USER GUIDE

invitrogen™
by life technologies™

pcDNA™3.1/His A, B, and C

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

life technologies™

Contents

Kit Contents and Storage

Shipping and Storage

pcDNA™ 3.1/His vectors are shipped on wet ice. Upon receipt, store vectors at –20ºC.

Kit Contents

10 µg each of pcDNA™3.1/His A, B, and C are supplied at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 20 µL.

10 µg of pcDNA™3.1/His/*lac*Z is supplied at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 20 µL.

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

Methods

Cloning into pcDNA™3.1/His A, B, and C

Description of the System

pcDNA™3.1/His A, B, and C are 5.5 kb vectors derived from pcDNA™3.1 and designed for high-level expression and purification of recombinant proteins in mammalian hosts. The vectors are supplied in three reading frames to facilitate in frame cloning with a polyhistidine metal-binding tag. The human cytomegalovirus (CMV) immediate-early promoter provides high-level expression in a wide range of mammalian cells. In addition, the vector replicates episomally in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS7). High-level stable and non-replicative transient expression can be carried out in most mammalian cells. The control plasmid, pcDNA™3.1/His/*lac*Z, is the pcDNA™3.1/His B vector with a 3.2 kb fragment containing the β-galactosidase gene cloned in frame with the N-terminal peptide. pcDNA™3.1/His/*lac*Z is included for use as a positive control for transfection, expression, and purification in the cell line of choice.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, see *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Maintaining pcDNA™3.1/His

Many *E. coli* strains are suitable for the growth of this vector. To propagate and maintain pcDNA™3.1/His A,B, and C, use the supplied stock solution to transform a *rec*A (recombination deficient), *end*A (endonuclease A deficient) *E. coli* strain like TOP10, TOP10F′, DH5α™-T1^R, DH10B™, or equivalent (see page 12 for ordering information). Select the transformants on LB plates containing 50–100 µg/mL ampicillin.

For long-term storage, prepare a glycerol stock of your plasmid-containing *E. coli* strain.

Continued on next page

Cloning into pcDNA™3.1/His A, B, and C, Continued

Kozak Sequence for Mammalian Expression

If you are recombining your entry clone with a destination vector for mammalian expression, 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**

Note

The pcDNA™3.1/His vectors are fusion vectors. To ensure proper expression of your recombinant protein, you must clone your gene in frame with the ATG at base pairs 920–922. This will create a fusion with the N-terminal polyhistidine tag, Xpress™ epitope, and the enterokinase cleavage site. The vector is supplied in three reading frames to facilitate cloning. See pages 3–5 to develop a cloning strategy.

If you wish to clone as close as possible to the enterokinase cleavage site, follow the guidelines below:

- Digest pcDNA™3.1/His A, B, or C with *Kpn* I
- Create blunt ends with T4 DNA polymerase and dNTPs
- Clone your blunt-ended insert in frame with the lysine codon (AAG) of the enterokinase recognition sequence.

Following enterokinase cleavage, no vector-encoded amino acid residues will be present in your protein.

Continued on next page

Cloning into pcDNA™3.1/His A, B, and C, Continued

Multiple Cloning Site of pcDNA™3.1/His A

Below is the multiple cloning site for pcDNA™3.1/His A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotide indicates the variable region. Note that there is a stop codon after the *Xba* I site and that the *Asp718 I* and *Kpn I* sites are in the same reading frame for all three vectors. The multiple cloning site has been confirmed by sequencing and functional testing. The sequence is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 13).

T7 promoter/priming site

```
839  CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT AGGGAGACCC AAGCTGGCTA
```

Hind III

Polyhistidine (6xHis) region

```
899  GCGTTTAAAC TTAAGCTTAC C ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT
                             Met Gly Gly Ser His His His His His His

950  GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC
     Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr
```

Xpress™ Epitope

*Asp*718 I *Kpn* I *Bam*H I

*Bst*X I* *Eco*R I

```
998  GAC GAT GAC GAT AAG GTA CCT AGG ATC CAG TGT GGT GGA ATT CTG CAG
     Asp Asp Asp Asp Lys Val Pro Arg Ile Gln Cys Gly Gly Ile Leu Gln
```

Enterokinase recognition sequence

EK cleavage site

*Eco*R V *Bst*X I* *Not* I *Xho* I *Xba* I *Apa* I

```
1046 ATA TCC AGC ACA GTG GCG GCC GCT CGA GTC TAG AGGGCCCGTT TAAACCCGCT
     Ile Ser Ser Thr Val Ala Ala Ala Arg Val ***
```

BGH reverse priming site

```
1099 GATCAGCCTC GACTGTGCCT TCTAGTTGCC AGCCATCTGT TGTTTGCCCC TCCCCCGTGC
```

BGH poly (A) site

```
1159 CTTCCTTGAC CCTGGAAGGT GCCACTCCCA CTGTCCTTTC CTAATAAAAT GAGGAAATTG
```

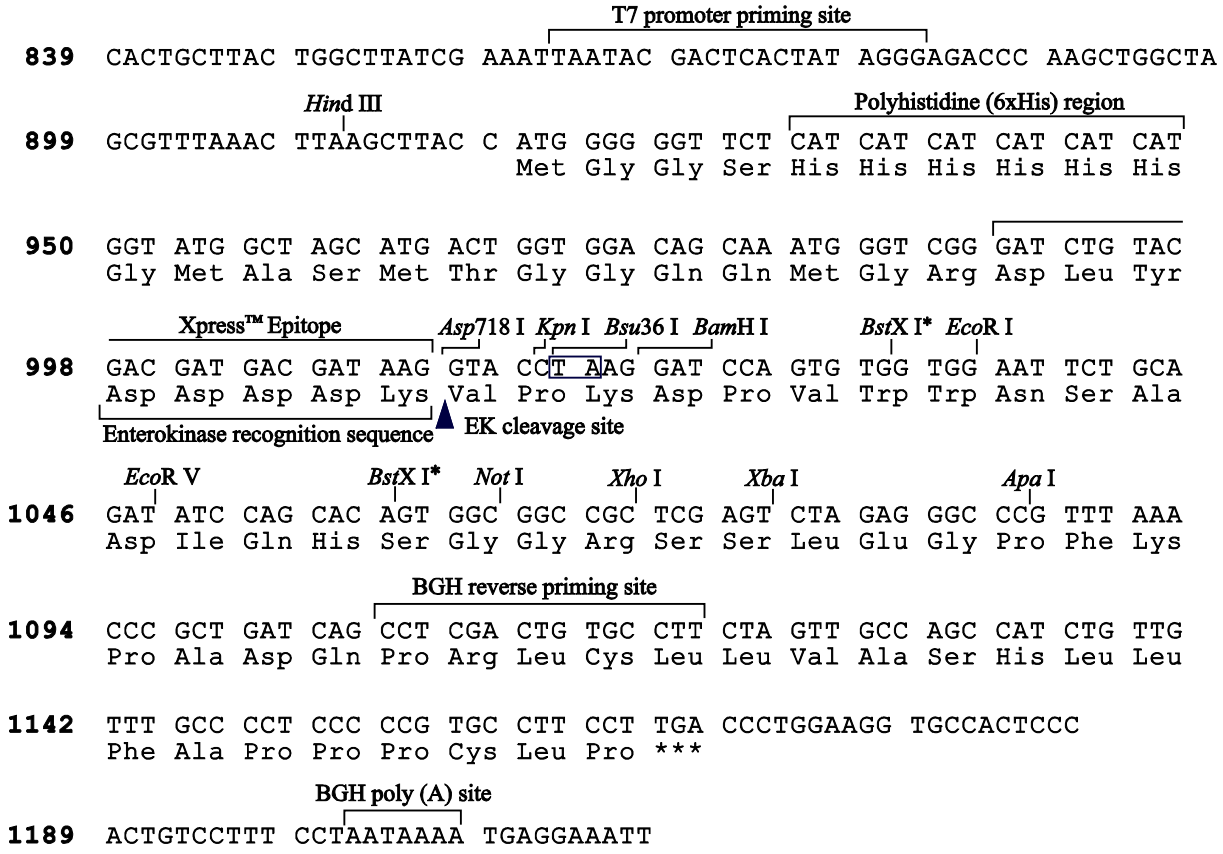
*Note that there are two *Bst*X I sites in the polylinker.

Continued on next page

Cloning into pcDNA™3.1/His A, B, and C, Continued

Multiple Cloning Site of pcDNA™3.1/His B

Below is the multiple cloning site for pcDNA™3.1/His B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. The multiple cloning site has been confirmed by sequencing and functional testing. Note that the *Asp718 I* and *Kpn I* sites are in the same reading frame for all three vectors. The sequence is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 13).



*Note that there are two *BstX* I sites in the polylinker.

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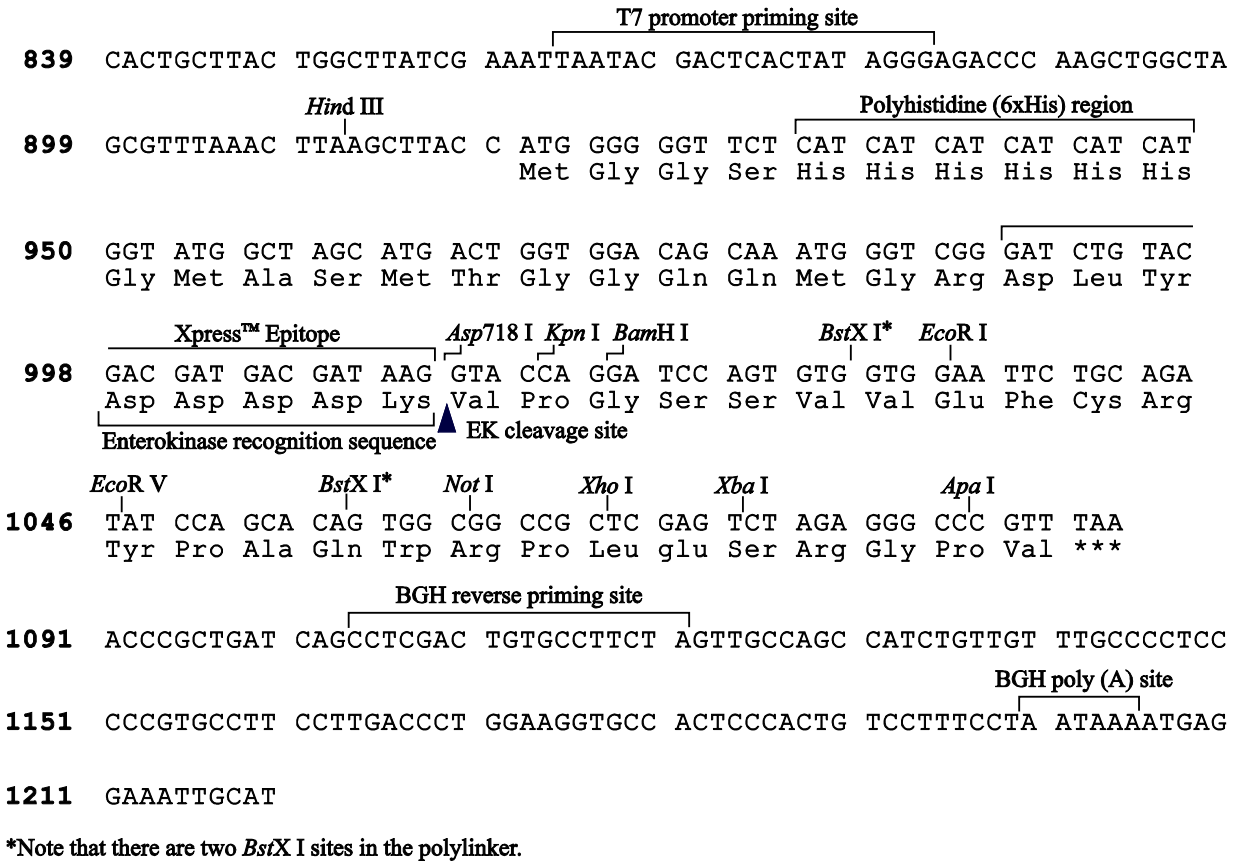
Cloning into pcDNA™3.1/His A, B, and C, Continued

Multiple Cloning Site of pcDNA™3.1/His C

Below is the multiple cloning site for pcDNA™3.1/His C. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. Note that the *Asp718 I* and *Kpn I* sites are in the same reading frame for all three vectors. The sequence is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 13).



*Note that there are two *BstX* I sites in the polylinker.

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Transformation and Transfection

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g., TOP10, TOP10F′, DH5α™-T1^R, DH10B™, see page 12) and select on LB plates containing 50–100 µg/mL ampicillin. Select 10–20 clones and analyze for the presence and orientation of your insert.

Applying Selective Pressure

We recommend taking some (if not all) of the following precautions to prevent your clone from being "overrun" by background contaminants:

- **Use carbenicillin instead of ampicillin.** Carbenicillin is more stable than ampicillin, and allows for a longer period of selective pressure, thus preserving your clones longer.
- **Increase the antibiotic concentration.** More antibiotic means that your clones will not be overwhelmed by β-lactamase buildup.
- **Periodically refresh plate media.** If you suspect that tubes/plates may be beginning to fail, spin them down, remove the old media, and replenish the wells with fresh LB media plus glycerol and antibiotic.

Streak clones on selective (preferably carbenicillin) LB agar plates. After about 12 hours, isolate colonies for downstream usage. This will isolate your desired clones from potential background contaminants.

We recommend that you sequence your construct with the T7 Forward and BGH Reverse primers (see page 12 for ordering information) to confirm that your gene is fused in frame with the N-terminal His tag and the enterokinase site. Refer to the diagrams on pages 3–5 for the sequences and locations of the priming sites.

Primer	Sequence
T7 Promoter	5′-TAATACGACTCACTATAGGG-3′
BGH Reverse	5′-TAGAAGGCACAGTCGAGG-3′

For your convenience, we offer a custom primer service. For more information, visit www.lifetechnologies.com or call Technical Support (see page 13).

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating DNA using the PureLink® HiPure Miniprep Kit or the PureLink® HiPure Midiprep Kit (see page 12 for ordering information) or CsCl gradient centrifugation.

Continued on next page

Transformation and Transfection, Continued

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. Precisely follow the protocol for your cell line, paying 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 cells, use Lipofectamine® 2000 Reagent available for purchase (see page 12). For more information on Lipofectamine® 2000 and other transfection reagents, visit www.lifetechnologies.com or contact Technical Support (see page 13).

Positive Control

pcDNA™3.1/His/*lac*Z is supplied as a positive control vector for mammalian transfection and expression (see page 11). pcDNA™3.1/His/*lac*Z may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the CMV promoter. A successful transfection results in β-galactosidase expression and can be easily assayed.

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. The β-Gal Assay Kit and the β-Gal Staining Kit are available for purchase (see page 12 for ordering information) for fast, easy detection of β-galactosidase expression.

Geneticin® Selective Antibiotic

For stable transfection, pcDNA™3.1/His A, B, and C contain the resistance factor to 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® Selective Antibiotic (Southern and Berg, 1982).

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Geneticin® Selection Guidelines

Geneticin® is available for purchase (see page 12 for ordering information). Use as follows:

1. Prepare Geneticin® in a buffered solution (e.g., 100 mM HEPES, pH 7.3).
2. Use 100 to 1,000 µg/mL of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug (check the lot label).
4. Test varying concentrations of Geneticin® on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin®.

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 4 weeks of growth in selective medium.

Preparing Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™ (see page 12 for ordering information). You will need 5×10^6 to 1×10^7 cells for purification of your protein on a 2 mL ProBond™ column (see ProBond™ Protein Purification manual).

Note: The N-terminal peptide adds approximately 5 kDa to the size of your recombinant fusion protein.

1. Seed cells in five T-75 flasks or 2 to 3 T-150 flasks.
2. Grow the cells in selective medium until they are 80–90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin, if necessary, and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at approximately 250 × *g* for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at −80°C until needed.

Lysing Cells

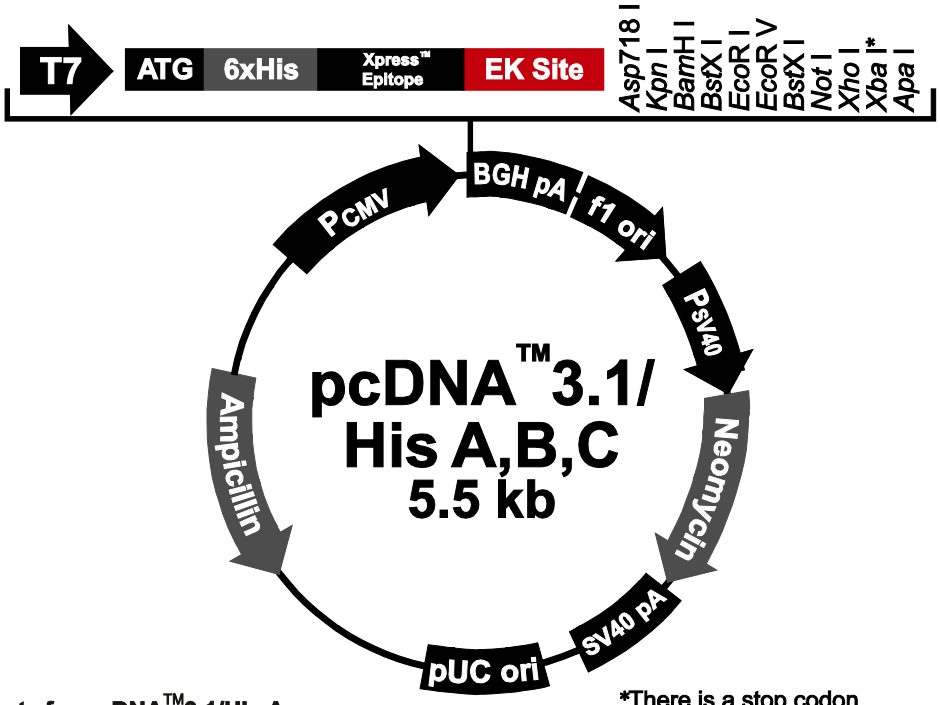
If you are using ProBond™ resin, refer to the ProBond™ Protein Purification manual for details about sample preparation for chromatography. If you are using other metal-chelating resin, refer to the manufacturer's instruction.

Appendix

Map of pcDNA™3.1/His A, B, and C Vectors

Map of pcDNA™3.1/His

The figure below summarizes the features of the pcDNA™3.1/His vectors. The sequences for pcDNA™3.1/His A, B, and C are available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 13). Details of the multiple cloning sites are shown on page 3 for pcDNA™3.1/His A, page 4 for pcDNA™3.1/His B, and page 5 for pcDNA™3.1/ His C.



*There is a stop codon following the *Xba* I site in version A.

Comments for pcDNA™3.1/His A
5514 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
ATG initiation codon: bases 920-922
Polyhistidine region: bases 932-949
Xpress™ epitope: bases 989-1012
Enterokinase recognition site: bases 998-1012
Multiple cloning site: bases 1012-1085
BGH reverse priming site: bases 1105-1122
BGH polyadenylation signal: bases 1104-1318
f1 origin: bases 1381-1794
SV40 early promoter and origin: bases 1859-2183
Neomycin resistance gene: bases 2219-3013
SV40 polyadenylation signal: bases 3029-3268
pUC origin: bases 3700-4373 (Complementary strand)
Ampicillin resistance gene: bases 4518-5379 (Complementary strand)

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Features of pcDNA™3.1/His A, B, and C Vectors

Features of pcDNA™3.1/His

pcDNA™3.1/His A (5,514 bp), pcDNA™3.1/His B (5,515 bp), and pcDNA™3.1/His C (5,513 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert.
N-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™.
Xpress™ epitope tag	Allows detection of your recombinant protein with the Anti-Xpress™ Antibody (Cat. no. R910-25).
Enterokinase cleavage site	Allows removal of the N-terminal polyhistidine tag from your recombinant protein using an enterokinase such as EKMax™ (Cat. no E180-01).
Multiple cloning site in three reading frames	Allows insertion of your gene and facilitates cloning in frame with the polyhistidine N-terminal tag.
BGH reverse priming site	Permits sequencing through the insert.
Bovine growth hormone (BGH) polyadenylation signal	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 efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing SV40 large T antigen.
Neomycin resistance gene	Selection of stable transfectants in mammalian cells (Southern and Berg, 1982).
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA.
pUC origin	High-copy number replication and growth in *E. coli*.
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*.

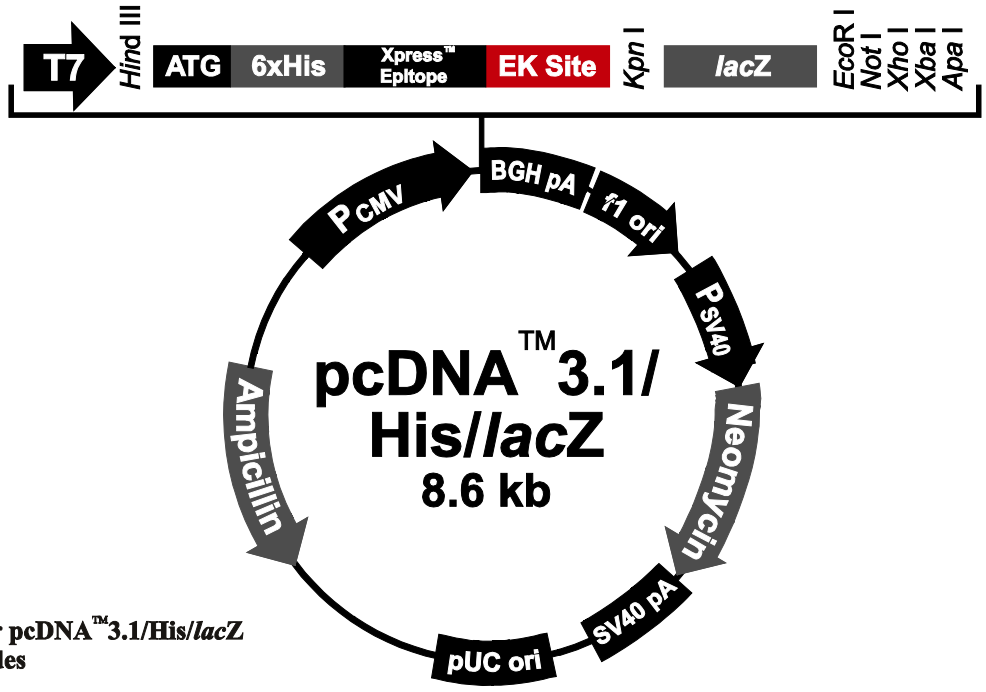
Map of pcDNA™3.1/His/*lacZ*

Description

pcDNA™3.1/His/*lacZ* is a 8,577 bp control vector containing the gene for β-galactosidase. pcDNA™3.1/His B was digested with *Bam*H I, blunted, and digested with *Eco*R I. A 3.2 kb blunt-*Eco*R I fragment containing the β-galactosidase gene was then ligated into pcDNA™3.1/His B in frame with the N-terminal peptide. The β-galactosidase protein expressed from pcDNA™3.1/His/*lacZ* has a size of approximately 120 kDa.

Map of Control Vector

The figure below summarizes the features of the pcDNA™3.1/His/*lacZ* vector. The nucleotide sequence for pcDNA™3.1/His/*lacZ* is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 13).



Comments for pcDNA™3.1/His/*lacZ*
8577 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
ATG initiation codon: bases 920-922
Polyhistidine region: bases 932-949
Xpress™ epitope: bases 989-1012
Enterokinase recognition site: bases 998-1012
LacZ ORF: bases 1033-4090
BGH reverse priming site: bases 4168-4185
BGH polyadenylation signal: bases 4167-4381
f1 origin: bases 4444-4857
SV40 promoter and origin: bases 4922-5246
Neomycin resistance gene: bases 5282-6076
SV40 polyadenylation signal: bases 6092-6331
pUC origin: bases 6763-7436 (Complementary strand)
Ampicillin resistance gene: bases 7581-8442 (Complementary strand)

Accessory Products

Additional Products

The following additional products may be used with the pcDNA™3.1/His vectors. For more information, visit www.lifetechnologies.com or contact Technical Support (see page 13).

Item	Quantity	Cat. no.
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
One Shot® TOP10F´ Chemically Competent *E. coli*	20 × 50 µL	C3030-03
One Shot® Max Efficiency® DH5α™ $T1^R$ Competent Cells	20 × 50 µL	12297-016
MAX Efficiency® DH10B™ Competent Cells	5 × 0.2 mL	18297-010
Electrocomp™ Kit	2 × 20 reactions	C66511
Ampicillin	200 mg	11593-027
Carbenicillin	5 g	10177-012
T7 promoter primer	2 µg	N560-02
BGH Reverse primer	2 µg	N575-02
PureLink® HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink® HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Lipofectamine® 2000 Reagent	1.5 mL	11668-019
β–Gal Assay Kit	1 kit	K1455-01
β–Gal Staining Kit	1 kit	K1465-01
Anti-Xpress™ Antibody	50 µL	R910-25
EKMax™ Enterokinase	250 U	E180-01
	1000 U	E180-02
Geneticin® Selective Antibiotic	1 g	11811-023
	5 g	11811-031
	20 mL (50 mg/mL)	10131-035
	100 mL (50 mg/mL)	10131-027

Purifying Fusion Proteins

The N-terminal polyhistidine tag can be used to purify the recombinant fusion protein with a metal-chelating resin such as ProBond™. Ordering information for ProBond™ resin is provided below.

Item	Quantity	Cat. no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Binding Resin (Precharged resin provided as a 50% slurry in 20% ethanol)	50 mL	R801-01
	150 mL	R801-15

Technical Support

Obtaining support

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

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

Certificate of Analysis

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

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Limited Use Label License 6x His Tag

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Notes

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