invitrogen™

pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST Gateway® Vectors

Gateway®-adapted destination vectors for cloning and expression of C-terminal V5 fusion proteins in mammalian cells

Catalog nos. 12489-019 and 12489-027

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User Manual

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Important Information

Gateway® Vectors

This manual is supplied with the following products.

Product	Catalog no.
pcDNA™3.2/V5-DEST Gateway® Vector	12489-019
pcDNA™6.2/V5-DEST Gateway® Vector	12489-027

Important

The pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST Gateway® Vectors have been renamed to be more descriptive and to better reflect the functionality of the vector.

Shipping and Storage

The pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST Gateway® Vectors are shipped on wet ice. Upon receipt, store at –20°C.

Contents

The pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST Gateway® Vector components are listed below.

Item	Concentration	Volume
Gateway® Destination Vector (pcDNA™3.2/V5-DEST **or** pcDNA™6.2/V5-DEST)	6 µg at 150 ng/µl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	40 µl
Control Plasmid (pcDNA™3.2/V5/GW/CAT **or** pcDNA™6.2/V5/GW/CAT)	10 µg at 0.5 µg/µl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	20 µl

Product Qualification

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

Accessory Products

Additional Products

Additional products that may be used with the pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST vectors are available from Invitrogen. Ordering information is provided below.

Product	Amount	Catalog no.
Gateway® LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
Tag-On-Demand™ Suppressor Supernatant	200 µl 5 x 200 µl	K400-01 K405-01
One Shot® TOP10 Chemically Competent Cells	10 reactions 20 reactions	C4040-10 C4040-03
PureLink™ HQ Mini Plasmid Purification Kit	100 preps	K2100-01
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Lipofectamine™ 2000	1.5 ml 0.75 ml	11668-019 11668-027
Geneticin®	1 g 5 g	11811-023 11811-031
Blasticidin	50 mg	R210-01

Detection of Recombinant Proteins

You can detect expression of your recombinant fusion protein using the Anti-V5 antibodies available from Invitrogen. The amount of antibody supplied is sufficient for 25 Western blots or 25 immunostaining reactions (FITC-conjugated antibody only).

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

Methods

Overview

Description

pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST are 7.7 kb and 7.3 kb vectors, respectively, that are adapted with the Gateway® Technology, and allow high-level, constitutive expression of the gene of interest in a variety of mammalian hosts. For more information on the Gateway® Technology, see the next page.

Features

pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression 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
- The V5 epitope tag for detection using Anti-V5 antibodies
- 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 neomycin (pcDNA™3.2/V5-DEST) or Blasticidin (pcDNA™6.2/V5-DEST) resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Neomycin (pcDNA™3.2/V5-DEST) or Blasticidin (pcDNA™6.2/V5-DEST) 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 (*bla*) resistance gene for selection in *E. coli*

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Overview, continued

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 a Gateway® destination vector (*e.g.* pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-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 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 25).

Generating an Entry Clone

Introduction

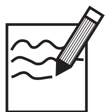
To recombine your gene of interest into pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST, you will need an entry clone containing the gene of interest (see below and the next page for recommendations). Many entry vectors including pENTR/D-TOPO® are available from Invitrogen to facilitate generation of entry clones. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25). Refer to the manual for the specific entry vector you are using for detailed instructions to construct an entry clone.

Tag-On-Demand™ System

The pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST vectors are 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, 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 our Web site (www.invitrogen.com) or contact Technical Service (page 25).



Note

If you wish to express a human gene of interest from pcDNA™3.2/V5-DEST or pcDNA™6.2/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™3.2/V5-DEST or pcDNA™6.2/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 available, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25).

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Generating an Entry Clone, continued

Kozak Consensus Sequence

Your insert 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 (shown in bold) illustrates the most commonly occurring consensus sequence.

Points to Consider Before Recombining

pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST are C-terminal fusion vectors; however, you may use these vectors to express native proteins or C-terminal fusion proteins. You may also use these vectors in the Tag-On-Demand™ System (see previous page). Consider the following when generating your entry clone.

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 6 for a diagram)
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 7 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, you will perform the LR recombination reaction to transfer the gene of interest into the pcDNA™3.2/V5-DEST or pcDNA™6.2/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 8-11) 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™3.2/V5-DEST or pcDNA™6.2/V5-DEST vector.
2. Transform the reaction mixture into a suitable *E. coli* host.
3. Select for expression clones (refer to pages 6-7 for a diagram of the recombination region of the resulting expression clones).

Propagating the Vectors

If you wish to propagate and maintain pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST, we recommend using One Shot® *ccd*B Survival™ 2 T1 Phage-Resistant Cells (Catalog no. A10460) from Invitrogen for transformation. The *ccd*B Survival™ 2 T1 Phage-Resistant *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.

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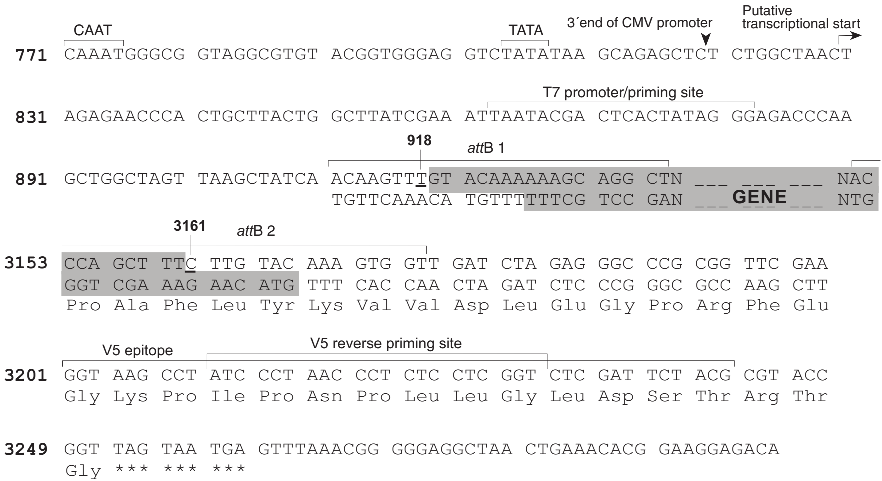
Creating an Expression Clone, continued

Recombination Region

The recombination region of the expression clone resulting from pcDNA™3.2/V5-DEST × entry clone or pcDNA™6.2/V5-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST by recombination. Non-shaded regions are derived from the pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST vector.
- The underlined nucleotides flanking the shaded region correspond to bases 918 and 3161 of the pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST vector sequence.



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Creating an Expression Clone, continued

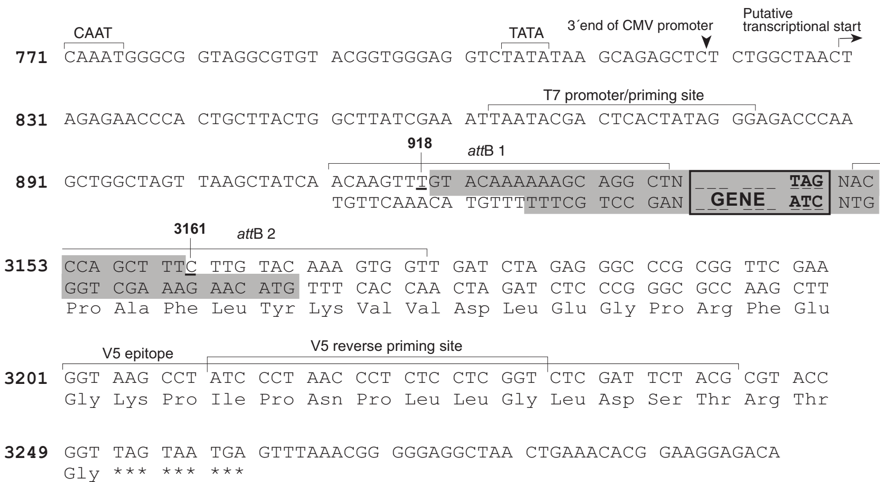
Recombination Region for Use in the Tag-On-Demand™ System

The recombination region of the expression clone resulting from pcDNA™3.2/V5-DEST × entry clone or pcDNA™6.2/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 3 for more information).

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST by recombination. Non-shaded regions are derived from the pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST vector.
- The underlined nucleotides flanking the shaded region correspond to bases 918 and 3161 of the pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-DEST vector sequence.



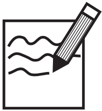
Performing the LR Recombination Reaction

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™3.2/V5-DEST or pcDNA™6.2/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

The presence of the EM7 promoter and the Blasticidin resistance gene in **pcDNA™6.2/V5-DEST** allows for selection of *E. coli* transformants using Blasticidin. For selection, 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.

Blasticidin is available separately from Invitrogen (see page vi for ordering information). Refer to page 21 for instructions on how to prepare and store Blasticidin.

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Performing the LR Recombination Reaction, continued

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.

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™3.2/V5-DEST or pcDNA™6.2/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
- LB agar plates containing 100 µg/ml ampicillin or Low Salt LB plates containing 100 µg/ml Blasticidin)

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Performing the LR Recombination Reaction, continued

Setting Up the LR Reaction

Follow this procedure to perform the LR reaction between your entry clone and a destination vector. 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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Creating an Expression Clone, 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, if desired. We suggest using the following primer sequences. Refer to the diagram on page 6 for the location of the primer binding sites.

For your convenience, Invitrogen offers a custom primer synthesis service. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25).

Primer	Sequence
T7 Promoter	5′-TAATACGACTCACTATAGGG-3′
V5 Reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′

Transfection

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™3.2/V5/GW/CAT or pcDNA™6.2/V5/GW/CAT) and a mock transfection (negative control) in your experiments to evaluate your results.

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 clean and free contamination with 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), the PureLink™ HiPure Plasmid Midiprep Kit (Catalog no. K2100-04), or CsCl gradient centrifugation.

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). 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, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25).

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Positive Control

pcDNA™3.2/V5/GW/CAT or pcDNA™6.2/V5/GW/CAT is provided as a positive control vector for mammalian cell transfection and expression (see page 24 for a map) and may be used to optimize recombinant protein expression levels in your cell line. These vectors allow expression of a C-terminally tagged chloramphenicol acetyl transferase (CAT) fusion protein that may be detected by Western blot or functional assay.

To propagate and maintain the plasmid:

1. Prepare a 1:50 dilution of the positive control vector in sterile water (i.e. 1 µl vector + 49 µl water) for a 10 ng/ul stock solution. Use 10 ng of the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, JM109, 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.

Expression and Analysis

Introduction

Expression of your gene of interest from the expression clone can be performed in either transiently transfected cells or stable cell lines (see page 16 for guidelines to create stable cell lines). You may use a functional assay or a Western blot analysis to detect your recombinant protein (see below).

Preparing Cell Lysates

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 are suitable. To lyse cells:

1. Wash cell monolayer (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS; Invitrogen 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 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 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.

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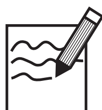
Polyacrylamide Gel Electrophoresis

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 our Web site (www.invitrogen.com) or contact Technical Service (page 25).

Detecting Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by Western blot analysis, you may use the Anti-V5 antibodies available from Invitrogen (see page vi for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope. 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. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 25).



Note

The C-terminal peptide containing the V5 epitope will add approximately 4 kDa to your protein.

Detecting CAT Protein

If you use the provided positive control vector in your experiment, you may assay for CAT expression using your method of choice. Note that CAT is fused to the C-terminal V5 epitope tag so you can use Western blot analysis and an Anti-V5 antibody to detect expression of CAT. Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT fusion protein is approximately 30 kDa.

Creating Stable Cell Lines

Introduction

The pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST vectors contain the neomycin and Blasticidin resistance genes, respectively, 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 Geneticin® **(pcDNA™3.2/V5-DEST only)** or Blasticidin **(pcDNA™6.2/V5-DEST only)**. General information and guidelines are provided below.

To obtain stable transfectants, we recommend that you linearize your pcDNA™3.2/V5-DEST or pcDNA™6.2/V5-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.

Geneticin®

Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phosphotransferase gene (APH), derived from Tn*5*, results in detoxification of Geneticin® (Southern and Berg, 1982).

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).

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Creating Stable Cell Lines, continued

Determining Antibiotic Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of antibiotic (Geneticin® or Blasticidin) required to kill your untransfected host cell line. Test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line. Refer to page 21 for instructions on how to prepare and store Blasticidin.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. For each antibiotic, prepare a set of 6–7 plates. Add the following concentrations of antibiotic to each plate:
   - For Blasticidin selection, test 0, 1, 3, 5, 7.5, and 10 μg/ml Blasticidin
   - For Geneticin® selection, test 0, 50, 125, 250, 500, 750, and 1000 μg/ml Geneticin®.
2. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
3. Count the number of viable cells at regular intervals to determine the appropriate concentration of antibiotic that prevents growth within 1–3 weeks after addition of the antibiotic.

Geneticin® Selection Guidelines

Once you have determined the appropriate Geneticin® concentration to use for selection, you can generate a stable cell line expressing your **pcDNA™3.2/V5-DEST** construct. Geneticin® is available separately from Invitrogen (see page vi for ordering information). Use as follows:

1. Prepare Geneticin® in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
2. Use the predetermined concentration of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Cells will divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 3 weeks of growth in selective medium.

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Creating Stable Cell Lines, continued

Blasticidin Selection Guidelines

Once you have determined the appropriate Blasticidin concentration to use for selection, you can generate a stable cell line expressing your **pcDNA™6.2/V5-DEST** construct. Blasticidin is available separately from Invitrogen (see page vi for ordering information). Use as follows:

1. Prepare a stock solution of 5–10 mg/ml of Blasticidin in sterile water. Filter-sterilize the solution.
2. Use the predetermined concentration of Blasticidin in complete medium.
3. Cells differ in their susceptibility to Blasticidin. Complete selection can take up to 10 days of growth in selective medium.

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

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.

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 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.

continued on next page

Recipes, continued

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:

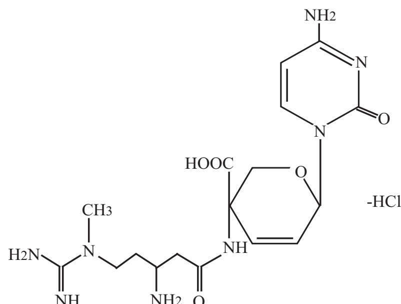
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

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

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

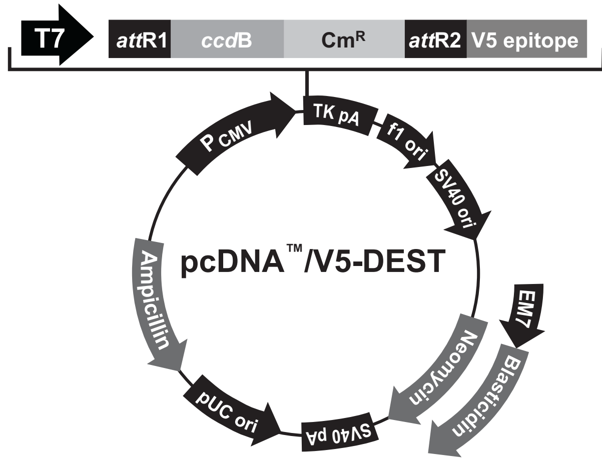
Blasticidin may be obtained separately from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

- 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 of pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST

Map

The map below shows the elements of pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST. DNA from the entry clone replaces the region between bases 918 and 3161. **The complete sequences of these vectors are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).**



Comments for:	**pcDNA™3.2/V5-DEST 7711 nucleotides**	**pcDNA™6.2/V5-DEST 7341 nucleotides**
CMV promoter:	232-819	232-819
T7 promoter/priming site:	863-882	863-882
*att*R1 site:	911-1035	911-1035
*ccd*B gene (c):	1464-1769	1464-1769
Chloramphenicol resistance gene (c):	2111-2770	2111-2770
*att*R2 site:	3051-3175	3051-3175
V5 epitope:	3201-3242	3201-3242
V5 reverse priming site:	3210-3230	3210-3230
TK polyadenylation signal:	3269-3540	3269-3540
f1 origin:	3576-4004	3576-4004
SV40 early promoter and origin:	4031-4339	4031-4339
Neomycin resistance gene:	4414-5208	---
EM7 promoter:	---	4394-4460
Blasticidin resistance gene:	---	4461-4859
SV40 early polyadenylation signal:	5384-5514	5017-5147
pUC origin (c):	5897-6570	5530-6200
Ampicillin (*bla*) resistance gene (c):	6715-7575	6345-7205
bla promoter (c):	7576-7674	7206-7304

(c) = complementary strand

continued on next page

Features of pcDNA™3.2/V5-DEST and pcDNA™6.2/V5-DEST

Features

pcDNA™3.2/V5-DEST (7711 bp) and pcDNA™6.2/V5-DEST (7341 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/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
*ccd*B gene	Allows negative selection of plasmid
Chloramphenicol resistance gene	Allows counterselection of plasmid
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 antibodies (Southern *et al.*, 1991).
V5 reverse priming 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
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin or Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene **(pcDNA™3.2/V5-DEST only)**	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
EM7 promoter **(pcDNA™6.2/V5-DEST only)**	Allows expression of the Blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene **(pcDNA™6.2/V5-DEST only)**	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*

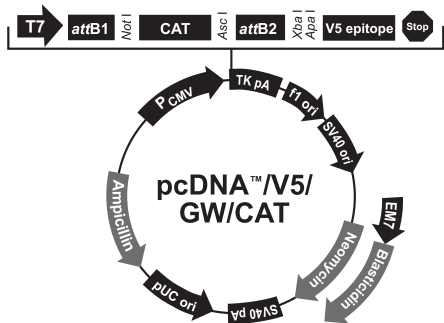
Map of pcDNA™3.2/V5/GW/CAT and pcDNA™6.2/V5/GW/CAT

Description

pcDNA™3.2/V5/GW/CAT (6188 bp) and pcDNA™6.2/V5/GW/CAT (5818 bp) are control vectors expressing chloramphenicol acetyltransferase (CAT). Each vector was constructed using the LR recombination reaction between an entry clone containing the CAT gene and the respective destination vector. **Note:** The CAT gene is in frame with the C-terminal V5 epitope and does not contain a stop codon. The molecular weight of the CAT fusion protein is ~30 kDa.

Map

The map below shows the elements of pcDNA™3.2/V5/GW/CAT and pcDNA™6.2/V5/GW/CAT. **The complete sequences of these vectors are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).**

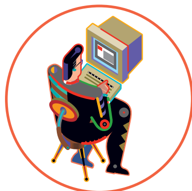


Comments for:	pcDNA™3.2/V5/GW/CAT 6188 nucleotides	pcDNA™6.2/V5/GW/CAT 5818 nucleotides
CMV promoter:	232-819	232-819
T7 promoter/priming site:	863-882	863-882
*att*B1 site:	911-935	911-935
CAT ORF:	955-1611	955-1611
*att*B2 site:	1628-1652	1628-1652
V5 epitope:	1678-1719	1678-1719
V5 reverse priming site:	1687-1707	1687-1707
TK polyadenylation signal:	1746-2017	1746-2017
f1 origin:	2053-2481	2053-2481
SV40 early promoter and origin:	2508-2816	2508-2816
Neomycin resistance gene:	2891-3685	---
EM7 promoter:	---	2871-2937
Blasticidin resistance gene:	---	2938-3336
SV40 early polyadenylation signal:	3861-3991	3494-3624
pUC origin (c):	4374-5047	4007-4677
Ampicillin (*bla*) resistance gene (c):	5192-6052	4822-5682
bla promoter (c):	6053-6151	5683-5781

(c) = complementary strand

Technical Service

World Wide Web



Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
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E-mail: techsupport@invitrogen.com	E-mail: eurotech@invitrogen.com

MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

continued on next page

Technical Service, continued

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product. CofAs are available on our website at www.invitrogen.com/support, and are searchable by product lot number, which is printed on each box.

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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**, page 29.

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Gateway® Clone Distribution Policy

Introduction

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