invitrogen™

GeneStorm® Expression-Ready Clones

Genes Expressed from pcDNA™3.1/GS

Cat. no. H-K1000

Version K

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25-0221

IMPORTANT!
Beginning in 2009, all Invitrogen clone manuals will only be available online at www.invitrogen.com

User Manual

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Kit Contents

Shipping/Storage

Each GeneStorm® Expression-Ready Human Clone is shipped on dry ice. Upon receipt:

- Store the GeneStorm® glycerol stock at –80°C
- Store the rest of the kit at 4°C

GeneStorm® Expression-Ready Clones

GeneStorm® Expression-Ready Clones (Cat. no. H-K1000) contain your gene of interest from the human genome. All clones are supplied transformed into GeneHogs® Electrocomp™ *E. coli*. All GeneStorm® Clones expressed from pcDNA™3.1/GS are supplied with Anti-V5-HRP Antibody and supercoiled pcDNA™3.1/GS.

Kit Contents

The table below describes each component included in the kit.

Item	Composition	Amount	Storage
GeneStorm® Clone	Supercoiled vector containing the gene of interest transformed into GeneHogs® Electrocomp™ *E. coli* (in LB media containing 10% glycerol and 25 µg/ml Zeocin™)	1.5 ml	–80°C
pcDNA™3.1/GS	Supercoiled vector lyophilized in TE, pH 8.0	50 ng	4°C
Anti-V5-HRP Antibody	~1 mg/ml in filter-sterilized PBS (see label on tube for actual concentration)	25 µl (12 westerns)	4°C

Genotype of GeneHogs®

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*Z M15 Δ*lac* 74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhu*A::IS2 (confers phage T1 resistance)

Anti-V5-HRP Antibody

This antibody detects a 14 amino acid epitope (GKPIPNPLLGLDST) derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991).

Accessory Products

Additional Products

Additional products are available from Invitrogen to help you characterize your GeneStorm® Expression-Ready Clone.

Item	Amount	Cat. no.
GeneHogs® Electrocomp™ *E. coli*	5 × 100 µl	C800-05
One Shot® GeneHogs® Electrocomp™ *E. coli*	11 × 50 µl	C8080-10
	21 × 50 µl	C8080-03
ChargeSwitch®-Pro Plasmid Miniprep Kit	10 preps	CS30010
	50 preps	CS30050
	250 preps	CS30250
PureLink™ HiPure Plasmid MiniPrep Kit	25 preps	K2100-02
PureLink™ HiPure Plasmid MidiPrep Kit	25 preps	K2100-04
Zeocin™	1 g	R250-01
	5 g	R250-05
imMedia™ Zeo Liquid	20 pouches	Q620-20
imMedia™ Zeo Agar	20 pouches	Q620-21
Lipofectamine™ 2000 Reagent	0.75 ml	11668-027
	1.5 ml	11668-019

Detection and Purification of Fusion Protein

Antibodies and ProBond™ resin are available from Invitrogen. See table below for ordering information.

Item	Amount	Cat. no.
Anti-V5-HRP Antibody	50 µl	R961-25
Anti-V5 Antibody	50 µl	R960-25
WesternBreeze® Chemiluminescent Immunodetection Kit	1 kit	WB7104
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Chelating Resin (Precharged resin as a 50% slurry in 20% ethanol)	50 ml	R801-01

Introduction

Overview

Introduction

GeneStorm® Expression-Ready Clones are expressed from the vector pcDNA™3.1/GS. This vector utilizes the strong immediate-early cytomegalovirus (CMV) promoter for high-level, constitutive expression in mammalian cell lines. Each GeneStorm® Clone is fused to a C-terminal peptide encoding the V5 epitope for detection with the Anti-V5-HRP Antibody and a 6×His tag for purification on metal-chelating resin (i.e. ProBond™). The vector also encodes the Zeocin™ resistance gene for selection in *E. coli* and for the creation of stable mammalian cell lines.
For a map of pcDNA™3.1/GS, see page 7. For more information on GeneStorm® Expression-Ready Clones, visit the website at www.invitrogen.com/clones.

Note

The GeneStorm® Clones are not guaranteed to exactly match GenBank sequences and may differ by one or more bases.

Experimental Outline

Use the following outline to express your GeneStorm® Clone from pcDNA™3.1/GS.

Step	Action	Page
1	Isolate plasmid DNA for transfection into the cell line of choice.	2
2	Transfect into the cell line of choice.	3
3	Prepare cell lysates for western blot analysis.	3
4	Test for expression of the gene by western blot analysis or functional assay.	4
5	Purify the fusion protein using a metal-chelating resin such as ProBond™	6

Methods

Isolating Plasmid DNA

Introduction

This section describes how to isolate plasmid DNA for transfection into the cell line of choice.

Important

To prepare plasmid DNA, you need to prepare **Low Salt LB medium with Zeocin™**. For your convenience Low Salt LB medium containing 25 µg/ml Zeocin™ is available as premixed, pre-sterilized *E. coli* growth medium (imMedia™), which contains everything you need in a convenient pouch (see page vi).

For Zeocin™ to be active, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.5. For selection in *E. coli*, it is **imperative** that you prepare LB broth and plates using the following recipe. Failure to use Low Salt LB medium will result in non-selection due to inactivation of the drug. Refer to the **Appendix** (page 8) for the recipe for Low Salt LB medium.

Preparing Glycerol Master Stocks

We recommend you prepare a set of master stocks prior to using your GeneStorm® clone.
To prepare 5–10 glycerol master stocks for long-term storage:

1. Streak a small portion of the glycerol stock you received out on a **Low Salt** LB plate containing 25 µg/ml Zeocin™ (see page 8).
2. Incubate the plate at 37°C overnight.
3. Isolate a single colony and inoculate into 5–10 ml of **Low Salt** LB containing 25 µg/ml Zeocin™.
4. Grow the culture to stationary phase (OD_{600} = 1–2).
5. Mix 0.80 ml of culture with 0.20 ml of sterile glycerol and transfer to a cryovial.
6. Store at –80°C. Use one master stock to create working stocks for regular use. We also recommend that you isolate and store a stock of plasmid DNA at –20°C.

Growing *E. coli* Cultures

To isolate plasmid DNA, you need to grow a culture of GeneHogs® containing your GeneStorm® clone of interest. Use Low Salt LB medium containing 25 µg/ml Zeocin™ (see recipe on page 8) to select single colonies or to grow a culture. Use a culture volume appropriate for the amount of plasmid needed using your plasmid isolation method of choice.

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 a resin based method, such as the ChargeSwitch®-Pro Plasmid Miniprep Kit (up to 20 µg DNA), PureLink™ HiPure Plasmid Miniprep Kit (up to 30 µg DNA), or PureLink™ HiPure Plasmid Midiprep Kit (100–350 µg DNA).
See page vi for ordering information.

Transfection

Introduction

Once you have isolated plasmid DNA, you are ready to transfect your cell line of choice. Supercoiled pcDNA™3.1/GS is included as a negative expression control. Once you have confirmed expression of the gene by transient expression, you may create stable cell lines using Zeocin™ as a selection agent. For more information, refer to the Zeocin™ manual available on our website at www.invitrogen.com or contact Technical Support (see page 9).

Methods of Transfection

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). Consult published literature or the supplier of your cell line for the recommended method of transfection and transfection reagent to use.

For high-efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent available from Invitrogen (see page vi for ordering information). For more information on a large selection of transfection reagents available from Invitrogen, refer to our website at www.invitrogen.com or contact Technical Support (see page 9).

Supercoiled pcDNA™3.1/GS is supplied as a negative control for expression in mammalian cells. We recommend that you include the negative control in your transfection experiments.

Detecting Fusion Proteins

The Anti-V5-HRP Antibody is included in the kit to detect expression of the gene. To detect the gene fusion protein by western blot, you will need to prepare a cell lysate from transfected cells (see next page). We recommend that you perform a time course to optimize expression of the fusion protein (*e.g.* 24, 48, 72 hours, etc. after transfection).

Cell Lysis

To lyse cells for western blot analysis:

1. Wash cell monolayers (~10^6 cells per 60 mm plate, 80–90% confluent) once with phosphate-buffered saline (PBS).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 × g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see recipe on page 8). Other lysis buffers may be suitable.
4. Incubate cell suspension at 37°C for 10 minutes to completely lyse the cells. You may also incubate at room temperature or on ice if you are concerned about protein degradation.
5. Centrifuge the cell lysate at 10,000 × g for 10 minutes at room temperature to pellet nuclei and transfer the supernatant to a fresh tube.
6. 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. Proceed to **Western Blot Analysis**, next page.

Western Blot Analysis

Recommended Antibody Dilution

For western blots, dilute the Anti-V5-HRP Antibody 1:5000 into Phosphate-Buffered Saline (PBS) containing 0.05% Tween-20 and 5% nonfat, dry milk (PBSTM).

If you use a different buffer for washing and blocking your blots, then dilute as described above with that buffer. You may use other blocking agents such as bovine serum albumin (BSA) or gelatin.

Suggested Solutions

We use chemiluminescence to detect binding of the Anti-V5-HRP Antibody to the recombinant protein. Other detection methods can be used to detect your protein. The following materials and solutions are needed for immunoblotting:

- Phosphate-Buffered Saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4)
- Phosphate-Buffered Saline + Tween 20 (PBST: PBS plus 0.05% Tween-20, v/v)
- Blocking buffer (PBST + 5% nonfat, dry milk, w/v)

Immunoblotting Protocol

Use the protocol below to prepare your lysate sample for SDS-PAGE and immunoblotting.

1. Add SDS sample buffer to a final concentration of 1X to the lysate and heat the sample for 5 minutes at 70°C.
2. Load 10–20 µg of protein onto an SDS-PAGE and electrophorese. Use the appropriate percentage of acrylamide to resolve the protein product. Proceed to western transfer.
   Note: We use Novex® 12% Tris-Glycine polyacrylamide gels.
3. Transfer proteins to nitrocellulose membrane electrophoretically. We use 25 mM Tris (pH 8.3), 192 mM glycine, 20% v/v methanol as a transfer buffer.
4. Run at 100V, 150 mA (100V, 240 mA at the finish) for 1 hour. Be sure to have a cooling system in place and operational with these electrophoretic settings. You may also transfer overnight at 30V, 40 mA (30V, 90 mA at the finish).
5. Remove the nitrocellulose membrane and incubate it in 10 ml blocking buffer. Gently agitate using a rocker platform for 1 hour at room temperature.
6. Wash the nitrocellulose membrane in 20 ml PBST 2X for 5 minutes each with gentle agitation.
7. Transfer the membrane to a tray containing the Anti-V5-HRP Antibody diluted 1:5000 in 10 ml blocking buffer (2 µl of Anti-V5 Antibody diluted into 10 ml blocking buffer). Incubate with gentle agitation for 1–2 hours.
   Note: Overnight incubation may be preferred, since longer incubations may increase the sensitivity of detection. Generally, 1 hour incubation is sufficient for detection.
8. Transfer membrane to a tray containing 20 ml PBST and wash for 2 × 5 minutes with gentle agitation. Proceed to detection, next page.

Continued on next page

Western Blot Analysis, Continued

Detection Reaction

We use chemiluminescence to detect the fusion proteins. The WesternBreeze® Chemiluminescent Immunodetection Kit is available from Invitrogen (Cat. no. WB7104). Follow the manufacturer's instructions. Other detection methods are suitable.

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine tag will add approximately 3 kDa to the size of your protein. In addition, posttranslational modification may cause the protein to migrate differently than expected.

The Next Step

Once you have confirmed expression of the GeneStorm® Clone, you may create stable cell lines or purify the protein. To select stable cell lines, refer to the Zeocin™ manual available on our website at www.invitrogen.com. To purify your protein, see the next page.

Purification

Introduction

To obtain the highest yields possible, we recommend that you purify your protein from a stable cell line.

ProBond™ is a metal-chelating resin that can be used to purify recombinant proteins containing a polyhistidine (6×His) tag. Ordering information on ProBond™ is on page vi. If you are using other metal-chelating resin, follow the manufacturer's recommended procedure to purify your protein.

Cell Preparation

You will need 5×10^6 to 1×10^7 **transfected** cells for purification of your protein on a 2 ml ProBond™ column (or other metal-chelating column). Refer to the manufacturer's instructions before attempting to purify your fusion protein. To prepare cells for lysis, follow the protocol below.

1. Seed cells in either five T-75 flasks or 2 to 3 T-175 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 by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1500 rpm for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1500 rpm for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at –70°C until needed.

Lysis of Cells

If you are using ProBond™ resin, refer to the ProBond™ Purification manual for details about sample preparation for chromatography. This manual is available for downloading from our website at www.invitrogen.com.

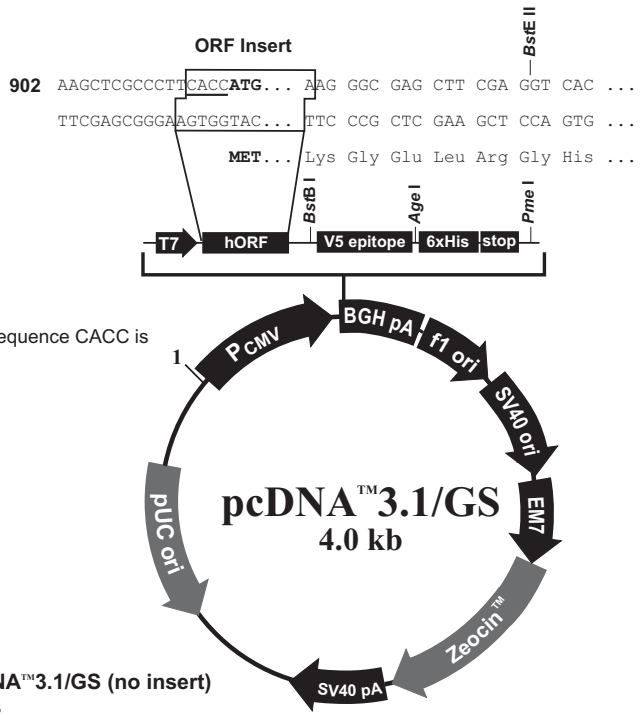
If you are using other metal-chelating resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

Map of pcDNA™3.1/GS

Map of pcDNA™3.1/GS

The following map shows the elements of pcDNA™3.1/GS. The full sequence of this vector (excluding the insert) is available on our website at www.invitrogen.com or by contacting Technical Support (see page 9).



Note: The underlined sequence CACC is not found in all clones.

Comments for pcDNA™3.1/GS (no insert)
4020 nucleotides

CMV promoter: bases 209-863
T7 promoter priming site: bases 863-882
hORF cloning site: between base 913 and 914
V5 epitope: bases 944-985
Polyhistidine tag: bases 995-1012
BGH Reverse priming site: bases 1035-1052
BGH polyadenylation signal: bases 1034-1248
f1 origin: bases 1311-1724
SV40 promoter and origin: bases 1789-2114
EM-7 promoter: bases 2130-2196
Zeocin™ resistance gene: bases 2197-2571
SV40 polyadenylation: bases 2580-2782
pUC origin: bases 3214-3887 (opposite strand)

Recipes

Low Salt LB Medium with Zeocin™

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 7.5 with 5 M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Thaw Zeocin™ on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/ml final concentration.
5. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1–2 weeks.

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.
   Note: Protease inhibitors may be added **fresh** at the following concentrations: 1 mM PMSF; 1 µg/ml pepstatin; 1 µg/ml leupeptin.

Technical Support

Web Resources

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
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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

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MSDS

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

Certificate of Analysis

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

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