USER GUIDE

invitrogen™
by life technologies™

pcDNA™3.1/Hygro (+)
pcDNA™3.1/Hygro (–)

For high-level stable and transient expression in mammalian hosts

Catalog numbers V870–20, V875–20

Revision date 19 January 2012
Publication Part number 28–0149

MAN0000650

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Kit Contents and Storage

Shipping and Storage

pcDNA™3.1/Hygro (+/–) vectors are shipped on wet ice. Upon receipt, store vectors at –20°C.

Kit Contents

Each catalog number contains the following vectors. All vectors are supplied in aliquot detailed below. **Store the vectors at –20°C.**

Catalog nos.	Vector	Quantity	Composition (supplied as)
V870-20	pcDNA™3.1/Hygro (+)	20 µg	40 µL of 0.5 µg/µL pcDNA™3.1/Hygro (+) vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
	pDNA™3.1/Hygro/*lac*Z control	20 µg	40 µL of 0.5 µg/µL pcDNA™3.1/Hygro/*lac*Z control vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
V875-20	pcDNA™3.1/Hygro (–)	20 µg,	40 µL of 0.5 µg/µL pcDNA™3.1/Hygro (–) vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0
	pDNA™3.1/Hygro/*lac*Z control	20 µg	40 µL of 0.5 µg/µL pcDNA™3.1/Hygro/*lac*Z control vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Intended Use

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

Accessory Products

Introduction

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

Item	Quantity	Catalog no.
One Shot® TOP10F´ (chemically competent cells)	21 × 50 µl	C3030–03
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040–10
One Shot® TOP10 Electrocomp™ *E. coli*	10 reactions 20 reactions	C4040–50 C4040–52
One Shot® MAX Efficiency® DH10B™ (chemically competent cells)	1 mL	18297–010
One Shot® MAX Efficiency® DH10α™ ($T1_R$ competent cells)	20 × 50 µL	12297–016
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
Hygromycin B	20 mL	10687-010
Bgl II	400 units	15213-010
Ssp	400 units	15458-011
β-Gal Assay Kit	1 kit	K1455-01
β-Gal Staining Kit	1 kit	K1465-01

Introduction

Overview

Introduction

pcDNA™3.1/Hygro (+) and pcDNA™3.1/Hygro (–) are 5.6 kb vectors derived from pcDNA™3.1 and are designed for high-level stable and transient expression in mammalian hosts. High-level stable and non-replicative transient expression can be carried out in most mammalian cells. These vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells
- Multiple cloning sites in the forward (+) and reverse (–) orientations to facilitate cloning
- Hygromycin resistance gene for selection of stable cell lines
- Episomal replication in cells lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g., COS–1, COS–7)

A control plasmid, pcDNA™3.1/Hygro/*lac*Z, is included for use as a positive control for transfection and expression in the cell line of choice.

CMV Promoter

pcDNA™3.1/ Hygro (+) and pcDNA™3.1/ Hygro (–) vectors contain the human CMV immediate early promoter to allow high-level, constitutive expression of the gene of interest in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). Although highly active in most mammalian cell lines, activity of the viral promoter can be down-regulated in some cell lines due to methylation (Curradi *et al.*, 2002), histone deacetylation (Rietveld *et al.*, 2002), or both.

Experimental Outline

Use the following outline to clone and express your gene of interest in pcDNA™3.1/Hygro (+/–).

1. Consult the multiple cloning sites described on pages 3–4 to design a strategy to clone your gene into pcDNA™3.1/Hygro.
2. Ligate your insert into the appropriate vector and transform into *E. coli*. Select transformants on LB plates containing 50–100 µg/mL ampicillin.
3. Analyze your transformants for the presence of insert by restriction digestion.
4. Select a transformant with the correct restriction pattern and sequence to confirm that your gene is cloned in the proper orientation.
5. Transfect your construct into the mammalian cell line of interest using your own method of choice. Generate a stable cell line, if desired.
6. Test for expression of your recombinant gene by western blot analysis or functional assay.

Methods

Cloning into pcDNA™3.1/Hygro (+/–)

Introduction

To recombine your gene of interest into pcDNA™3.1/Hygro (+/–), you will need to ligate your gene of interest into pcDNA™3.1/Hygro (+) or pcDNA™3.1/Hygro (–). Diagrams of the multiple cloning sites for each vector are provided on pages 3–4. General considerations for cloning and transformation are listed below.

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, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Strain

Many *E. coli* strains are suitable for the propagation of pcDNA™3.1/Hygro (+/–) including TOP10F´, DH5α, and TOP10 (see page v for ordering information). We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A-deficient (*end*A).

For your convenience, TOP10F´ is available as chemically competent or electrocompetent cells from Life Technologies.

Transformation Method

You may use any method of your choice for transformation. Chemical transformation is the most convenient for most researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of pcDNA™3.1/ Hygro (+/–)

To propagate and maintain pcDNA™3.1/Hygro (+/–), we recommend using 10 ng of the vector to transform a *rec*A, *end*A *E. coli* strain such as TOP10, TOP10F´, DH5α™, or equivalent (see page v for ordering information) using your method of choice. Select transformants on LB plates containing 50–100 µg/mL ampicillin.

For long-term storage, be sure to prepare a glycerol stock (page 5) of your plasmid-containing *E. coli* strain.

Points to Consider Before Recombining into pcDNA™3.1/ Hygro (+/–)

pcDNA™3.1/Hygro (+) and pcDNA™3.1/Hygro (–) are nonfusion vectors. Your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). 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)NNATG**G**

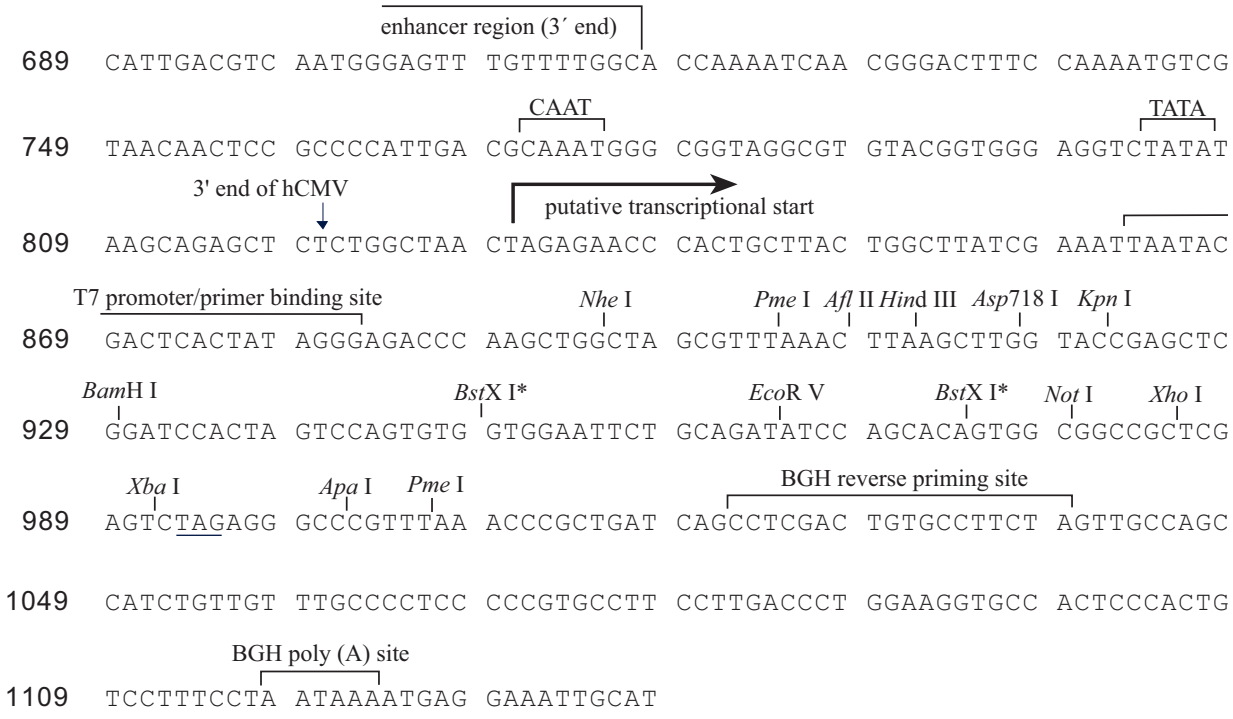
Your insert must also contain a stop codon for proper termination of your gene. Note that the *Xba* I site contains an internal stop codon (TCTAGA).

Continued on next page

Cloning into pcDNA™3.1/Hygro, continued

Multiple Cloning Site of pcDNA™3.1/ Hygro (+)

Below is the multiple cloning site for pcDNA™3.1/Hygro (+). Restriction sites are labeled to indicate the cleavage site. The *Xba* I site contains an internal stop codon (TCTAGA). The multiple cloning site has been confirmed by sequencing and functional testing. The complete sequence of pcDNA™3.1/Hygro (+) is available for downloading from our web site at **www.lifetechnologies.com** or from **Technical Support** (see page 13).



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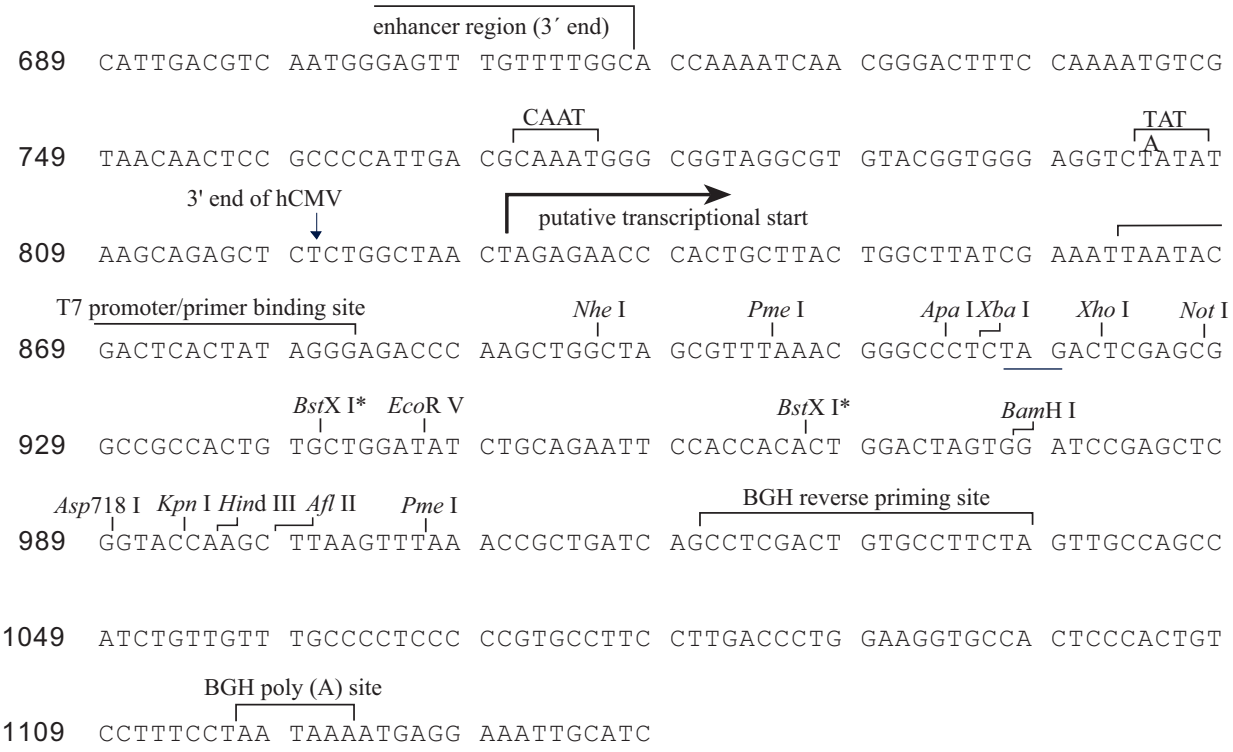
Cloning into pcDNA™3.1/Hygro, continued

Multiple Cloning Site of pcDNA™3.1/ Hygro (–)

Below is the multiple cloning site for pcDNA™3.1/Hygro (–). Restriction sites are labeled to indicate the cleavage site. The *Xba* I site contains an internal stop codon (TCTAGA). The multiple cloning site has been confirmed by sequencing and functional testing. The complete sequence of pcDNA™3.1/Hygro (–) is available for downloading from our web site at **www.lifetechnologies.com** or from **Technical Support** (see page 13).



E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10F´, DH5α, TOP10, page v) 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.

We recommend that you sequence your construct with the T7 Promoter and BGH Reverse primers (page v) to confirm that your gene is in the correct orientation for expression, and contains an ATG initiation codon and a stop codon. Refer to the diagrams on pages 3–4 for the sequences and location of the priming sites.

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

For your convenience, Life Technologies offers a custom primer synthesis service. Visit **www.lifetechnologies.com** for more details.

Preparing a Glycerol Stock for Long-Term Storage

Once you have identified the correct clone, purify the colony and make a glycerol stock for long-term storage. You should keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB plate containing 50 µg/mL ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL ampicillin.
3. Grow the culture to mid-log phase (OD_{600} = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

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Transfection

Introduction

Once you have verified that your gene is cloned in the correct orientation and contains an initiation ATG and a stop codon, you are ready to transfect your cell line of choice. We recommend that you include the positive control vector and a mock transfection (negative control) to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipids, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® HiPure Miniprep Kit or the PureLink® HiPure Midiprep Kit (see page v for ordering information), 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 the protocol for your cell line, exactly. 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 & Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner & Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa & Dower, 1988). For high efficiency transfection in a broad range of mammalian cells, we recommend using Lipofectamine® 2000 Reagent available from Life Technologies. For more information on Lipofectamine® 2000 and other transfection reagents available, visit our web site at **www.lifetechnologies.com** or contact **Technical Support** (page 13).

Positive Control

pcDNA™3.1/Hygro/*lac*Z is provided as a positive control vector for mammalian transfection and expression (see page 12) and may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase (*lac*Z) is expressed in mammalian cells under the control of the CMV promoter. A successful transfection will result in β-galactosidase expression that can be easily assayed (see below).

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. Life Technologies offers the β-Gal Assay Kit and the β-Gal Staining Kit (see page v for ordering information) for fast and easy detection of β-galactosidase expression.

Creation of Stable Cell Lines

Introduction

The pcDNA™3.1/Hygro (+) and pcDNA3.1/Hygro (–) vectors contain the hygromycin resistance gene for selection of stable cell lines using hygromycin B. We recommend that you test the sensitivity of your mammalian host cell to hygromycin B, as natural resistance varies among cell lines. General information and guidelines are provided in this section for your convenience.

Hygromycin-B Activity

Hygromycin-B (527.5 MW) is an aminocyclitol that inhibits protein synthesis by disrupting translocation and promoting mistranslation. Hygromycin-B-phosphotransferase detoxifies hygromycin-B by phosphorylation.

- Hygromycin is light sensitive. Store the liquid stock solution at 4°C protected from exposure to light.
- Hygromycin is toxic. Do not ingest solutions containing the drug.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling hygromycin and hygromycin-containing solutions.

Determining Antibiotic Sensitivity

To successfully generate a stable cell line expressing your gene of interest from pcDNA™3.1/Hygro, you need to determine the minimum concentration of hygromycin B required to kill your untransfected host cell line. Typically, concentrations ranging from 10 to 400 g/mL hygromycin are sufficient to kill most untransfected mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your host cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of hygromycin (0, 10, 25, 50, 100, 200, 400 g/mL hygromycin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of hygromycin that prevents growth within 2–3 weeks after addition of hygromycin.

Note: Cells will divide once or twice in the presence of lethal doses of hygromycin, so the effects of the drug may take several days to become apparent. Complete inhibition of cell growth can take 2–3 weeks of growth in selective medium.

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Possible Sites for Linearization of pcDNA™3.1/ Hygro (+)

Prior to transfection, we recommend that you linearize the pcDNA™3.1/Hygro (+) vector. Linearizing the pcDNA™3.1/Hygro (+) will decrease the likelihood of the vector integrating into the genome in a way that disrupts the gene of interest or other elements required for expression in mammalian cells. The table below lists unique restriction sites that may be used to linearize your construct prior to transfection. **Other unique restriction sites are possible.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Location	Supplier
Bgl II	12	Upstream of CMV promoter	Life Technologies (page v)
Mfe I	161	Upstream of CMV promoter	New England Biolabs
Sap I	3668	Backbone	New England Biolabs
*Eam*1105 I	4674	Ampicillin gene	AGS*, Fermentas, Takara
Ssp I	5478	Backbone	Life Technologies (page v)

*Angewandte Gentechnologie Systeme

Possible Sites for Linearization of pcDNA™3.1/ Hygro (–)

The table below lists unique restriction sites that may be used to linearize your pcDNA™3.1/Hygro (–) construct prior to transfection. **Other unique restriction sites are possible.** Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.

Enzyme	Restriction Site (bp)	Location	Supplier
Bgl II	12	Upstream of CMV promoter	Life Technologies (page v)
Mfe I	161	Upstream of CMV promoter	New England Biolabs
Sap I	3667	Backbone	New England Biolabs
*Eam*1105 I	4673	Ampicillin gene	AGS*, Fermentas, Takara
Ssp I	5477	Backbone	Life Technologies (page v)

*Angewandte Gentechnologie Systeme

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Selection of Stable Integrants

Once you have determined the appropriate hygromycin concentration to use for selection in your host cell line, you can generate a stable cell line expressing your gene of interest.

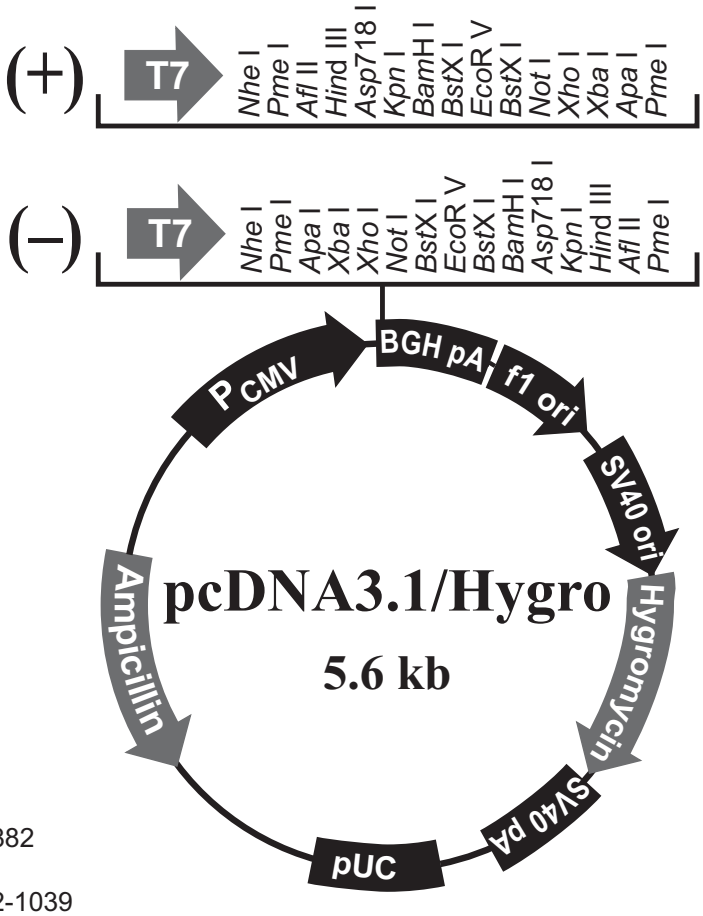
1. Transfect your mammalian host cell line with your pcDNA™3.1/Hygro construct using the desired protocol. Remember to include a plate of untransfected cells as a negative control and the pcDNA™3.1/Hygro/*lacZ* plasmid as a positive control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing hygromycin at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent.
4. Feed the cells with selective medium every 3–4 days until hygromycin-resistant foci can be identified.
5. Pick and expand colonies in 96- or 48-well plates.

Appendix

Map of pcDNA™3.1/Hygro (+) and pcDNA™3.1/Hygro (–) Vectors

Map of pcDNA™3.1/ Hygro (+/–)

The figure below summarizes the features of the pcDNA™3.1/Hygro (+) and pcDNA™3.1/Hygro (–) vectors. The complete sequences for pcDNA™3.1/Hygro (+) and pcDNA™3.1/Hygro (–) are available for downloading from our web site at **www.lifetechnologies.com** or from **Technical Support** (see page 13).



Comments for pcDNA™3.1/Hygro (+): 5597 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1021-1235
f1 origin: bases 1298-1711
SV40 promoter and origin: bases 1776-2100
Hygromycin resistance gene: bases 2118-3141
SV40 early polyadenylation signal: bases 3154-3526
pUC origin: bases 3786-4456 (complementary strand)
Ampicillin resistance gene: bases 4601-5461 (complementary strand)

Features of pcDNA™3.1/Hygro (+) and pcDNA™3.1/Hygro (–) Vectors

Features of pcDNA™3.1/ Hygro (+/–)

pcDNA™3.1/Hygro (+) (5597 bp) and pcDNA™3.1/Hygro (–) (5596 bp) contain the following elements. All features have been functionally tested.

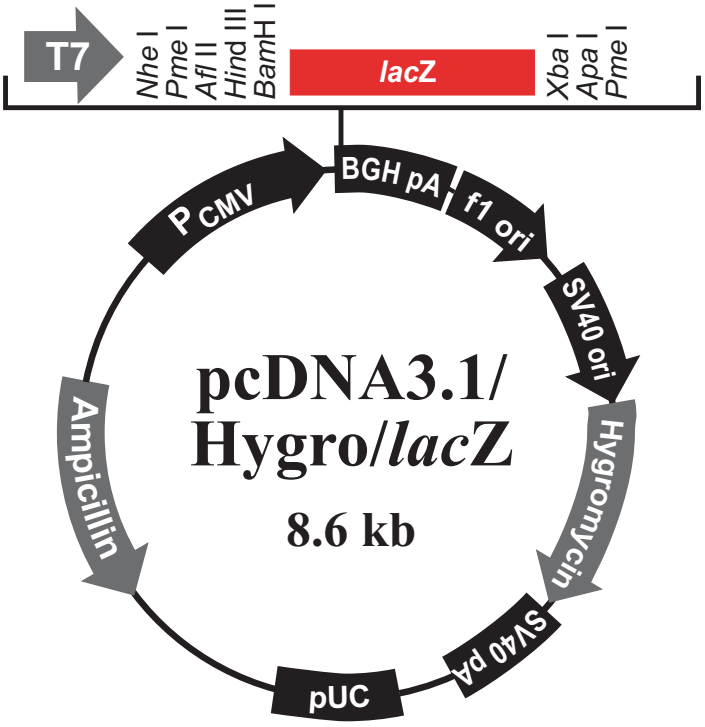
Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
Multiple cloning site in forward or reverse orientation	Allows insertion of your gene and facilitates cloning
BGH reverse priming site	Allows sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA (Goodwin & Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 promoter and origin	Allows efficient, high-level expression of the hygromycin resistance gene and episomal replication in cells expressing SV40 large T antigen
Hygromycin resistance gene (Hygromycin-B-phosphotransferase)	Permits selection of stable transfectants in mammalian cells (Gritz & Davies, 1983; Palmer *et al.*, 1987)
SV40 early polyadenylation signal	Permits efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Permits selection of vector in *E. coli*

Map of pcDNA™3.1/Hygro/*lacZ*

Map of pcDNA™3.1/ Hygro/*lacZ*

pcDNA™3.1/Hygro/*lacZ* is an 8648 bp control vector containing the gene for β-galactosidase. It was constructed by cloning a 3.2 kb *Hin*d III-*Xho* I fragment containing the *lacZ* gene into pcDNA™3.1/Hygro (+).

The figure below summarizes the features of the pcDNA™3.1/Hygro/*lacZ* vector. The complete nucleotide sequence for pcDNA™3.1/Hygro/*lacZ* is available for downloading from our web site at **www.lifetechnologies.com** or from **Technical Support** (see page 13).



Comments for pcDNA™3.1/Hygro (+)/*lacZ*
8648 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
LacZ ORF: bases 972-4044
BGH reverse priming site: bases 4073-4090
BGH polyadenylation signal: bases 4072-4286
f1 origin: bases 4349-4762
SV40 promoter and origin: bases 4827-5151
Hygromycin resistance gene: bases 5169-6192
SV40 early polyadenylation signal: bases 6205-6577
pUC origin: bases 6837-7507 (complementary strand)
Ampicillin resistance gene: bases 7652-8512 (complementary strand)

Technical Support

Obtaining support

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

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 Sata Sheets (SDS)

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

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.

Limited warranty

Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies General Terms and Conditions of Sale found on the Life Technologies web site at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies.

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Purchaser Notification

Introduction

Use of the pcDNA™3.1/Hygro (+/–) vectors is covered under a number of different licenses including those detailed below.

Limited Use Label License No. 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

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Notes

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