invitrogen™

Vivid Colors™ pcDNA™ 6.2/ EmGFP-Bsd/V5-DEST Mammalian Expression Vector Kit

Gateway-adapted destination vector for simultaneous expression and fluorescence-based detection of V5 fusion proteins in mammalian cells

Catalog No. V366-20
Version C

29 October 2010

25-0859

User Manual

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Kit Contents and Storage

Shipping and Storage

The pcDNA™6.2/EmGFP-Bsd/V5-DEST and pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT vectors are shipped at room temperature. Upon receipt, store lyophilized vectors at -20°C.

Contents

The pcDNA™6.2/EmGFP-Bsd/V5-DEST Mammalian Expression Vector Kit includes a destination vector and a corresponding expression control plasmid containing the CAT gene as listed below:

Item	Concentration	Amount
pcDNA™6.2/EmGFP-Bsd/V5-DEST	Lyophilized in TE, pH 8.0	6 µg
pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT	Lyophilized in TE, pH 8.0	10 µg

Quality Control

Each vector is qualified by restriction endonuclease digestion. In addition, the functionality of the destination vector is qualified in an LR recombination assay using Gateway® LR Clonase™ II Enzyme Mix. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

Accessory Products

Additional Products

Additional products that may be used with the pcDNA™6.2/EmGFP-Bsd/V5-DEST Mammalian Expression Vector are available from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
Ultimate™ ORF Clones	1 glycerol stock	HORF01
Gateway® LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
One Shot® TOP10 Chemically Competent Cells	10 reactions 20 reactions	C4040-10 C4040-03
One Shot® TOP10 Electrocompetent Cells	10 reactions 20 reactions	C4040-50 C4040-52
One Shot® *ccd*B Survival T1^R Chemically Competent Cells	10 reactions	C7510-03
Tag-On-Demand™ Suppressor Supernatant	200 µl 5 x 200 µl	K400-01 K405-01
PureLink™ HQ Plasmid Miniprep Kit	100 reactions	K2100-01
Lipofectamine™ 2000	0.75 ml 1.5 ml	11668-027 11668-019
Blasticidin	50 mg	R210-01
CAT Antiserum	50 µl	R902-25
Anti-V5 Antibody	50 µl	R960-25
Anti-V5-HRP Antibody	50 µl	R961-25
Anti-V5-AP Antibody	50 µl	R962-25
WesternBreeze® Chemiluminescent Kit, Anti-Rabbit	20 reactions	WB7106
WesternBreeze® Chemiluminescent Kit, Anti-Mouse	20 reactions	WB7104
WesternBreeze® Chromogenic Kit, Anti-Rabbit	20 reactions	WB7105
WesternBreeze® Chromogenic Kit, Anti-Mouse	20 reactions	WB7103

Introduction

Overview

Description

pcDNA™6.2/Em-GFP-Bsd/V5-DEST is an 8.1 kb vector that is adapted with the Gateway® Technology that allows high-level, constitutive expression of a gene of interest in mammalian cells. Users can make a V5 epitope tagged expression clone by performing an LR reaction between a Gateway® entry vector containing the gene of choice and the pcDNA™6.2/Em-GFP-Bsd/V5-DEST vector. After transfection of the expression clone into mammalian cells, the protein of interest can be identified by Western blot or other functionally relevant assay.

A separate transcriptional unit on pcDNA™6.2/EmGFP-Bsd/V5-DEST allows high-level constitutive expression of Emerald Green Fluorescent Protein (EmGFP) fused to the Blasticidin resistance gene, allowing non-invasive monitoring of transfection efficiency by fluorescence microscopy as well as selection of stable cell lines using Blasticidin.

A control expression plasmid, pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT, is also included for transfection and expression optimization.

Features of the Vector

The pcDNA™6.2/EmGFP-Bsd/V5-DEST vector contains the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression of your gene of interest in a wide range of mammalian cells
- 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
- The *ccd*B gene located between the two *att*R sites for negative selection
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- C-terminal V5 epitope tag for detection of recombinant protein using Anti-V5 antibodies
- Murine PGK promoter for high level expression of the EmGFP-Blasticidin gene fusion
- Emerald Green Fluorescent Protein (EmGFP, derived from *Aequorea victoria* GFP) fused to the Blasticidin resistance gene for fluorescent detection of transfected cells and for selection in both *E. coli* and mammalian cell lines
- 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*
- pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- Ampicillin (*bla*) resistance gene for selection in *E. coli*

For the map and features of the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector, see page 22.

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Overview, continued

PGK Promoter

The pcDNA™6.2/EmGFP-Bsd/V5-DEST and pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT vectors contain the murine phosphoglycerate kinase-1 (PGK) promoter to drive high-level mammalian expression of the EmGFP-Blasticidin fusion. In some mammalian cell types, the activity of viral promoters may become significantly reduced over time due to promoter silencing. The PGK promoter is a ubiquitous housekeeping promoter (Adra *et al.*, 1987) that has been shown to promote long-term persistent expression (Hamaguchi *et al.*, 2000), and may provide consistent expression in cells that are susceptible to promoter silencing from methylation (Curradi *et al.*, 2002) or histone deacetylation (Rietveld *et al.*, 2002), such as undifferentiated embryonic stem (ES) cells (Hamaguchi *et al.*, 2000), (Gerolami *et al.*, 2000).

Green Fluorescent Protein (GFP)

Green Fluorescent Protein (GFP) is a naturally occurring bioluminescent protein derived from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962). GFP emits fluorescence upon excitation, and the gene encoding GFP contains all of the necessary information for posttranslational synthesis of the luminescent protein. GFP is often used as a molecular beacon because it requires no species-specific cofactors for function, and is easily detected using fluorescence microscopy and standard filter sets. Commonly, GFP is fused to a protein of interest, and upon expression, the localization of the fusion protein can be detected in cells. GFP can also function as a reporter gene downstream of a promoter of interest.

GFP and Spectral Variants

Modifications have been made to the wild-type GFP to enhance its expression in mammalian systems. These modifications include nucleic acid substitutions that correspond to the codon preference for mammalian use, and mutations that increase the brightness of the fluorescent signal, resulting in "enhanced" GFP (Zhang *et al.*, 1996). Mutations have also arisen or have been introduced into GFP that further enhance and shift the spectral properties of GFP such that these proteins will emit fluorescent color variations (reviewed in Tsien, 1998). The Emerald GFP (EmGFP) is a variant of enhanced GFP.

EmGFP

The EmGFP variant has been described in a published review (Tsien, 1998) and the amino acid changes are summarized in the table below. The mutations are represented by the single letter abbreviation for the amino acid in the consensus GFP sequence, followed by the codon number and the single letter amino acid abbreviation for the substituted amino acid.

Fluorescent Protein	GFP Mutations*
EmGFP	S65T, S72A, N149K, M153T, I167T

*Mutations listed are as described in the literature. When examining the actual sequence, the vector codon numbering starts at the first amino acid **after** the initiation methionine of the fluorescent protein, so that mutations appear to be increased by one position. For example, the S65T mutation actually occurs in codon 66 of EmGFP.

Continued on next page

Overview, continued

Spectral Properties of EmGFP Fluorescence

EmGFP expressed from the pcDNA6.2/EmGFP-Bsd/V5-DEST vector has the following excitation and emission wavelengths, as published in the literature (Tsien, 1998):

Fluorescent Protein	Excitation (nm)	Emission (nm)
EmGFP	487	509

Filter Set for Detecting EmGFP Fluorescence

The fluorescent signal from EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescent signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for each of the fluorescent proteins. This filter set and the manufacturer is listed below:

Fluorescent Protein	Filter Set for Fluorescence Microscopy	Manufacturer
EmGFP	Omega XF100	Omega (www.omegafilters.com)

For information on obtaining this filter set, contact Omega Optical, Inc. (www.omegafilters.com).

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 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 pcDNA™6.2/EmGFP-Bsd/V5-DEST.
3. Transfect your expression clone into the cell line of choice for transient or stable expression of your gene of interest and the EmGFP-Blasticidin fusion.

For more information on the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page **Error! Bookmark not defined.**).

Methods

Generating an Entry Clone

Introduction

To recombine your gene of interest into pcDNA™6.2/EmGFP-Bsd/V5-DEST, you will need an entry clone containing the gene of interest. This section provides guidelines for obtaining or generating an entry clone.

Many entry vectors are available from Invitrogen to facilitate generation of entry clones.

Entry Vector	Catalog Number
pENTR™/D-TOPO®	K2400-20
pENTR™/SD/D-TOPO®	K2420-20
pENTR™/TEV/D-TOPO®	K2525-20

For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page **Error! Bookmark not defined.**). Refer to the manual for the specific entry vector you are using for detailed instructions to construct an entry clone.

Tag On Demand™

The pcDNA™6.2/EmGFP-Bsd/V5-DEST vector is compatible with the Tag-On-Demand™ System which allows expression of both native and C-terminally-tagged recombinant protein from the same expression construct.

The System is based on stop suppression technology originally developed by RajBhandary and colleagues (Capone *et al.*, 1985) and consists of a recombinant adenovirus expressing a $tRNA^{ser}$ suppressor. When an expression vector encoding a gene of interest with the TAG (amber stop) codon is transfected into mammalian cells expressing the $tRNA^{ser}$ suppressor, the stop codon will be translated as serine, allowing translation to continue and resulting in production of a C-terminally-tagged fusion protein.

For more information, refer to the Tag-On-Demand™ Suppressor Supernatant manual. This manual is available for downloading from www.invitrogen.com or contact Technical Service (page **Error! Bookmark not defined.**).

Note

If you wish to express a human gene of interest from pcDNA™6.2/EmGFP-Bsd/V5-DEST, we recommend using an Ultimate™ Human ORF (hORF) Clone available from Invitrogen. Each Ultimate™ hORF Clone is a fully sequenced clone provided in a Gateway® entry vector that is ready-to-use in an LR recombination reaction with pcDNA™6.2/EmGFP-Bsd/V5-DEST. In addition, each Ultimate™ hORF Clone contains a **TAG** stop codon, making it fully compatible for use in the Tag-On-Demand™ System. For more information about the Ultimate™ hORF Clones, refer to www.invitrogen.com or contact Technical Service (page **Error! Bookmark not defined.**).

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Generating an Entry Clone, continued

Kozak Consensus

If you are recombining into the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector, the design for your entry clone should contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). 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).

V5 Epitope

If you are recombining into the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector, you may express your protein as a fusion to the C-terminal V5 epitope by omitting a stop codon in your gene of interest. The V5 epitope is a 14 amino acid epitope (GKPIPNPLLGLDST) derived from the P and V proteins of the SV5 paramyxovirus (Southern *et al.*, 1991). Fusion proteins containing the V5 epitope can be detected using specific antibodies (see page vi for ordering information).

Points to Consider Before Recombining

pcDNA™6.2/EmGFP-Bsd/V5-DEST is a C-terminal fusion vector; however, you may use this vector to express a native protein or a V5 fusion protein. You may also use this vector in the Tag-On-Demand™ System (see previous page).

Consider the following points when generating your entry clone. For more information on the recombination region of pcDNA™6.2/EmGFP-Bsd/V5-DEST, refer to pages 7-8.

If you wish to…	Then your insert…
include the V5 epitope tag and **NOT** use the Tag-On-Demand™ System	• should **NOT** contain a stop codon • should be in frame with the V5 epitope tag after recombination (see page 7 for a map of the recombination region)
include the V5 epitope tag for use in the Tag-On-Demand™ System	• should contain a **TAG** stop codon • should be in frame with the V5 epitope tag after recombination (see page 8 for a map of the recombination region)
not include the V5 epitope tag	• should contain a stop codon

Creating an Expression Clone

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector to create your expression clone. To ensure that you obtain the best results, we recommend that you read this section and the next section entitled **Performing the LR Recombination Reaction** (pages 6-11) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing pcDNA™6.2/EmGFP-Bsd/V5-DEST vector.
2. Transform the reaction mixture into a suitable *E. coli* host.
3. Select for expression clones (refer to the next pages for diagrams of the recombination regions of the resulting expression clones).

Resuspending the Vector

The pcDNA™6.2/EmGFP-Bsd/V5-DEST vector is supplied as 6 µg of plasmid, lyophilized in TE, pH 8.0. To use, resuspend the plasmid in 40 µl of sterile water to a final concentration of 150 ng/µl.

Propagating the Vector

If you wish to propagate and maintain pcDNA™6.2/EmGFP-Bsd/V5-DEST, we recommend using One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli* (Catalog no. C7510-03) from Invitrogen for transformation. The *ccd*B Survival T1^R *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.

Continued on next page

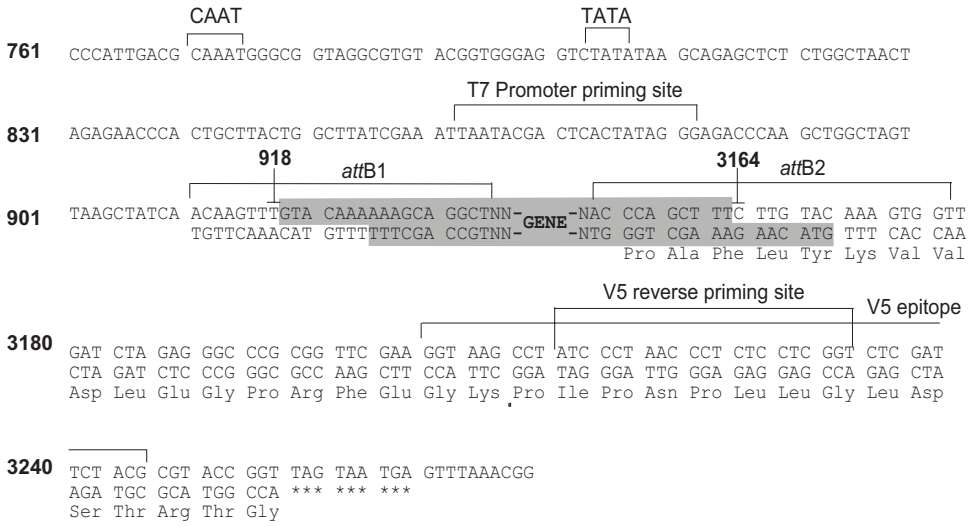
Creating an Expression Clone, continued

Recombination Region

The recombination region of the expression clone resulting from pcDNA™6.2/EmGFP-Bsd/V5-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions between the *att*B sites correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/EmGFP-Bsd/V5-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector.
- The overlined nucleotides flanking the shaded region correspond to bases 918 and 3164 of the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector sequence.



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Creating an Expression Clone, continued

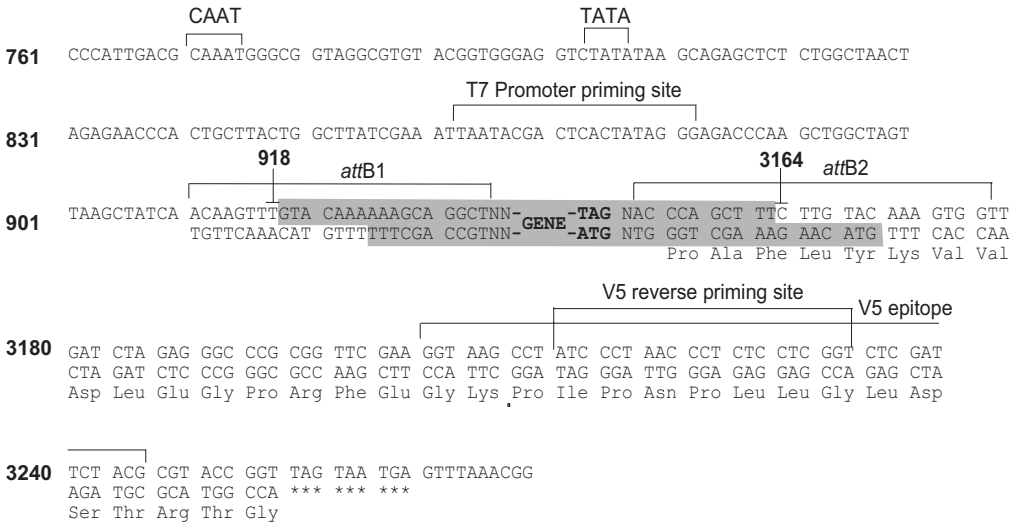
Recombination Region for Use in the Tag-On-Demand™ System

The recombination region of the expression clone resulting from pcDNA™6.2/EmGFP-Bsd/V5-DEST × entry clone is shown below.

Note: The gene of interest must contain a **TAG** stop codon for use in the Tag-On-Demand™ System (see page 4 for more information).

Features of the Recombination Region:

- Shaded regions between the *att*B sites correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/EmGFP-Bsd/V5-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector.
- The overlined nucleotides flanking the shaded region correspond to bases 918 and 3164 of the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector sequence.



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 pcDNA™6.2/EmGFP-Bsd/V5-DEST, and transform the reaction mixture into a suitable *E. coli* host (see below) to select for an expression clone. We recommend including a negative control (no LR Clonase™ II) in your experiment 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 (see page vi for ordering information). **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

Most commonly, ampicillin is used to select for *E. coli* transformants; however the presence of the EM7 promoter and the Blasticidin resistance gene in pcDNA™6.2/EmGFP-Bsd/V5-DEST allows for selection of *E. coli* transformants using Blasticidin instead of ampicillin, if preferred.

For selection using Blasticidin, use Low Salt LB agar plates containing 100 µg/ml Blasticidin (see 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. Refer to page 21 for instructions on how to prepare and store Blasticidin.

LR Clonase™ II Enzyme Mix

LR Clonase™ II enzyme mix is available separately from Invitrogen (Catalog no. 11791-020) to catalyze the LR recombination reaction. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase™ Reaction Buffer previously supplied as separate components in LR Clonase™ enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 10 to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix as reaction conditions differ.

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Performing the LR Recombination Reaction, continued

Materials Needed

You should have the following materials on hand before beginning:

- Purified plasmid DNA of your entry clone (50-150 ng/µl in TE, pH 8.0)
- pcDNA™6.2/EmGFP-Bsd/V5-DEST (150 ng/µl in TE, pH 8.0)
- LR Clonase™ II enzyme mix (Invitrogen, Catalog no. 11791-020; keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with LR Clonase™ II enzyme mix; thaw and keep on ice until use)
- pENTR™-gus (supplied with LR Clonase™ II enzyme mix; use as a control for the LR reaction; 50 ng/µl)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- Selective LB agar plates containing 100 µg/ml ampicillin or Low Salt LB plates containing 100 µg/ml Blasticidin

Setting Up the LR Reaction

Follow this procedure to perform the LR reaction between your entry clone and pcDNA™6.2/EmGFP-Bsd/V5-DEST. To include a negative control, set up a second sample reaction, but omit the LR Clonase™ II enzyme mix.

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

Component	Sample	Positive Control
Entry clone (50-150 ng/rxn)	1-7 µl	--
Destination vector (150 ng/µl)	1 µl	1 µl
pENTR™-gus (50 ng/µl)	--	2 µl
TE Buffer, pH 8.0	to 8 µl	5 µl

2. Remove the LR Clonase™ II enzyme mix from -20°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 2 µl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return LR Clonase™ II enzyme mix to -20°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 1 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.

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Performing the LR Recombination Reaction, continued

What You Should See

If you use *E. coli* cells with a transformation efficiency of ≥ 1 x 10^8 cfu/µg, the LR reaction should give > 5,000 colonies if the entire 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.

Sequencing

To confirm that your gene of interest is in frame with the C-terminal V5 epitope, you may sequence your expression construct. We suggest using the following primer sequences. Refer to the diagrams on pages 7-8 for the location of the primer binding sites.

Primer	Sequence
T7 promoter primer	5′-TAATACGACTCACTATAGGG-3′
V5 reverse primer	5′-ACCGAGGAGAGGGTTAGGGAT-3′

For your convenience, Invitrogen offers a custom primer synthesis service. For more information, go to www.invitrogen.com or contact Technical Service (page **Error! Bookmark not defined.**).

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 also recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on an LB plate containing 100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 µg/ml ampicillin.
3. Grow at 37°C with shaking until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol.
5. Transfer to a cryovial and store at -80°C.

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 the positive control vector (pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT, below) and a mock transfection (negative control) in your experiments to evaluate your results.

Positive Control Plasmid

The pcDNA™6.2/EmGFP-Bsd/V5-DEST Mammalian Expression Vector Kit contains a positive control plasmid expressing CAT and the EmGFP-Blasticidin fusion (see page 24 for map). This vector allows the expression of a V5 C-terminally tagged chloramphenicol acetyl transferase (CAT) fusion protein that may be detected by Western blot and the expression of EmGFP to monitor transfection efficiency by fluorescence microscopy.

The control vector is supplied lyophilized. Resuspend the vector in 10 µl TE or sterile water to a final concentration of 1µg/µl. You can transfect mammalian cells with this stock or propagate and maintain the plasmid as described below:

1. Use 1 µl of the control vector to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™, JM109, or equivalent.
2. Select transformants on LB agar plates containing 100 µg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage (see page 11 for a protocol for preparing glycerol stocks).

Plasmid Preparation

Once you have generated your expression vector, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be clean and free of contamination 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 (Catalog no. K2100-01). Other methods of obtaining high quality plasmid DNA may be suitable.

Methods of Transfection

For established cell lines, consult the 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). For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen. For more information about Lipofectamine™ 2000 and other transfection reagents, go to www.invitrogen.com or contact Technical Service (page **Error! Bookmark not defined.**).

Creating Stable Cell Lines

Introduction

The pcDNA™6.2/EmGFP-Bsd/V5-DEST vector contains the Blasticidin resistance gene to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your expression construct into the mammalian cell line of choice and select for stable transfectants using Blasticidin. General information and guidelines are provided below.

Linearizing the Plasmid

To obtain stable transfectants, we recommend that you linearize your pcDNA™6.2/EmGFP-Bsd/V5-DEST expression 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. Cut at a unique site that is not located within a critical element or within your gene of interest. Restriction site information for plasmid vectors is available at www.invitrogen.com.

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 nontoxic deaminohydroxy derivative (Izumi *et al.*, 1991). Blasticidin is available separately from Invitrogen (see page vi for ordering information). For information on preparing and handling Blasticidin see the Appendix, page 21.

Determining Blasticidin Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you first need to determine the minimum concentration of Blasticidin required to kill your untransfected host cell line. Most mammalian cells are killed by 2-10 µg/ml Blasticidin. Test a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line (see protocol below). Refer to page 21 for instructions on how to prepare and store Blasticidin.

1. Prepare 6 plates of cells so that each plate will be approximately 25% confluent.
2. Replace the growth medium with fresh growth medium containing a range of Blasticidin concentrations: 0, 1, 3, 5, 7.5, and 10 µg/ml.
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.

Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that kills your cells within 1-3 weeks after addition of Blasticidin.

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Creating Stable Cell Lines, continued

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/EmGFP-Bsd/V5-DEST expression construct.

1. Transfect the mammalian cell line of interest with the pcDNA™6.2/EmGFP-Bsd/V5-DEST expression construct using your transfection method of choice.
2. 24 hours after transfection, wash the cells and add fresh growth medium without Blasticidin.
3. 48 hours after transfection, split the cells into fresh growth medium without Blasticidin 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 (see previous page).
6. Feed the cells with selective media every 3-4 days until Blasticidin-resistant colonies can be identified.
7. Pick at least 10 Blasticidin-resistant colonies and expand them to assay for recombinant protein expression.

Refer to page 21 for instructions on how to prepare and store Blasticidin.

Detecting Fluorescence

Introduction

After transfecting your cells with the pcDNA™6.2/EmGFP-Bsd/V5-DEST expression construct, you can monitor transfection efficiency by detecting EmGFP fluorescence. You may detect EmGFP expression directly in cells by fluorescence microscopy or other methods that use light excitation and detection of emission. The following sections provide guidelines for detecting the EmGFP fluorescent signal in transfected cells.

Detecting EmGFP

EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescent signal, you may use a filter set which is optimized for detection within the excitation and emission ranges of EmGFP This optimized filter set and the excitation/emission properties of EmGFP are listed below:

Fluorescent Protein	Excitation/Emission (nm)	Optimized Filter Set for Fluorescence Microscopy
EmGFP	487/509	Omega XF100

For information on obtaining this filter set, contact Omega Optical, Inc. (www.omegafilters.com)

EmGFP Expression

In general, EmGFP fluorescence can be visualized at 24-48 hours post transfection, although this will vary depending on the cell line used. Based on the number of fluorescent cells observed, you can estimate the transfection efficiency and normalize further assays for your gene of interest.

Detecting Recombinant Protein

Introduction

After transfecting your cells with the pcDNA™6.2/EmGFP-Bsd/V5-DEST expression construct, you can detect your protein of interest by Western blot of cell lysates using an antibody.

Note

Note that the expression of EmGFP does not necessarily correlate with expression of the recombinant protein due to the fact that the genes are under control of separate promoters. We recommend that you perform a time course to determine the optional time to assay for transient expression of your protein of interest. Optimal times may vary depending on your cell line.

Antibodies for Detection of Recombinant Protein

You may detect expression of your recombinant fusion protein using an antibody against your protein of interest, or if you have expressed the V5 epitope at the C-terminal end of your protein by an Anti-V5 antibody available from Invitrogen:

Antibody	Description	Catalog Number
Anti-V5	Mouse monoclonal IgG_{2a}	R960-25
Anti-V5 HRP	Mouse monoclonal IgG_{2a}, conjugated to HRP	R961-25
Anti-V5-AP	Mouse monoclonal IgG_{2a}, conjugated to AP	R692-25

Preparing Cell Lysates for Western Blot Detection

To detect your fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols may be suitable. To lyse cells:

1. Wash cell monolayer (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS; Catalog no. 10010-023).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see page 20 for a recipe). Other cell lysis buffers may be suitable. Vortex.
4. To lyse cells, perform 3 freeze thaw cycles by incubating the samples in a dry ice/ethanol bath for 2 minutes, then incubating the sample in a 37°C water bath for one minute. Perform this step 3 times to ensure complete cell lysis without protein degradation.
5. Centrifuge the cell lysate at 10,000 x 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.

Continued on next page

Detecting Recombinant Protein, continued

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 from Invitrogen. For more information, refer to www.invitrogen.com or contact Technical Service (page **Error! Bookmark not defined.**).

Western Blot Detection of Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by Western blot analysis, you may use an antibody to your protein of interest, or an Anti-V5 antibody (see previous page). The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods (see page vi for ordering information).

Note

The fusion peptide including the V5 epitope will add approximately 4 kDa to your protein.

Detecting CAT Protein

If you use the pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT vector in your experiment, you may assay for CAT expression. Note that CAT is fused to the C-terminal V5 epitope tag so you can use Western blot analysis and an Anti-V5 or an Anti-CAT antibody to detect expression of CAT (See page vi for ordering information). The molecular weight of the CAT fusion protein is approximately 30 kDa.

Troubleshooting

Introduction

The table below lists some potential problems and solutions for troubleshooting protein expression from your pcDNA™6.2/EmGFP-Bsd/V5-DEST expression construct. For more information on troubleshooting within the Gateway® system, refer to the Gateway® Technology with Clonase™ II manual, which is available from www.invitrogen.com or by contacting Technical Service.

Problem	Possible Cause	Solution
Recombinant protein not expressed	No Kozak consensus sequence added to C-terminal fusion	• Make sure a Kozak consensus sequence is present in your entry clone design (see page 5).
	The C-terminal V5 tag adversely affects protein expression	• Insert a stop codon at the end of your gene or use Tag-On Demand™ to express native protein as described on page 4.
Recombinant protein not detectable with Anti-V5 antibodies	Stop codon inserted	• Make sure no stop codon is at the end of your gene (see page 5).
	Gene of interest not in frame with V5 epitope sequence	• Make sure that the gene of interest is in frame with the V5 epitope sequences as shown on page 7.
No fluorescent signal detected after transfecting control or experimental expression clone	Incorrect filters used to detect fluorescence	Be sure to use the recommended filter set (see page 3).
	Transfection efficiency too low to allow detection of transfected cells	• Make sure cells are healthy before transfection. • Use Lipofectamine™ 2000 for high transfection efficiency into a wide range of mammalian cell types.
	Too soon to detect fluorescence	Maximal fluorescence is usually observed 24-48 hours after transfection, but may vary with cell type.
Fluorescent signal detected after transfecting control but not after transfecting experimental expression clone	Poor DNA quality of the experimental expression vector	Make sure DNA is purified with a high-quality prep kit such as the PureLink™ HQ Plasmid Miniprep Kit.
After selection for stable expression in mammalian cells, little or no expression of gene of interest	Not enough clones screened	• Screen 30-40 clones to obtain high expression of gene of interest.
	Plasmid not linearized before transfection and gene of interest is disrupted	• Linearize the plasmid in a non-essential region prior to transfection as recommended on page 13.

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 if needed.
4. Store at room temperature or at +4°C.

For 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 if needed, and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

Low Salt LB Plates with Blasticidin

Composition:

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 and 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. Let harden, then invert and store at +4°C.

Store plates at +4°C in the dark. Plates containing Blasticidin S HCl are stable for up to 2 weeks.

Continued on next page

Recipes, continued

Cell Lysis Buffer

Composition:

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:

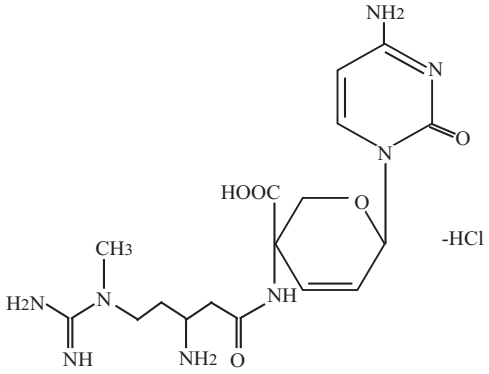
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

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

Blasticidin

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 from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Use sterile water to prepare stock solutions of 5 to 10 mg/ml.

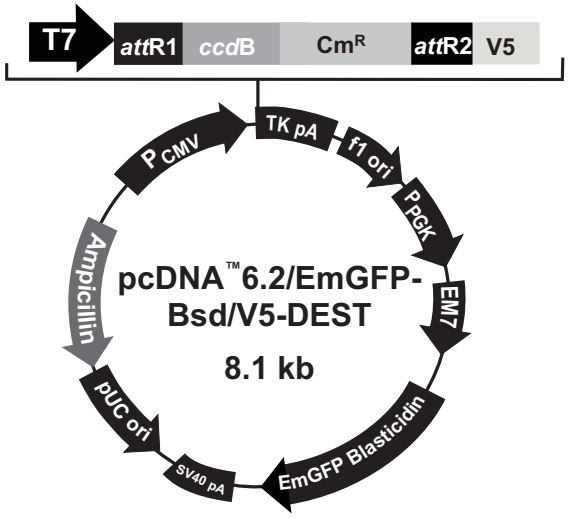
- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot solution in small volumes suitable for one time use (see next to last point below) and freeze at -20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks 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/EmGFP-Bsd/V5-DEST

Map

The map below shows the elements of the pcDNA™6.2/EmGFP-Bsd/V5-DEST vector (8198 bp). DNA from the entry clone replaces the region between bases 918 and 3164 after performing the LR reaction. **The complete sequence of this vector is available for downloading from www.invitrogen.com or by contacting Technical Service (page** Error! Bookmark not defined.**).**



Comments for: pcDNA™6.2/EmGFP-Bsd/V5-DEST 8198 nucleotides

CMV promoter: 235-822
T7 promoter/primer: 863-882
*att*R1 site: 911-1035
*ccd*B gene: 1771-1991
Chloramphenicol resistance (Cm^R) gene: 2114-2773
*att*R2 site: 3054-3178
V5 epitope: 3204-3245
TK polyadenylation signal: 3272-3543
f1 origin: 3579-5007
PGK promoter: 4017-4523
EM7 promoter: 4534-4600
EmGFP/Blasticidin fusion: 4601-5713
EmGFP: 4601-5317
Blasticidin resistance gene: 5321-5713
SV40 early polyadenylation signal: 5871-6001
pUC origin (c): 6384-7057
Ampicillin (*bla*) resistance gene (c): 7202-8062
bla promoter (c): 8057-8161

(c) = complementary strand

Continued on next page

Map and Features of pcDNA™6.2/EmGFP-Bsd/V5-DEST

Features

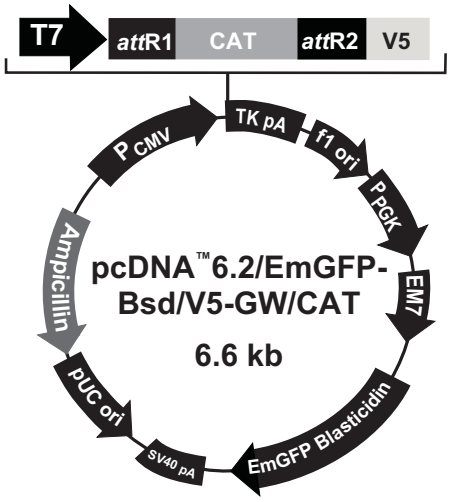
The pcDNA™6.2/EmGFP-Bsd/V5-DEST (8198 bp) vector contains the following elements. All features have been functionally tested and the vector has been fully sequenced.

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/primer binding site	Allows sequencing of the insert
*att*R1 and *att*R2 sites	Allows recombinatorial cloning of the gene of interest from an entry clone
*Ccd*B gene	Allows negative selection of plasmid
Chloramphenicol resistance gene	Allows counterselection of plasmid
V5 epitope	Allows detection of recombinant fusion protein with Anti-V5 antibodies (Southern *et al.*, 1991)
V5 reverse primer binding site	Allows sequencing of the insert
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985)
f1 origin	Allows rescue of single-stranded DNA
PGK promoter	Allows high-level expression of the Blasticidin-EmGFP fusion in mammalian cell lines
EM7 promoter	Allows expression of the Blasticidin-EmGFP fusion in *E. coli*
EmGFP-Blasticidin (*bsd*) resistance gene fusion	Allows visual detection of transfected mammalian cells using fluorescence microscopy 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 (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Map and Features of pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT

Map

The map below shows the elements of the pcDNA™6.2/EmGFP-Bsd/V5-GW/CAT vector. The plasmid was generated by performing an LR recombination reaction between an entry vector containing the CAT gene and the pcDNA™6.2/EmGFP-Bsd/V5-DESTvector. **The complete sequence of this vector is available for downloading from www.invitrogen.com or by contacting Technical Service (page** Error! Bookmark not defined.**).**



Comments for: **pcDNA™6.2/ EmGFP-Bsd/V5-GW/CAT 6667 nucleotides**

CMV promoter: 235-822
T7 promoter/primer: 863-882
*att*B1 site: 911-935
CAT gene: 965-1647
*att*B2 site: 1623-1647
V5 epitope: 1673-1714
TK polyadenylation signal: 1741-2012
f1 origin: 2048-2478
PGK promoter: 2486-2992
EM7 promoter: 3003-3786
EmGFP/Blasticidin fusion: 3070-4182
EmGFP: 3070-3786
Blasticidin resistance gene: 3790-4182
SV40 early polyadenylation signal: 4340-4182
pUC origin (c): 4853-5526
Ampicillin (*bla*) resistance gene (c): 5671-6531
bla promoter (c): 6526-6630

(c) = complementary strand

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

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Purchaser Notification

Introduction

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy** on page 28.

Continued on next page

Purchaser Notification, Continued

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Gateway® Clone Distribution Policy

Introduction

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

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Invitrogen 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. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen 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. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

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 Invitrogen 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 Invitrogen's 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 Invitrogen's licensing department at 760-603-7200.

References

Adra, C., Boer, P. H., and McBurney, M. W. (1987). Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. Gene *60*, 65-74.

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 (New York: Greene Publishing Associates and Wiley-Interscience).

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.

Capone, J. P., Sharp, P. A., and RajBhandary, U. L. (1985). Amber, Ochre and Opal Suppressor tRNA Genes Derived from a Human Serine tRNA Gene. EMBO J. *4*, 213-221.

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

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.

Curradi, M., Izzo, A., Badaracco, G., and Landsberger, N. (2002). Molecular Mechanisms of Gene Silencing Mediated by DNA Methylation. Mol. Cell. Biol. *22*, 3157-3173.

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.

Gerolami, R., Uch, R., F, Chapel, S., Bagnis, C., Brechot, C., and Mannoni, P. (2000). Gene transfer to hepatocellular carcinoma: transduction efficacy and transgene expression kinetics by using rertoviral and lentiviral vectors. Cancer Gene Therapy *7*, 1286-92.

Hamaguchi, I., Woods, N.-B., Panagopoulos, I., Andersson, E., Mikkola, H., Fahlman, C., Zufferey, R., Carlsson, L., Trono, D., and Karlsson, S. (2000). Lentivirus Vector Gene Expression during ES-Cell Derived Hematopoietic Development In Vitro. J Virology *74*, 10778-10784.

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.

References

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

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

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

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

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.

Rietveld, L. E., Caldenhoven, E., and Stunnenberg, H. G. (2002). In vivo Repression of an Erythroid-Specific Gene by Distinct Corepressor Complexes. EMBO J. *21*, 1389-1397.

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.

Shimomura, O., Johnson, F. H., and Saiga, Y. (1962). Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous hHydromedusan, Aequorea. Journal of Cellular and Comparative Physiology *59*, 223-239.

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.

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.

Tsien, R. Y. (1998). The Green Fluorescent Protein. Annu. Rev. Biochem. *67*, 509-544.

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.

Zhang, G., Gurtu, V., and Kain, S. (1996). An Enhanced Green Fluorescent Protein Allows Sensitive Detection of Gene Transfer in Mammalian Cells. Biochem. Biophys. Res. Comm. *227*, 707-711.

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Corporate Headquarters
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech.service@invitrogen.com

For country-specific contact information visit our web site at **www.invitrogen.com**