USER GUIDE

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Gateway® pcDNA™-DEST40 Vector

A destination vector for cloning and expression of C-terminal fusion proteins in mammalian cells

Catalog Number 12274-015

Document Part Number 25-0409
Publication Number MAN0000223
Revision 3.0

For Research Use Only. Not for use in diagnostic procedures.

life technologies™

Contents

Important Information

Shipping and Storage

pcDNA™-DEST40 and pcDNA™/GW-40/*lacZ* are shipped on wet ice. Upon receipt, store at –20°C. Products are guaranteed for six months from date of shipment when stored properly.

Contents

The pcDNA™-DEST40 Gateway® Vector components are listed below.

Item	Concentration	Amount
pcDNA™-DEST40 Vector	40 µL of vector at 150 ng/µL in TE, pH 8.0 (10 mM Tris-HCl, 1mM EDTA, pH 8.0)	6 µg
pcDNA™/GW-40/ *lacZ* Control Plasmid	20 µL of vector at 0.5 µg/µL in TE, pH 8.0	10 µg

Product Use

For Research Use Only. Not for use in diagnostic procedures.

Accessory Products

Additional Products

Additional products that may be used with the pcDNA™-DEST40 Gateway® Vector are available from Life Technologies. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 15). Ordering information is provided below.

Product	Quantity	Cat. no.
Gateway® LR Clonase® II Enzyme Mix	20 reactions	11791-020
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
	20 reactions	C4040-52
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells	10 reactions	A10460
Lipofectamine® 2000 Reagent	1.5 mL	11668-019
	0.75 mL	11668-027
Geneticin®	1 g	11811-023
	5 g	11811-031
	20 mL (50 mg/mL)	10131-035
	100 mL (50 mg/mL)	10131-027
pENTR/D-TOPO® Cloning Kit	20 reactions	K2400-20
PureLink® HiPure Plasmid Miniprep Kit	25 preps 100 preps	K2100–02 K2100–03
PureLink® HiPure Plasmid Midiprep Kit	25 preps 50 preps	K2100–04 K2100–05
Phosphate-Buffered Saline (PBS), pH 7.4, (1X), liquid	500 mL	10010-023
β-Gal Antiserum	25 western blots	R901-25
β-Gal Assay Kit	1 kit	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
Novex® Tris-Glycine SDS Sample Buffer (2X)	20 mL	LC2676

Continued on next page

Accessory Products, continued

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The amount of antibody supplied is sufficient for 25 western blot experiments.

Product	Epitope	Cat. 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-His (C-term) Antibody	Detects the C-terminal polyhistidine (6×His) tag; requires the free carboxyl group for detection (Lindner *et al.*, 1997). HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25
Positope™ Control Protein	53 kDa positive control protein for antibody function.	R900-50

Purification of Recombinant Fusion Protein

If your gene of interest is in frame with the C-terminal peptide containing the V5 epitope and the polyhistidine (6×His) tag, you may use Immobilized Metal Affinity Chromatography (IMAC) to purify your recombinant fusion protein. The ProBond™ Purification System and bulk ProBond™ resin are available separately from Life Technologies. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 15).

Product	Quantity	Cat. no.
ProBond™ Nickel-chelating Resin	50 mL	R801-01
	150 mL	R801-15
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 kit	K854-01
Purification Columns (10 mL polypropylene columns)	50	R640-50

Introduction

Overview

Description

pcDNA™-DEST40 is a 7.1 kb vector derived from the pcDNA™3.1/V5-His™ vector and adapted for use with the Gateway® Technology. It is designed to allow high-level, constitutive expression of the gene of interest in a variety of mammalian hosts. For more information on Gateway® Technology, see the next page.

Features

pcDNA™-DEST40 contains the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells
- Two recombination sites, attR1 and attR2, downstream of the CMV promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two attR sites for counterselection
- The ccdB gene located between the two attR sites for negative selection
- The V5 epitope and 6×His tag for detection and purification (optional)
- Bovine growth hormone (BGH) polyadenylation sequence 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 resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Neomycin resistance gene for selection of stable cell lines using Geneticin®
- 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*

For a map of pcDNA™-DEST40, see page 12.

Continued on next page

Overview, continued

The Gateway® Technology

Gateway® is a universal cloning technology 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™-DEST40).
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® System, refer to the Gateway® Technology Manual. This manual is available for downloading at **www.lifetechnologies.com** or by contacting Technical Support (page 15).

Methods

Using pcDNA™-DEST40

Important

The pcDNA™-DEST40 vector is supplied as a supercoiled plasmid. Although Life Technologies has previously recommended using a linearized destination vector for more efficient recombination, further testing has found that linearization of this vector is **NOT** required to obtain optimal results for any downstream application.

Propagating pcDNA™-DEST40

If you wish to propagate and maintain pcDNA™-DEST40, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (see page v) from Life Technologies. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

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

Entry Clone

To recombine your gene of interest into pcDNA™-DEST40, you should have an entry clone containing your gene of interest. For your convenience in generating your entry vector, Life Technologies offers the pENTR/D-TOPO® Cloning Kit for 5 minute, directional TOPO® cloning your gene of interest (see page v for ordering information). For more information on entry vectors available from Life Technologies, refer to **www.lifetechnologies.com** or contact Technical Support (page 15).

For detailed information on constructing an entry clone, refer to the specific entry vector manual. For detailed information on performing the LR recombination reaction, refer to the Gateway® Technology Manual.

Continued on next page

Using pcDNA™-DEST40, continued

Points to Consider Before Recombining

Your insert should contain a Kozak consensus 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. 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 sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

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

If you wish to include the V5 epitope and 6×His tag, your gene in the entry clone should **not** contain a stop codon. The gene should also be designed to be in frame with the C-terminal epitope tag after recombination. Refer to the **Recombination Region**, see page 12.

If you do NOT wish to include the V5 epitope and 6×His tag, your gene should contain a stop codon in the entry clone.

Recombining Your Gene of Interest

Each entry clone contains *att*L sites flanking the gene of interest. Genes in an entry clone are transferred to the destination vector backbone by mixing the DNAs with the Gateway® LR Clonase® II enzyme mix (see page v for ordering information). The resulting recombination reaction is then transformed into *E. coli* and the expression clone selected. Recombination between the *att*R sites on the destination vector and the *att*L sites on the entry clone replaces the *ccd*B gene and the chloramphenicol (Cm^R) gene with the gene of interest and results in the formation of *att*B sites in the expression clone.

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

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Using pcDNA™-DEST40, continued

Confirming the Expression Clone

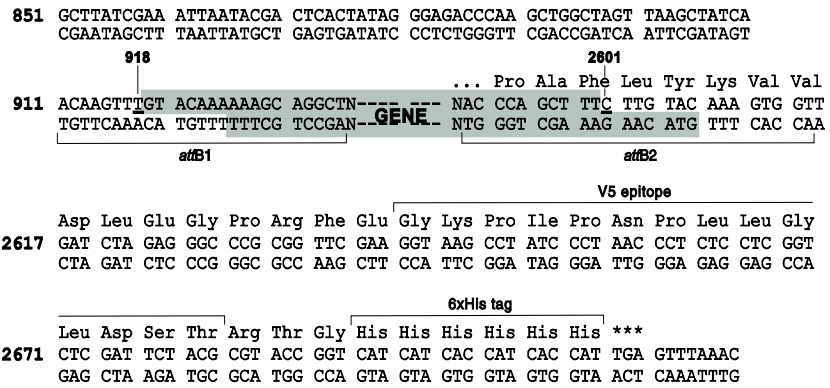
The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. 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.

Recombination Region

The recombination region of the expression clone resulting from pcDNA™-DEST40 × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into pcDNA™-DEST40 by recombination. Non-shaded regions are derived from the pcDNA™-DEST40 vector.
- The underlined nucleotides flanking the shaded region correspond to bases 918 and 2601, respectively, of the pcDNA™-DEST40 vector sequence.



Transfection

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™/GW-40/*lacZ* 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 very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® HiPure Plasmid Miniprep Kit (10–15 µg DNA), the PureLink® HiPure Plasmid Midiprep Kit (10–200 µg DNA), or CsCl gradient centrifugation. See page v for ordering information.

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). If you wish to use a lipid-based reagent for transfection, we recommend using Lipofectamine® 2000 Reagent available from Life Technologies (see page v for ordering). For more information, refer to **www.lifetechnologies.com** or contact Technical Support (page 15).

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Transfection, continued

Positive Control

pcDNA™ /GW-40/*lacZ* is provided as a positive control vector for mammalian cell transfection and expression (see page 14 for a map) and may be used to optimize recombinant protein expression levels in your cell line. The vector allows expression of a C-terminally tagged β-galactosidase fusion protein that may be detected by western blot or functional assay.

To propagate and maintain the plasmid:

1. Use 10 ng of the vector supplied in stock solution (0.5 µg/µL in TE, pH 8.0) 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 transiently transfected cells or stable cell lines (see page 10 for guidelines to create stable cell lines). A sample protocol to detect your fusion protein by Western blot is provided below. Other protocols are suitable.

Preparing Cell Lysates

1. Wash cell monolayers (~5×10^5 to 1×10^6 cells) once with phosphate-buffered saline (PBS, available from Gibco®; see page v for ordering information).
2. Scrape cells into 1 mL PBS and pellet the cells at $1500 \times g$ for 5 minutes.
3. Resuspend in 50 µL Cell Lysis Buffer (see page 11 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 $10{,}000 \times g$ for 10 minutes at 4°C to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration. 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 1X SDS-PAGE sample buffer (see page v for ordering) 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.

SDS-PAGE

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 Life Technologies. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (page 15).

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Detecting Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Life Technologies (see page vi for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein is available from Life Technologies for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6×His) tag. See page vi for ordering information. For more information on detecting your recombinant fusion protein, refer to **www.lifetechnologies.com** or contact Technical Support (see page 15).

Assay for β-Gal

If you use the pcDNA™/GW-40/*lacZ* plasmid as a positive control vector, you may assay for β-galactosidase expression by western blot analysis or activity assay using cell-free lysates (Miller, 1972). Life Technologies offers β-Gal Antiserum, the β-Gal Assay Kit, and the β-Gal Staining Kit for fast and easy detection of β-galactosidase expression.

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine tag will add approximately 5 kDa to your protein.

Purifying Recombinant Fusion Proteins

The presence of the C-terminal polyhistidine (6×His) tag in your recombinant fusion protein allows use of a metal-chelating resin such as ProBond™ to purify your fusion protein. The ProBond™ Purification System and bulk ProBond™ resin are available from Life Technologies. Refer to the ProBond™ Purification System manual for protocols to purify your fusion protein. Life Technologies also offers Ni-NTA Agarose for purification of proteins containing a polyhistidine (6×His) tag. See page vi for ordering information.

Note: Other metal-chelating resins and purification methods are suitable.

Creating Stable Cell Lines

Introduction

The pcDNA™-DEST40 vector contains the neomycin resistance gene to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your construct into the mammalian cell line of choice and select for foci using Geneticin®. General guidelines are provided below.

To obtain stable transfectants, we recommend that you linearize your pcDNA™-DEST40 construct before transfection. While linearizing the vector may not improve transfection efficiency, 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 neither located within a critical element nor within your gene of interest.

Geneticin® Selective Antibiotic

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

Geneticin® Selection Guidelines

Geneticin® selective antibiotic is available from Life Technologies. Use as follows:

1. Prepare Geneticin® antibiotic in a buffered solution (e.g., 100 mM HEPES, pH 7.3).
2. Use 100–1000 µg/mL of Geneticin® antibiotic in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Test varying concentrations of Geneticin® antibiotic on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin® selective antibiotic.

Cells will divide once or twice in the presence of lethal doses of Geneticin® Selective Antibiotic, 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.

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.

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

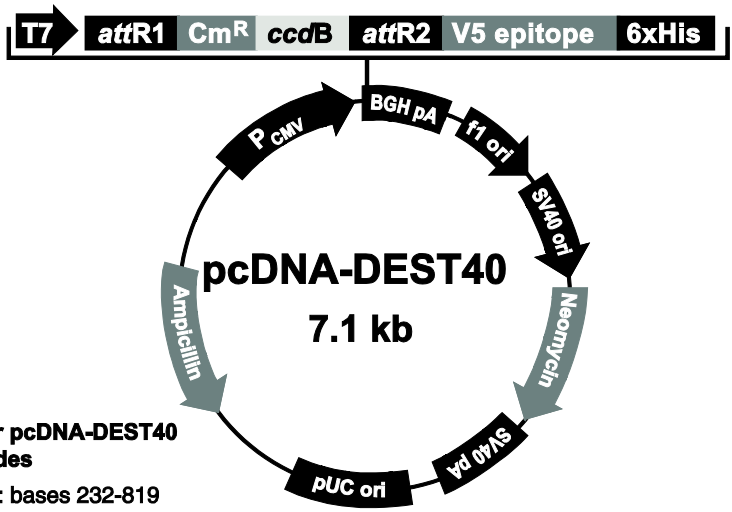
2. Bring the volume up to 90 mL with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 mL. Store at room temperature.

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

Map and Features of pcDNA™-DEST40

Map of pcDNA™-DEST40

The map below shows the elements of pcDNA™-DEST40. DNA from the entry clone replaces the region between bases 918 and 2601. **The complete sequence of pcDNA™-DEST40 is available at www.lifetechnologies.com or by contacting Technical Support (page 15).**



Comments for pcDNA-DEST40
7143 nucleotides

CMV promoter: bases 232-819
T7 promoter: bases 863-882
*att*R1 recombination site: bases 911-1035
Chloramphenicol resistance gene: bases 1144-1803
*ccd*B gene: bases 2145-2450
*att*R2 recombination site: bases 2491-2615
V5 epitope: bases 2641-2682
6xHis tag: bases 2692-2709
BGH polyadenylation region: bases 2735-2962
f1 origin: bases 3008-3436
SV40 early promoter and origin: bases 3463-3771
Neomycin resistance ORF: bases 3846-4640
SV40 early polyadenylation region: bases 4816-4946
pUC origin: bases 5329-6002
Ampicillin (*bla*) resistance ORF: bases 6147-7007 (complementary strand)
bla promoter: bases 7008-7106 (complementary strand)

Continued on next page

Map and Features of pcDNA™-DEST40, continued

Features of pcDNA™-DEST40

pcDNA™-DEST40 (7143 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter	Allows *in vitro* transcription in the sense orientation
*att*R1 and *att*R2 sites	Allows recombinational cloning of the gene of interest from an entry clone
Chloramphenicol resistance gene	Allows counterselection of expression clones
*ccd*B gene	Allows negative selection of expression clones
V5 epitope	Allows detection of recombinant fusion proteins by the Anti-V5 antibodies (Southern *et al.*, 1991)
C-terminal polyhistidine tag	Allows purification of recombinant proteins on metal-chelating resin such as ProBond™. Allows detection of the recombinant protein by the Anti-His (C-term) antibodies (Lindner *et al.*, 1997)
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin resistance gene	Allows selection of transformants in *E. coli*

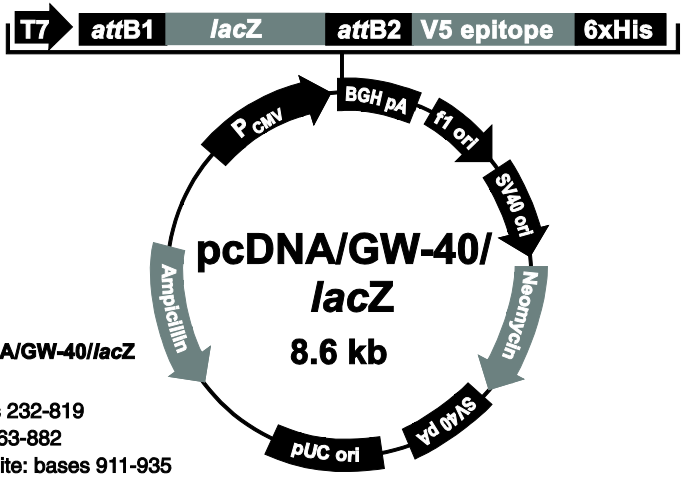
Map of pcDNA™/GW-40/*lacZ*

Description

pcDNA™/GW-40/*lacZ* is an 8584 bp control vector containing the gene for β-galactosidase. pcDNA™/GW-40/*lacZ* was created with Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pcDNA™-DEST40. β-galactosidase is expressed as a fusion to the C-terminal tag. The MW of the fusion protein is approximately 120 kDa.

Map of pcDNA™/ GW-40/*lacZ*

The map below shows the elements of pcDNA™/GW-40/*lacZ*. **The complete sequence of pcDNA™/GW-40/*lacZ* is available at www.lifetechnologies.com or by contacting Technical Support (page 15).**



Comments for pcDNA/GW-40/*lacZ*
8584 nucleotides

CMV promoter: bases 232-819
T7 promoter: bases 863-882
*att*B1 recombination site: bases 911-935
*lac*Z ORF: bases 956-4015
*att*B2 recombination site: bases 4032-4056
V5 epitope: bases 4082-4123
6xHis tag: bases 4133-4150
BGH polyadenylation region: bases 4176-4403
f1 origin: bases 4449-4877
SV40 early promoter and origin: bases 4904-5212
Neomycin resistance ORF: bases 5287-6081
SV40 early polyadenylation region: bases 6257-6387
pUC origin: bases 6770-7443
Ampicillin (*bla*) resistance ORF: bases 7588-8448 (complementary strand)
bla promoter: bases 8449-8547 (complementary strand)

Technical Support

Obtaining Support

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

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- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
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- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
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- Obtain information about customer training
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28 February 2013