination vector for more efficient recombination, further testing has found that linearization of pcDNA™6/BioEase™-DEST is **not** required to obtain optimal results for any downstream application.

Propagating the Vector

To propagate and maintain the pcDNA™6/BioEase™-DEST vector, we recommend using One Shot® *ccdB* Survival™ 2 T1^R Phage-Resistant Cells (see page 21 for ordering information) for transformation. The One Shot® ccdB Survival™ 2 T1^R Phage-Resistant Cells *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50–100 µg/mL ampicillin and 15–30 µg/mL chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5α for propagation and maintenance as these strains are sensitive to CcdB effects.

Entry Clone

To recombine your gene of interest into pcDNA™6/BioEase™-DEST, you should have an entry clone containing your gene of interest. Refer to the Gateway® Technology manual for details on choosing a Gateway® entry vector and constructing an entry clone. The Gateway® Technology manual is available from **www.lifetechnologies.com/manuals** or by contacting Technical Support (see page 22).

Points to Consider Before Recombining into pcDNA™6/ BioEase™-DEST

pcDNA™6/BioEase™-DEST is an N-terminal fusion vector and contains an ATG initiation codon in the context of a Kozak consensus sequence to ensure optimal translation initiation (Kozak, 1987; Kozak, 1991; Kozak, 1990). Your gene of interest in the entry clone must:

- Be in frame with the N-terminal BioEase™ tag after recombination
- Contain a stop codon

Refer to the diagram of the recombination region of pcDNA™6/BioEase™-DEST on the page 9 for more information.

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Recombining Your Gene of Interest

Each entry clone contains *att*L sites flanking the gene of interest. Genes in an entry clone are transferred to the destination vector backbone by mixing the DNAs with the Gateway® LR Clonase™ Enzyme Mix (see page 21 for ordering information). The resulting LR recombination reaction is then transformed into *E. coli* (e.g. TOP10 or DH5α™-T1^R) and the expression clone selected using ampicillin. Recombination between the *att*R sites on the destination vector and the *att*L sites on the entry clone replaces the chloramphenicol (Cm^R) gene and the *ccd*B gene with the gene of interest and results in the formation of *att*B sites in the expression clone.

Follow the instructions in the Gateway® Technology manual to set up the LR recombination reaction, transform *E. coli*, and select for the expression clone.

Note

The presence of the blasticidin resistance gene in pcDNA™6/BioEase™-DEST allows selection of *E. coli* transformants using blasticidin. For selection, use Low Salt LB agar plates containing 100 µg/mL blasticidin (see page 20 for a recipe). Note that for blasticidin to be active, the salt concentration of the bacterial medium must remain low (< 90 mM) and the pH must be 7.0.

Blasticidin is available separately (see page 21 for ordering information). For more information about blasticidin, see the **Appendix**, page 19.

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Recombination Region of pcDNA™6/BioEase™-DEST

The recombination region of the expression clone resulting from pcDNA™6/BioEase™-DEST x entry clone is shown in the following graphic.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pcDNA™6/BioEase™-DEST vector by recombination. Non-shaded regions are derived from the pcDNA™6/BioEase™-DEST vector.
- Bases 1191 and 2853 of the pcDNA™6/BioEase™-DEST sequence are marked.
- The biotin binding site is labeled with a *.
- Potential stop codons are underlined.

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 should not grow in the presence of chloramphenicol.

Sequencing

To confirm that your gene of interest is in frame with the BioEase™ tag, sequence your expression construct, if desired. We recommend using the priming sites indicated in the diagram above (i.e. BioEase™ forward and BGH reverse) to help you sequence your insert. Life Technologies has a custom primer synthesis service. For more information, call Technical Support (see page 22).

Transfection

Introduction

After generating your expression clone, you are ready to transfect the plasmid into the mammalian cells of choice. You may perform transient transfection experiments or use blasticidin selection to generate stable cell lines.

Plasmid Preparation

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 lipids decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® HiPure Miniprep Kit or the PureLink® HiPure Midiprep Kit (see page 21 for ordering information).

Positive Control

pcDNA™6/BioEase™-GW/lacZ is provided as a positive control vector for mammalian transfection and expression (see page 18 for a map) and may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the control of the CMV promoter. A successful transfection will result in β-galactosidase expression that can be easily assayed.

To propagate and maintain the pcDNA™6/BioEase™-GW/lacZ plasmid:

1. Transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™-T1^R, or equivalent. Use 10 ng of plasmid for transformation.
2. Select transformants on LB agar plates containing 50–100 µg/mL ampicillin or Low Salt LB agar plates containing 100 µg/mL blasticidin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

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 and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Life Technologies offers a large selection of reagents for transfection including Lipofectamine™ 2000 and the Calcium Phosphate Transfection Kit (see page 21 for ordering information). Other transfection reagents are also available. For more information, see **www.lifetechnologies.com** or call Technical Support (see page 22).

Generate Stable Cell Lines

Introduction

The pcDNA™6/BioEase™-DEST vector contains the blasticidin resistance gene to allow selection of stable cell lines using blasticidin (see the following guidelines).

We recommend linearizing your pcDNA™6/BioEase™-DEST expression construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector will not integrate in a way that disrupts elements necessary for expression in mammalian cells. To linearize your construct, use a unique restriction site that is not located within a critical element or within your gene of interest.

Determine Blasticidin Sensitivity

To generate a stable cell line, you need to determine the minimum concentration of blasticidin required to kill your untransfected mammalian cell line. Typically, concentrations ranging from 2.5–10 µg/mL blasticidin are sufficient to kill most untransfected mammalian cell lines. We recommend testing a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line. Refer to the **Appendix**, page 19 for instructions to prepare and store blasticidin.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 6 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of blasticidin (0, 1.25, 2.5, 5, 7.5, 10 µg/mL blasticidin).
3. Replenish the selective medium every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular interval to determine the appropriate concentration of blasticidin that kills the cells within 10 days after addition of blasticidin.

Select Stable Transfectants

Use the following guidelines to generate a stable cell line with your pcDNA™6/BioEase™-DEST expression construct.

1. Transfect mammalian cells with your pcDNA™6/BioEase™-DEST construct using the desired protocol and transfection reagent. Remember to include the pcDNA™6/BioEase™-GW/lacZ plasmid as a positive control and a plate of untransfected cells as a negative control.
2. 48 hours after transfection, split the cells into fresh medium. Split the cells such that they are no more than 25% confluent.
3. Incubate the cells at 37°C for 2–3 hours to allow attachment to the culture dish.
4. Remove the medium and add fresh medium containing blasticidin at the pre-determined concentration required for your cell line.
5. Replenish the selective medium every 3–4 days until foci can be identified. **Note:** Selection generally takes up to 10 days after addition of blasticidin.
6. Pick 10–20 blasticidin-resistant foci and expand them to assay for recombinant protein expression.

Expression and Analysis of Recombinant Fusion Protein

Introduction

You may assay for expression of your recombinant fusion protein from either transiently transfected cells or stable cell lines. Because your recombinant fusion protein is biotinylated, you may take advantage of the strong association between biotin and avidin (and its analogs including streptavidin) to easily detect your recombinant protein.

Note: You may also use your biotinylated protein for ELISA-related applications or for affinity purification by immobilization on a solid support.

Detect Recombinant Fusion Proteins

To detect expression of your biotinylated, recombinant fusion protein, you may perform:

- Western blot analysis using the Streptavidin-HRP conjugate supplied with the kit (see page 14 for a protocol), another streptavidin conjugate, or an antibody to your protein
- Immunofluorescence using the Streptavidin-Fluorescein Isothiocyanate (FITC) conjugate available from Life Technologies (see page 21 for ordering information)
- Functional analysis

For more information about the streptavidin conjugates above and other conjugates available, refer to **www.lifetechnologies.com** or call Technical Support (see page 22).

Assay for β-galactosidase

If you use the pcDNA™6/BioEase™-GW/lacZ positive control vector for your expression studies, you may assay for β-galactosidase expression by western blot analysis or activity assay using cell-free lysates (Miller, 1972). Life Technologies offers the β-Gal Staining Kit and the β-Gal Assay Kit (see page 21 for ordering information) for fast and easy detection of β-galactosidase expression.

Note: You may also detect β-galactosidase expression using any of the streptavidin conjugates mentioned in the preceding section.

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Prepare Cell Lysates

To detect your fusion recombinant protein by western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided following this paragraph. Other protocols are suitable. To lyse cells:

1. Wash cell monolayers (~5×10^5 to 1×10^6 cells) once with phosphate-buffered saline (PBS, see page 21 for ordering information).
2. Scrape cells into 1 mL PBS and pellet the cells at 1500 × *g* for 5 minutes.
3. Resuspend the cells in 50 µL Cell Lysis Buffer (see the **Appendix**, page 20 for a recipe). Other cell lysis buffers are suitable. Vortex.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells. **Note:** You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 × g for 10 minutes at 4°C to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration. **Note:** Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer (see page 20 for a recipe) to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available. In addition, Life Technologies carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to **www.lifetechnologies.com** or call Technical Support (see page 22).

Solutions Required for Immunoblotting (Western Blotting)

The following materials and solutions are needed for western blotting:

- Tris-Buffered Saline (TBS: 20 mM Tris-HCl, 140 mM NaCl, pH 7.5)
- Tris-Buffered Saline with Tween 20 (TBST: TBS with 0.1% Tween-20, v/v)
- Blocking Buffer (TBS with 5% nonfat dry milk, w/v)
- Dilution Buffer (TBST with 1% nonfat dry milk, w/v)
- Streptavidin-HRP conjugate (supplied with the kit; recommended dilution for western blotting is 1:5000)
- Appropriate reagents to detect streptavidin conjugates using colorimetric or chemiluminescent methods.

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Immunoblotting Protocol

Before beginning, prepare an SDS polyacrylamide gel (either Tris/Glycine or Tris/Tricine) designed to resolve your recombinant fusion protein or use a pre-cast SDS polyacrylamide gel.

1. Load your samples (from Step 7, **Prepare Cell Lysates**) and electrophorese your SDS polyacrylamide gel.
2. Transfer proteins to nitrocellulose or any other suitable membrane electrophoretically using 25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol as a transfer buffer. Other transfer buffers are suitable.

   Note: If you are using NuPAGE® gels or other types of pre-cast gels, other transfer buffers may be required. Refer to the manufacturer's instructions to transfer proteins to the membrane of choice.
3. Run at 100V, 150 mA (100V, 240 mA at the finish) for 1 hour. Be sure to have a cooling system in place. You may also transfer overnight at 30V, 40 mA (30V, 90 mMA at the finish).
4. Remove membrane and incubate it in 10 mL of Blocking Buffer. Gently agitate using a rocker platform for 1 hour at room temperature.

   Note: You may store the blot overnight at this step, if needed. Keep the blot in Blocking Buffer and store at 4°C covered with plastic wrap.
5. Wash membrane in 20 mL TBST for 5 minutes with gentle agitation. Repeat twice.
6. Transfer the membrane to a tray containing the streptavidin-HRP conjugate diluted 1:5000 in 10 mL of Dilution Buffer. Incubate with gentle agitation for at least 1 hour at room temperature. Longer incubations may be performed, but may increase background.
7. Wash membrane in:
   - 20 mL TBST for 3 × 5 minutes with gentle agitation
   - 20 mL TBS for 3 × 5 minutes with gentle agitation
   - 20 mL sterile water for 5 minutes with gentle agitation.
8. Proceed to detect the streptavidin-HRP conjugate using the appropriate colorimetric or chemiluminescence reagents.

Note

The N-terminal peptide containing the BioEase™ tag and EK recognition site will add approximately 9.8 kDa to your protein.

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Purify the Recombinant Fusion Protein

To purify your recombinant fusion protein, you may use Streptavidin-Agarose (see page 21 for ordering information). Proteins may be purified under native or denaturing conditions. For more information, refer to **www.lifetechnologies.com** or call Technical Support (see page 22).

Cleavage of the BioEase™ Tag

pcDNA™6/BioEase™-DEST contains an enterokinase (EK) recognition site to allow removal of the BioEase™ tag from your recombinant fusion protein, if desired. Note that after digestion with enterokinase, 12 amino acids will remain at the N-terminus of your protein (see diagram on page 9).

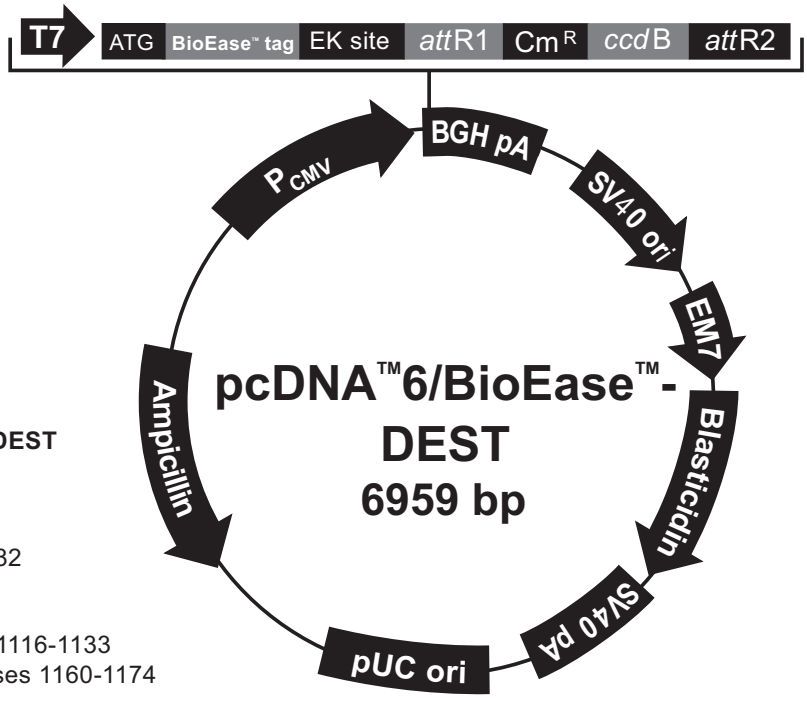
A recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) is available (see page 21 for ordering information) to remove the BioEase™ tag from your recombinant fusion protein. Instructions for digestion are included with the product. For more information, see **www.lifetechnologies.com** or contact Technical Support (see page 22).

Appendix

Map and Features of pcDNA™6/BioEase™-DEST

Map of pcDNA™6/BioEase™-DEST

The following map shows the elements of pcDNA™6/BioEase™-DEST. DNA from the entry clone replaces the region between bases 1191 and 2853. The sequence for pcDNA™6/BioEase™-DEST is available from **www.lifetechnologies.com** or by contacting Technical Support (see page 22).



Comments for pcDNA™6/BioEase™-DEST
6959 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
ATG initiation codon: bases 920-922
BioEase™ tag: bases 923-1138
BioEase™ forward priming site: bases 1116-1133
Enterokinase (EK) recognition site: bases 1160-1174
*att*R1: bases 1184-1308
Chloramphenicol resistance gene (Cm^R): bases 1417-2076
*ccd*B gene: bases 2397-2702
*att*R2: bases 2743-2867
BGH reverse priming site: bases 2914-2931
BGH polyadenylation signal: bases 2920-3144
f1 origin: bases 3190-3617
SV40 early promoter and origin: bases 3645-3954
EM7 promoter: bases 4009-4075
Blasticidin resistance gene: bases 4076-4474
SV40 early polyadenylation signal: bases 4632-4762
pUC origin: bases 5145-5818 (complementary strand)
bla promoter: bases 6824-6922 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 5963-6823 (complementary strand)

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Map and Features of pcDNA™6/BioEase™-DEST, Continued

Features of pcDNA™6/BioEase™-DEST

The pcDNA™6/BioEase™-DEST vector (6959 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant fusion protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert.
BioEase™ tag	Allows biotinylation of recombinant fusion protein (Schwarz *et al.*, 1988).
BioEase™ forward priming site	Permits sequencing through the insert.
Enterokinase (EK) recognition site (Asp-Asp-Asp-Asp-Lys)	Allows removal of the BioEase™ tag from your recombinant fusion protein using an enterokinase such as EKMax™ (Catalog no. E180-01).
*att*R1 and *att*R2 sites	Bacteriophage -derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
BGH reverse priming site	Permits sequencing through the insert.
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992).
f1 origin	Allows single strand rescue of DNA.
SV40 early promoter and origin	Permits efficient, high-level expression of the blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Permits expression of the blasticidin resistance gene in *E. coli*.
Blasticidin (*bsd*) resistance gene	Permits 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	Permits high-copy replication and maintenance in *E. coli*.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.

Map of pcDNA™6/BioEase™-GW/lacZ

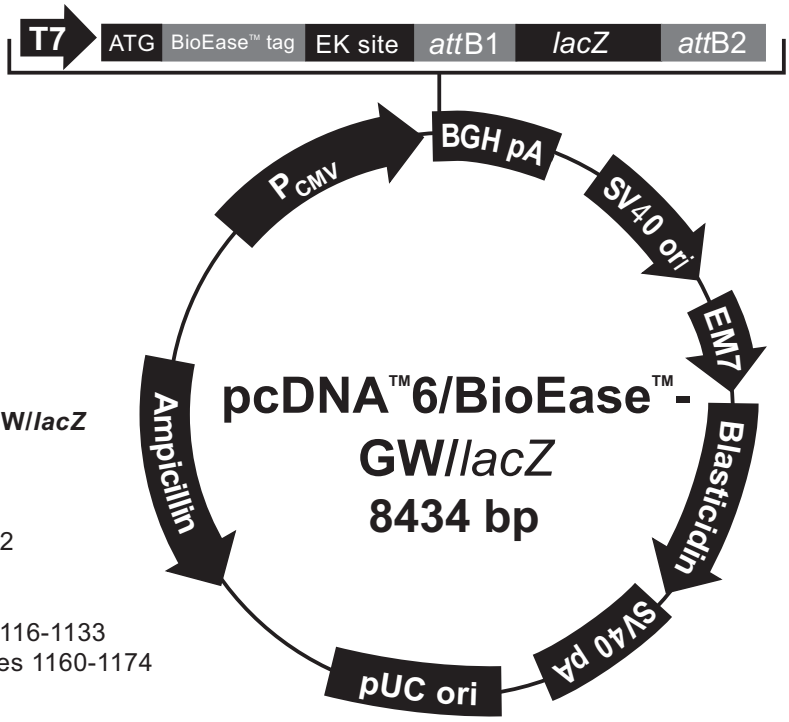
Description

pcDNA™6/BioEase™-GW/lacZ is a 8434 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pcDNA™6/BioEase™-DEST. β-galactosidase is expressed as an N-terminal fusion protein with a molecular weight of approximately 129 kDa.

Note: The β-galactosidase fusion protein includes amino acids contributed by the *att*B1 and *att*B2 sites.

Map of the Vector

The map below shows the elements of pcDNA™6/BioEase™-GW/lacZ. The sequence of the vector is available from **www.lifetechnologies.com** or by calling Technical Support (see page 22).



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8434 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
ATG initiation codon: bases 920-922
BioEase™ tag: bases 923-1138
BioEase™ forward priming site: bases 1116-1133
Enterokinase (EK) recognition site: bases 1160-1174
*att*B1: bases 1184-1208
lacZ ORF: bases 1229-4285
*att*B2: bases 4318-4342
BGH reverse priming site: bases 4389-4406
BGH polyadenylation signal: bases 4395-4619
f1 origin: bases 4665-5093
SV40 early promoter and origin: bases 5120-5429
EM7 promoter: bases 5484-5550
Blasticidin resistance gene: bases 5551-5949
SV40 early polyadenylation signal: bases 6107-6237
pUC origin: bases 6620-7293 (complementary strand)
bla promoter: bases 8299-8397 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 7438-8298 (complementary strand)

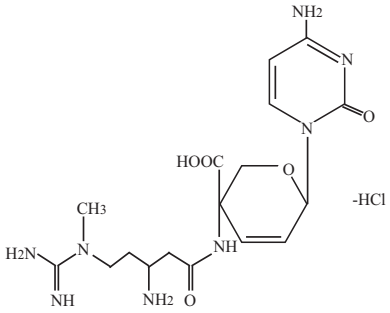
Blasticidin

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 following diagram 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 21 for ordering information) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5–10 mg/mL. Use the following guidelines to prepare and store a stock blasticidin solution:

- 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.
- The 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.

Recipes

Low Salt LB Medium with Blasticidin

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 the pH to 7.0 with 5 M NaOH. Bring the volume up to 1 L. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C and add 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.

Cell Lysis Buffer

50 mM Tris, pH 7.8

150 mM NaCl

1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 mL, combine

1 M Tris base	5 mL
5 M NaCl	3 mL
Nonidet P-40	1 mL

2. Bring the volume up to 90 mL with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 mL. Store at room temperature.

To prevent proteolysis, you may add 1 mM PMSF, 1 µM leupeptin, or 0.1 µM aprotinin before use.

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	5 mL
Glycerol (100%)	4 mL
β -mercaptoethanol	0.8 mL
Bromophenol Blue	0.04 g
SDS	0.8 g

2. Bring the volume to 10 mL with sterile water.
3. Aliquot and freeze at –20°C until needed.

Accessory Products

Additional Products

Additional streptavidin-HRP conjugate and other products that may be used with the pcDNA™6 BioEase™ Gateway® Biotinylation System are available separately. For more information, refer to **www.lifetechnologies.com** or call Technical Support (see page 22).

Product	Amount	Catalog no.
Gateway® LR Clonase® Enzyme Mix	20 reactions	11791-019
One Shot® ccdB Survival™ 2 T1^R Phage-Resistant Cells	10 transformations	A10460
One Shot® TOP10 Chemically Competent *E. coli*	20 x 50 µL	C4040-03
	40 x 50 µL	C4040-06
PureLink® HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink® HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Lipofectamine® 2000 Reagent	1.5 mL	11668-019
	0.75 mL	11668-027
Calcium Phosphate Transfection Kit	75 reactions	K2780-01
Blasticidin HCl	50 mg	R210-01
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL	10010-023
EKMax™	250 units	E180-01
	1000 units	E180-02
ß-Gal Assay Kit	80 mL	K1455-01
ß-Gal Staining Kit	1 kit	K1465-01
Streptavidin-HRP Conjugate	50 µL*	SA100-01
Streptavidin-FITC Conjugate	50 µL*	SA100-02
Streptavidin-Agarose	5 mL	SA100-04

*The amount of conjugate supplied is sufficient for 25 western blots.

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