invitrogen™

# GeneSwitch™ System

## A Mifepristone-Regulated Expression System for Mammalian Cells

**Catalog nos. K1060-01, K1060-02**

**Version F**
19 October 2010
*25-0313*

**User Manual**

# Table of Contents

# Important Information

## Introduction

The GeneSwitch™ System manual is supplied with the kits listed below. The Core System includes vectors, primers for sequencing, and the inducing agent, mife-pristone. The Complete System includes the Core System plus selection agents. See below for a detailed description of the contents of each GeneSwitch™ System.

| GeneSwitch™ System | Catalog no. |
|---|---|
| Complete System | K1060-01 |
| Core System | K1060-02 |

## Shipping/Storage

The GeneSwitch™ Core System is shipped at room temperature. Store at -20°C.

The GeneSwitch™ Complete System is shipped in 2 boxes. Store as described below:

- Box 1 contains vectors, primers, mifepristone, and hygromycin B and is shipped at room temperature. Upon receipt, remove the vectors, primers, and mifepristone, and store at -20°C. Store the bottle of hygromycin B liquid at +4°C protected from exposure to light.
- Box 2 contains Zeocin™ and is shipped on blue ice. Store at -20°C protected from exposure to light.

## Kit Contents

Both the GeneSwitch™ Complete and the GeneSwitch™ Core Systems include the following regulatory vector, sequencing primers, and inducing agent. **Store all reagents at -20°C.**

| Reagent | Amount | Comments |
|---|---|---|
| pSwitch | 20 µg, lyophilized in TE, pH 8.0 | Regulatory vector for expression of the *GAL4-DBD/hPR-LBD/p65-AD* gene fusion |
| pGene Forward Primer (19-mer) | 2 µg (353 pmoles), lyophilized in TE, pH 8.0 | 5´-CTGCTATTCTGCTCAACCT-3´ |
| BGH Reverse Primer (18-mer) | 2 µg (358 pmoles), lyophilized in TE, pH 8.0 | 5´-TAGAAGGCACAGTCGAGG-3´ |
| Mifepristone | 100 µg, lyophilized in ethanol | Inducing agent |

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# Important Information, continued

## Expression Vectors

Each GeneSwitch™ Complete and Core System also includes an inducible expression vector for cloning your gene of interest and a corresponding positive control vector containing the *lacZ* gene as described below. The inducible expression vector is supplied in three reading frames to facilitate cloning the gene of interest in frame with a C-terminal peptide containing the V5 epitope and a polyhistidine tag. **Store the vectors at -20°C.**

| Vector | Amount |
|---|---|
| pGene/V5-His A, B, C | 20 µg each, lyophilized in TE, pH 8.0 |
| pGene/V5-His/*lacZ* | 20 µg, lyophilized in TE, pH 8.0 |

## Selection Agents

In addition to the vectors and primers provided in the GeneSwitch™ Core System, the GeneSwitch™ Complete System also includes the selection agents listed below. Zeocin™ is supplied in 8 x 1.25 ml aliquots at a concentration of 100 mg/ml. Store the Zeocin™ liquid at -20°C protected from exposure to light. Hygromycin B is supplied in a 10 ml aliquot at a concentration of 100 mg/ml. Store the hygromycin B liquid at +4°C protected from exposure to light.

| Reagent | Amount Supplied | Comments |
|---|---|---|
| Zeocin™ | 1 g | Selection agent for the pGene/V5-His plasmid |
| Hygromycin B | 1 g | Selection agent for the pSwitch regulatory plasmid |

# Accessory Products

## Introduction

The products listed in this section are intended for use with the GeneSwitch™ System. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 33).

## GeneSwitch™ System Components

Many of the reagents used in the GeneSwitch™ System are available separately from Invitrogen. See the table below for ordering information.

| Item | Amount | Catalog no. |
|---|---|---|
| BGH Reverse Primer | 2 µg, lyophilized in TE, pH 8.0 | N575-02 |
| Mifepristone | 100 µg, lyophilized in ethanol | H110-01 |
| Zeocin™ | 1 g | R250-01 |
| | 5 g | R250-05 |
| Hygromycin B | 1 g | R220-05 |

## Detection of Fusion Protein

A number of antibodies are available from Invitrogen to detect expression of your fusion protein from pGene/V5-His. Horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection in western blots using colorimetric or chemiluminescent detection methods. The amount of antibody supplied is sufficient for 25 westerns.

| Antibody | Epitope | Catalog no. |
|---|---|---|
| Anti-V5 | 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 | | R961-25 |
| Anti-V5-AP | | R962-25 |
| Anti-His(C-term) | Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH | R930-25 |
| Anti-His(C-term)-HRP | | R931-25 |
| Anti-His(C-term)-AP | | R932-25 |

## Purification of Fusion Protein

The polyhistidine (6xHis) tag allows purification of the recombinant fusion protein using metal-chelating resins such as ProBond™ or Ni-NTA. Ordering information for ProBond™ and Ni-NTA is provided below.

| Item | Quantity | Catalog no. |
|---|---|---|
| ProBond™ Purification System | 6 purifications | K850-01 |
| ProBond™ Purification System with Anti-V5-HRP Antibody | 1 kit | K854-01 |
| ProBond™ Nickel-Chelating Resin | 50 ml | R801-01 |
| | 150 ml | R801-15 |
| Ni-NTA Purification System | 6 purifications | K950-01 |
| Ni-NTA Purification System with Anti-V5-HRP Antibody | 1 kit | K954-01 |
| Ni-NTA Agarose | 10 ml | R901-01 |
| | 25 ml | R901-15 |
| Polypropylene Columns | 50 columns | R640-50 |

# Introduction

## The GeneSwitch™ System

### Overview

The GeneSwitch™ System is a mifepristone-inducible mammalian expression system originally developed by Wang *et al.* (1994) that uses a combination of regulatory components derived from various human and yeast transcription factors. Mifepristone regulation in the GeneSwitch™ System is based on an autoregulatory feedback loop that involves the binding of a GAL4 regulatory fusion protein (see below) to GAL4 upstream activating sequences (UAS) in both the promoter controlling expression of the GAL4 regulatory fusion protein and the promoter controlling expression of the gene of interest. The major components of the GeneSwitch™ System include:

- An inducible expression plasmid, pGene/V5-His, for expression of your gene of interest under the control of a hybrid promoter containing GAL4 upstream activating sequences (UAS) and the adenovirus E1b TATA box (see pages 27-28 for more information)
- A regulatory plasmid, pSwitch, which encodes a fusion protein consisting of the yeast GAL4 DNA binding domain (DBD), a truncated human progesterone receptor ligand binding domain (hPR-LBD), and the human p65 activation domain (AD) from NF-κB (see pages 29-31 for more information)
- Mifepristone for inducing expression
- A control expression plasmid containing the *lacZ* gene, pGene/V5-His/*lacZ*, which when cotransfected with pSwitch, expresses β-galactosidase upon induction with mifepristone.

### Description of the GeneSwitch™ System

The GeneSwitch™ System exploits the fact that transcription factors are composed of functional domains (*e.g.* DNA binding domain (DBD) or activation domain (AD)). In the GeneSwitch™ System, a hybrid regulatory protein containing a DBD from the yeast GAL4 protein, a truncated ligand binding domain (LBD) from the human progesterone receptor, and an AD from the human NF-κB protein is expressed. This hybrid regulatory protein binds to the synthetic steroid, mifepristone, and functions as a ligand-dependent transcription factor to induce expression of the gene of interest as well as its own expression.

The first major component of the GeneSwitch™ System is the pGene/V5-His inducible expression plasmid. Expression of your gene of interest from the inducible expression plasmid is controlled by a hybrid promoter consisting of *Saccharomyces cerevisiae* GAL4 upstream activating sequences (UAS) (Giniger *et al.*, 1985; Wang *et al.*, 1994) linked to the TATA box sequence from the adenovirus major late E1b gene (Lillie and Green, 1989). Contained within the GAL4 UAS are 6 copies of the 17 nucleotide sequence, 5′-(T/C)GGAGTACTGTCCTCCG-3′ that constitute the binding site for the yeast GAL4 transcription factor. Each 17 nucleotide sequence serves as the binding site for two molecules of the GAL4 DBD (Marmorstein *et al.*, 1992). For more information about the specific features of the pGene/V5-His vector, refer to the **Appendix**, pages 27-28.

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# The GeneSwitch™ System, continued

## Description of the GeneSwitch™ System, continued

The second major component of the GeneSwitch™ System is the pSwitch regulatory vector which expresses the GAL4-DBD/hPR-LBD/p65-AD regulatory fusion protein under the control of a hybrid promoter consisting of a GAL4 UAS (containing 4 copies of the GAL4 binding site) linked to a minimal promoter from the Herpes Simplex Virus thymidine kinase (TK) gene. For more information about the pSwitch vector, refer to the **Appendix**, pages 29-31. For more information about the GAL4-DBD/hPR-LBD/p65-AD regulatory fusion protein, see pages 4 and 31.

## Mechanism of Autoregulation and Induction

The pSwitch regulatory vector and your pGene/V5-His construct will be introduced into the mammalian cell line of choice by cotransfection. In the absence of mifepristone, low basal transcription of the *GAL4-DBD/hPR-LBD/p65-AD* fusion gene from pSwitch occurs from the minimal thymidine kinase (TK) promoter. Once translated into protein, the GAL4-DBD/hPR-LBD/p65-AD regulatory protein (GeneSwitch™ protein) is predominantly localized in the nucleus in an inactive form.

Upon addition, mifepristone binds with high affinity to the truncated hPR-LBD in the GeneSwitch™ protein ($K_d \sim 3 \times 10^{-9}$ M) (Vegeto *et al.*, 1992) and causes a conformational change in the hPR-LBD, resulting in dimerization of the GeneSwitch™ protein and conversion to an active form. The ligand-bound GeneSwitch™ homodimer then interacts with the GAL4 binding sites in the GAL4 UAS of both pGene/V5-His and pSwitch and activates transcription of both the gene of interest from the E1b TATA box and the regulatory fusion gene from the minimal TK promoter (see figure on the next page). Expression of the gene of interest is further amplified through initiation of an autoregulatory feedback loop whereby the ligand-bound GeneSwitch™ protein upregulates expression of its own gene (see figure on the next page). Newly synthesized GeneSwitch™ protein binds to mifepristone, undergoes a conformational change to an active state, and induces transcription of the gene of interest as well as its own gene.

For more information about the individual components comprising the GeneSwitch™ protein, see pages 4 and 31. For more information about the progesterone receptor, its mechanism of action, and the ligand-receptor interaction, refer to published reviews and articles (DeFranco, 1998; Gasc *et al.*, 1989; Guiochon-Mantel *et al.*, 1989; Simons, 1998; Ylikomi *et al.*, 1992).

**Note:** Refer to the figure on the next page for an illustrated depiction of the GeneSwitch™ System.

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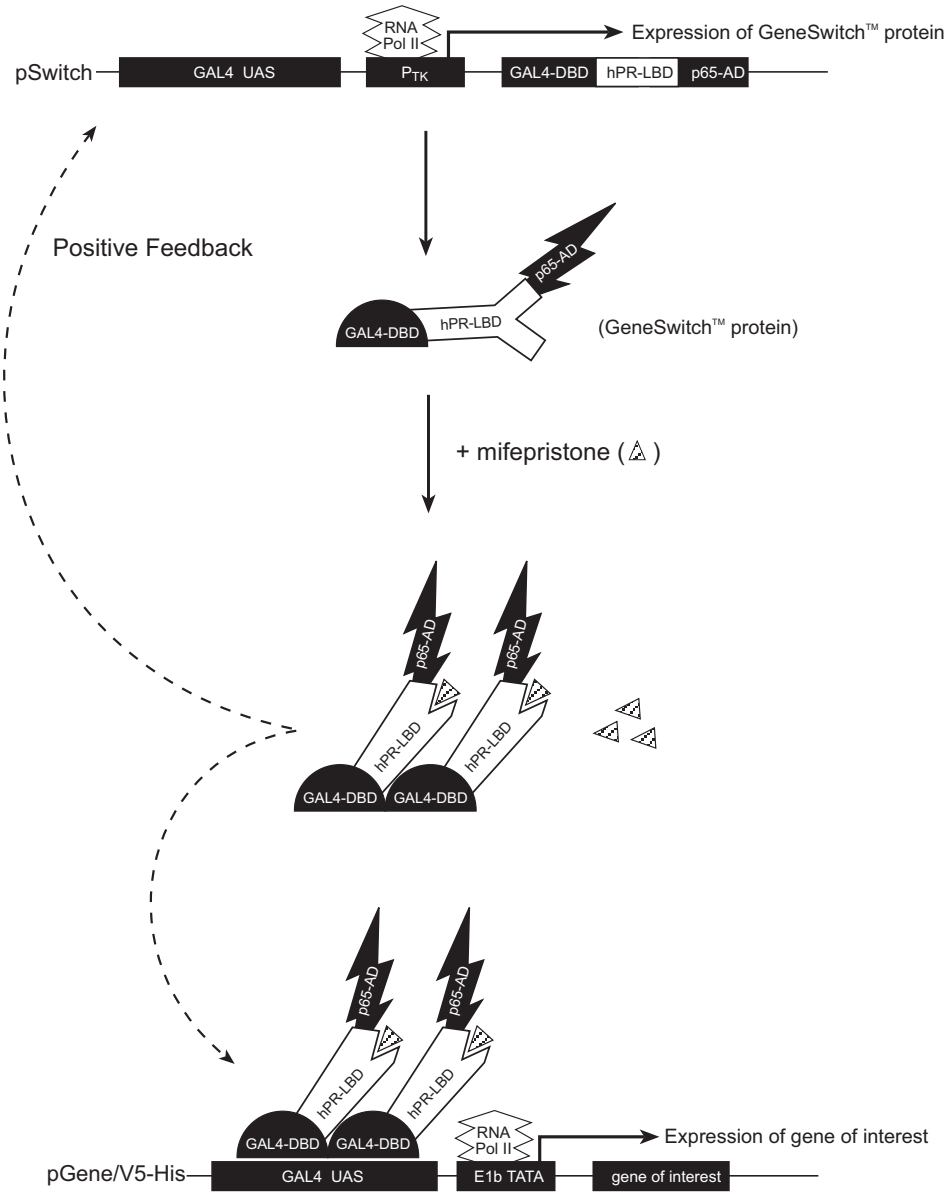
# The GeneSwitch™ System, continued

## The GeneSwitch™ System

The figure below illustrates the components of the GeneSwitch™ System.




## Web Site

The GeneSwitch™ System is a modified version of the gene switch regulatory system originally described by Wang, et al. (1994). For additional information and publications relating to the gene switch technology, refer to the Web site administered by Valentis, Inc. (www.geneswitch.com).

For specific information pertaining to the GeneSwitch™ System, see our Web site (www.invitrogen.com) or call Technical Service (see page 33).

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# The GeneSwitch™ System, continued

## The GAL4-DBD/ hPR-LBD/p65-AD Regulatory Protein

The GAL4-DBD/hPR-LBD/p650-AD regulatory fusion protein (GeneSwitch™ protein) expressed from the pSwitch plasmid is a 73 kDa hybrid protein composed of the specified regulatory domains from the three transcription factors listed below. For more information about the individual components comprising the GeneSwitch™ protein, see the **Appendix**, page 31.

| Transcription Factor | Regulatory Domain | Benefit of Regulatory Domain | References |
|---|---|---|---|
| *Saccharomyces cerevisiae* GAL4 | DNA binding domain (DBD)<br>Amino acids 1-93 in native protein | Allows GeneSwitch™ protein to bind to DNA via GAL4 binding sites in the GAL4 UAS | (Giniger *et al.*, 1985; Laughon and Gesteland, 1984; Marmorstein *et al.*, 1992) |
| Human progesterone receptor (hPR) | Truncated ligand binding domain (LBD)<br>Amino acids 640-914 in native protein | Allows mifepristone-specific binding and activation of the GeneSwitch™ protein | (Kastner *et al.*, 1990; Misrahi *et al.*, 1987; Vegeto *et al.*, 1992; Wang *et al.*, 1997) |
| Human p65 subunit of NF-κB | Activation domain (AD)<br>Amino acids 283-551 in native protein | Permits activation of gene transcription | (Burcin *et al.*, 1999; Deloukas and Loon, 1993; Ruben *et al.*, 1991; Schmitz and Baeuerle, 1991) |

In the GeneSwitch™ System, the GeneSwitch™ protein functions as a ligand-dependent transcription factor to activate expression of both the gene of interest and its own gene via binding to GAL4 sites within the GAL4 UAS (see figure on the previous page). The combination of functional domains from the transcription factors described above allows the GeneSwitch™ protein to exhibit the following characteristics:

- Because the GAL4 DBD is derived from a yeast protein, the GeneSwitch™ protein has no effect on endogenous genes and can only activate transcription of genes whose expression is controlled by a GAL4 UAS (*i.e.* the gene of interest and the regulatory fusion gene).
- The GAL4 DBD binds to an individual 17 nucleotide GAL4 binding site as a homodimer (Carey *et al.*, 1989; Marmorstein *et al.*, 1992). The pGene/V5-His and pSwitch plasmids contain 6 and 4 copies of the GAL4 binding site, respectively, although it is not known if all of the GAL4 binding sites are occupied at any given time.
- The truncated hPR-LBD contains a 19 amino acid deletion from its C-terminal end that abolishes its ability to bind to progesterone, other endogenous steroid hormones, or other progesterone agonists, but still enables it to bind with high affinity to mifepristone (Vegeto *et al.*, 1992; Wang *et al.*, 1994; Wang *et al.*, 1997).
- The p65 AD is a strong transcriptional activator but is derived from a human protein to minimize possible toxic or pleiotrophic effects associated with viral transactivation domains (Abruzzese *et al.*, 1999; Burcin *et al.*, 1999).

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# The GeneSwitch™ System, continued

## Hallmarks of Mifepristone Induction

The synthetic steroid, mifepristone, is used as an inducing agent in the GeneSwitch™ System (see pages 13 and 24 for more information). The hallmarks of mifepristone induction in this system are listed below:

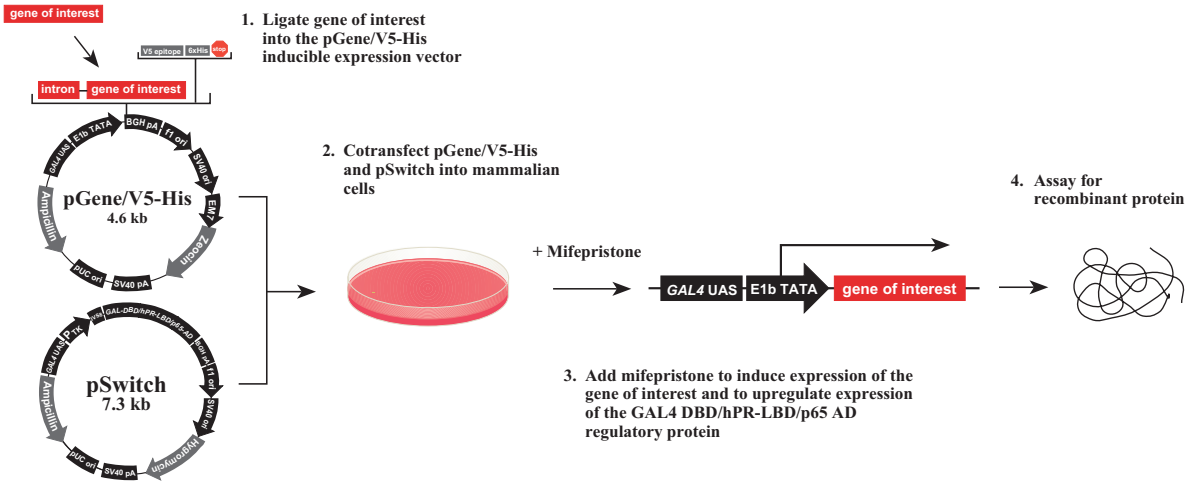
- Induction of gene expression requires extremely low concentrations of mifepristone (*i.e.* 1 x $10^{-8}$ M mifepristone)
- The concentration of mifepristone required for half-maximal induction in the GeneSwitch™ System is approximately $10^{-10}$ M
- The concentrations of mifepristone used for gene induction exert no known toxic or pleiotrophic effects on mammalian cells that lack endogenous progesterone and glucocorticoid receptors (Vegeto *et al.*, 1992; Wang *et al.*, 1994)

## Experimental Outline

To express your gene of interest using the GeneSwitch™ System, you will perform the following steps (see below for a diagram):

1. Ligate your gene of interest into the multiple cloning site of the pGene/V5-His inducible expression vector.
2. Cotransfect your pGene/V5-His construct and the pSwitch regulatory plasmid into the mammalian cell line of choice.
3. Add mifepristone to induce expression of the gene of interest via an autoregulatory feedback loop involving the GAL4-DBD/hPR-LBD/p65-AD regulatory fusion protein.
4. Assay for expression of your recombinant protein of interest.

**Note:** The positive control vector containing the *lacZ* gene (pGene/V5-His/*lacZ*) can be transiently cotransfected into mammalian cells with pSwitch to demonstrate that the system is working properly in your cell line. Stable cell lines expressing the GAL4 DBD/hPR-LBD/p65 AD regulatory fusion protein from pSwitch can be established to serve as hosts for inducible expression vector-based constructs.



# Methods

## Propagation and Maintenance of Plasmids

### Introduction

This section contains guidelines to propagate the GeneSwitch™ vectors.

### *E. coli* Strain

Many *E. coli* strains are suitable for the propagation of the GeneSwitch™ vectors including TOP10 (Catalog no. C610-00) and DH5α. We recommend propagating the GeneSwitch™ vectors in recombination deficient (*rec*A) and endonuclease A deficient (*end*A) *E. coli* strains. For your convenience, TOP10 and DH5α *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen.

| Item | Quantity | Catalog no. |
|---|---|---|
| One Shot® TOP10 Chemically Competent *E. coli* | 20 x 50 µl | C4040-03 |
| One Shot® TOP10 Electrocomp™ *E. coli* | 20 x 50 µl | C4040-52 |
| One Shot® MAX Efficiency® DH5α™-T1$^R$ Chemically Competent *E. coli* | 20 x 50 µl | 12297-016 |

### Transformation Method

You may use any method of choice for transformation. Chemical transformation is the most convenient, but electroporation is the most efficient and the method of choice for large plasmids.

### Maintenance of Plasmids

The GeneSwitch™ vectors contain the ampicillin resistance gene to allow selection of the plasmid using ampicillin (see pages 27-32 for more information). The pGene/V5-His A, B, C and pGene/V5-His/*lacZ* vectors also contain the Zeocin™ resistance gene for selection of the plasmids using Zeocin™. To propagate and maintain the GeneSwitch™ plasmids, use the following procedure:

1. Resuspend each vector in 20 µl sterile water to prepare a 1 µg/µl stock solution. Store the stock solution at -20°C.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, or equivalent.
3. Select transformants on the appropriate plates as follows:
   - For the pGene/V5-His A, B, C and pGene/V5-His/*lacZ* plasmids, select on LB plates containing 50 to 100 µg/ml ampicillin or 25 to 50 µg/ml Zeocin™ in Low Salt LB (see page 22 for a recipe)
   - For the pSwitch plasmid, select on an LB plate containing 50 to 100 µg/ml ampicillin

   **Note:** For fast and easy microwaveable preparation of Low Salt LB agar containing ampicillin or Zeocin™, imMedia™ Amp Agar (Catalog no. Q601-20) or imMedia™ Zeo Agar (Catalog no. Q621-20) is available from Invitrogen. For more information, see our Web site (www.invitrogen.com) or call Technical Service (see page 33).
4. Prepare a glycerol stock from each transformant containing plasmid for long-term storage (see page 11).

# Cloning into pGene/V5-His A, B, and C

## Introduction

You will express your gene of interest from the pGene/V5-His inducible expression vector. The pGene/V5-His vector is supplied with the multiple cloning site in three reading frames (A, B, and C) to facilitate cloning your gene of interest in frame with the C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag. Use the diagrams provided on pages 8-10 to help you design a strategy to clone your gene of interest in frame with the C-terminal peptide. General considerations for cloning and transformation are discussed in this section.

## General Molecular Biology Techniques

For assistance with *E. coli* transformations, restriction enzyme analysis, DNA biochemistry, and plasmid preparation, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

## Cloning Considerations

Your insert should contain a Kozak translation initiation sequence with an ATG start codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Please note that other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is shown underlined.

(**G/A**)NNATG**G**

To express your gene as a recombinant fusion protein, you must clone your gene in frame with the C-terminal peptide. The vector is supplied in three reading frames to facilitate cloning. See pages 8-10 to develop a cloning strategy.

If you wish to express your protein WITHOUT the C-terminal peptide, be sure to include a stop codon.

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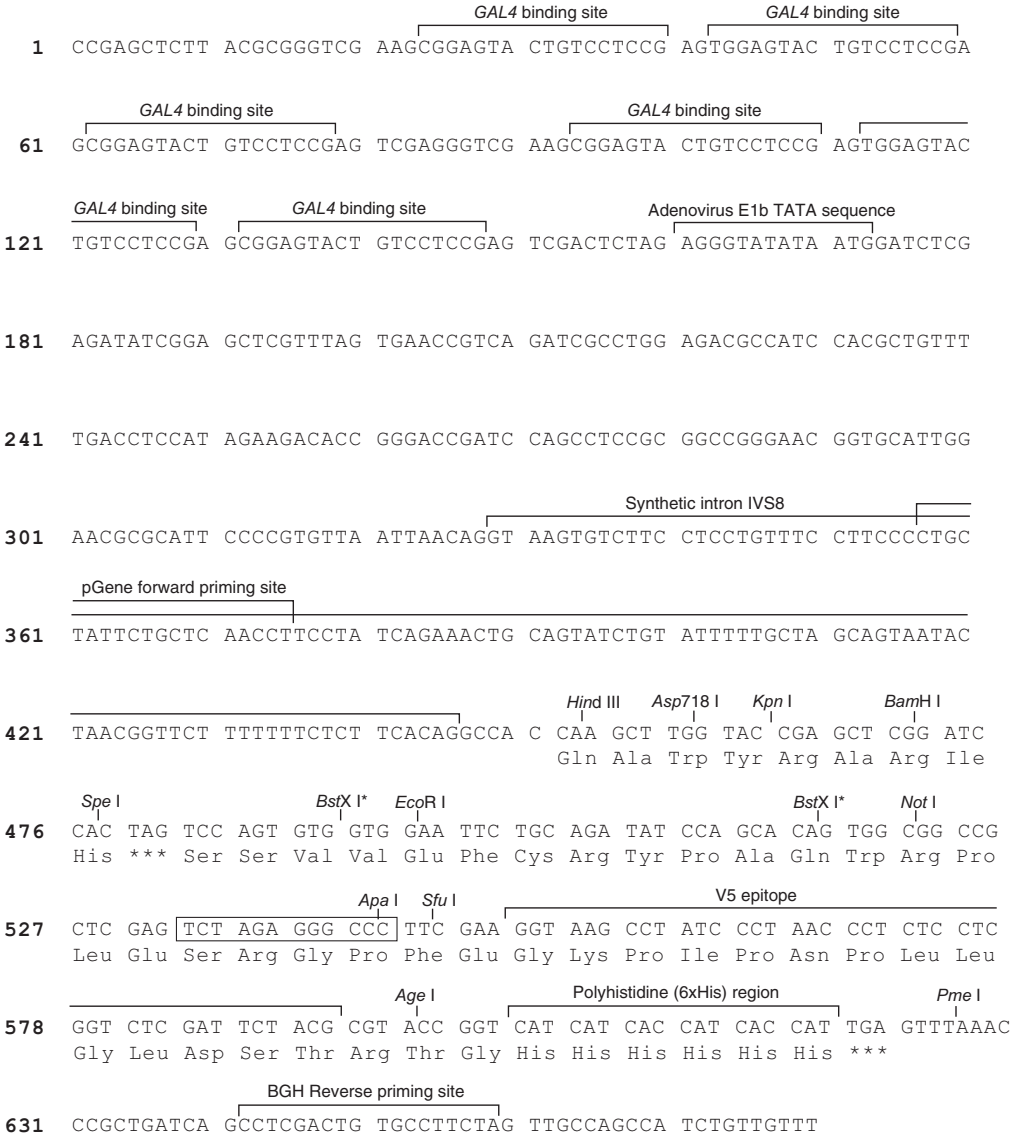
# Cloning into pGene/V5-His A, B, and C, continued

## Multiple Cloning Site of Version A

Below is the multiple cloning site for pGene/V5-His A. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Note that there is a stop codon between the *Spe* I and *Bst*X I sites in the polylinker. Also, note that the sequences of the GAL4 binding sites are not identical. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pGene/V5-His A is available for downloading from our Web site (**www.invitrogen.com**) or from Technical Service (see page 33).** For a map and a description of the features of pGene/V5-His, refer to the **Appendix**, pages 27-28.




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

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# Cloning into pGene/V5-His A, B, and C, continued

## Multiple Cloning Site of Version B

Below is the multiple cloning site for pGene/V5-His B. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Note that the sequences of the GAL4 binding sites are not identical. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pGene/V5-His B is available for downloading from our Web site (**www.invitrogen.com**) or from Technical Service (see page 33).** For a map and a description of the features of pGene/V5-His, refer to the **Appendix**, pages 27-28.


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

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# Cloning into pGene/V5-His A, B, and C, continued

## Multiple Cloning Site of Version C

Below is the multiple cloning site for pGene/V5-His C. Restriction sites are labeled to indicate the cleavage site. The boxed nucleotides indicate the variable region. Note that the sequences of the GAL4 binding sites are not identical. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of pGene/V5-His C is available for downloading from our Web site (**www.invitrogen.com**) or from Technical Service (see page 33).** For a map and a description of the features of pGene/V5-His, refer to the **Appendix**, pages 27-28.


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

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# Cloning into pGene/V5-His A, B, and C, continued

### *E. coli* Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (*e.g.* TOP10, DH5α) and select on LB agar plates containing 50 to 100 µg/ml ampicillin or 25 to 50 µg/ml Zeocin™ in Low Salt LB (see page 22 for a recipe). Select 10-20 clones and analyze for the presence and orientation of your insert.

### Important

Any *E. coli* strain that contains the complete *Tn*5 transposable element (*i.e.* DH5αF′IQ, SURE, SURE2) encodes the *ble* (bleomycin) resistance gene. These strains will confer resistance to Zeocin™. For the most efficient selection with Zeocin™, we recommend that you choose an *E. coli* strain that does not contain the *Tn*5 gene (*i.e.* TOP10, TOP10F′).

### Zeocin™ Selection in *E. coli*

To facilitate selection of Zeocin™-resistant *E. coli*, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.5. Prepare Low Salt LB broth and plates using the recipe in the **Appendix**, page 22.

**Failure to lower the salt content of your LB medium will result in non-selection due to inactivation of the Zeocin™.**


We recommend that you sequence your construct with the pGene Forward and BGH Reverse primers provided with the kit to confirm that your gene contains an ATG start codon and is cloned in frame with the C-terminal peptide (if desired). Refer to the diagrams on pages 8-10 for the sequences and location of the priming sites.

### Preparing a Glycerol Stock

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to 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 or 25-50 µg/ml Zeocin™ in Low Salt LB. 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 or 25-50 µg/ml Zeocin™ in Low Salt LB.
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.

# Transfection and Analysis

## Introduction

Once you have cloned your gene of interest into pGene/V5-His and have prepared clean plasmid preparations of your pGene/V5-His construct and pSwitch, you are ready to cotransfect the plasmids into the mammalian cell line of choice. We recommend that you include the positive control vector (see below) and a mock transfection to evaluate your results. General guidelines are provided on the next page for cotransfection and induction.

## 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 S.N.A.P.™ MiniPrep Kit (10-15 µg DNA, Catalog no. K1900-01), the S.N.A.P.™ MidiPrep Kit (10-200 µg DNA, Catalog no. K1910-01), or CsCl gradient centrifugation.

## Methods of Transfection

For established cell lines (*e.g.* HeLa, COS-1), 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). Invitrogen offers the Calcium Phosphate Transfection Kit and cationic lipid-based reagents including Lipofectamine™ 2000 for transfection. For more information about the transfection reagents available, see our Web site (www.invitrogen.com) or call Technical Service (see page 33).

| Item | Catalog no. |
|---|---|
| Calcium Phosphate Transfection Kit | K2780-01 |
| Lipofectamine™ 2000 | 11668-027 |

## Positive Control

pGene/V5-His/*lacZ* is provided as a positive control vector for mammalian cell transfection and expression (see page 32) and may be used to optimize induction conditions for your cell line. Cotransfection of the positive control vector and pSwitch results in the induction of β-galactosidase expression upon addition of mifepristone. A successful cotransfection will result in β-galactosidase expression that can be easily detected (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. Invitrogen offers the β-Gal Assay Kit (Catalog no. K1455-01) and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

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### Mifepristone

In the GeneSwitch™ System, the synthetic steroid, mifepristone, is used as an inducing agent to activate transcription of the gene of interest as well as transcription of the GeneSwitch™ regulatory protein. Although mifepristone generally behaves as a progesterone antagonist (Philibert *et al.*, 1985), the compound actually functions as an **agonist** in the GeneSwitch™ System through binding to the truncated hPR-LBD in the GeneSwitch™ protein (Vegeto *et al.*, 1992; Wang *et al.*, 1994; Wang *et al.*, 1997). Once bound to mifepristone, the hPR-LBD undergoes a conformational change that converts the GeneSwitch™ protein from an inactive to an active form. The active regulatory protein then acts as a ligand-dependent transcription factor to activate expression of the gene of interest and its own gene. Extremely low doses of mifepristone (nanomolar range) are required to activate gene transcription. For more information about mifepristone and instructions to prepare mifepristone, refer to the **Appendix**, page 24. For more information about the hPR-LBD and the GeneSwitch™ protein, see page 4.

**Note:** Mifepristone can bind to progesterone and glucocorticoid receptors in cell lines that contain the native receptors. In these cell lines (*e.g.* some Chinese Hamster Ovary (CHO) and HeLa cell lines), mifepristone may exert antagonistic effects against the native receptors. However, the concentrations of mifepristone used to induce gene expression in the GeneSwitch™ System exert no known toxic or pleiotrophic effects on mammalian cells that lack endogenous progesterone and glucocorticoid receptors.


- Mifepristone is toxic. Do not ingest or inhale the powder or solutions containing the drug.
- Use caution when handling large quantities of mifepristone. At high doses (> 100 mg), mifepristone may impair fertility and may cause harm to the unborn child.
- Always wear gloves, a laboratory coat, and safety glasses or goggles when handling mifepristone and mifepristone-containing solutions.

**Important**

We have found that by varying the ratio of pSwitch and pGene/V5-His plasmids that we transiently cotransfect into mammalian cells, we can minimize basal transcription and optimize mifepristone regulation of the gene of interest from the pGene/V5-His expression plasmid. **In general, we recommend that you cotransfect your mammalian host cell line with a ratio of at least 1:4 (w/w) pSwitch:pGene/V5-His plasmid DNA.** Note that results may vary depending on the cell line and the gene of interest; therefore, you may want to empirically determine an optimal ratio of pSwitch and pGene/V5-His to use for cotransfection of your particular cell line.

*continued on next page*

# Transfection and Analysis, continued

**Note**

Although the GeneSwitch™ System is designed for use in a wide range of mammalian cell lines, there may be some host cell lines that are not suitable due to detectable basal transcription or low inducibility of the gene of interest. We have observed detectable basal expression as well as low inducibility of the *lacZ* gene from pGene/V5-His/*lacZ* (after cotransfection with pSwitch) in Chinese Hamster Ovary (CHO) cells; therefore, we **do not** recommend using CHO cells to perform transient transfection experiments with the GeneSwitch™ System.

## Cotransfection and Induction with Mifepristone

General guidelines are provided below to cotransfect your pGene/V5-His construct (or the control plasmid) and pSwitch into the mammalian cell line of your choice and to induce expression of your protein of interest with mifepristone. Since every cell line is different and may require a different method of transfection, some empirical experimentation may be needed to determine the optimal conditions for inducible expression.

- Use cells that are approximately 60% confluent for transfection.
- Cotransfect the pSwitch plasmid and your pGene/V5-His construct at a ratio of at least 1:4 (w:w) or at your optimally-determined ratio into the cell line of choice using your preferred method. Absolute amounts of plasmid will vary depending on the method of transfection and the cell line used.
- After transfection, add fresh medium and allow the cells to recover for 24 hours before induction.
- Remove medium and add fresh medium containing the appropriate concentration of mifepristone to the cells. In general, we recommend that you add mifepristone to a final concentration of $1 \times 10^{-8}$ M (10 µl of a 10 µM stock per 10 ml of medium) to the cells and incubate the cells for 24 hours at 37°C.

*continued on next page*

### Detection of Recombinant Fusion Proteins

You may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen to detect expression of your recombinant fusion protein from pGene/V5-His if you have cloned your gene in frame with the C-terminal peptide (see page vii for ordering information). 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 and a polyhistidine (6xHis) tag. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 33).

To detect your fusion protein by western blot, you will need to prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of the fusion protein (*e.g.* 12, 24, 36, 48 hours etc. after mifepristone induction). To assay for β-galactosidase expression from the pGene/V5-His/*lacZ* control plasmid, we generally harvest cells after 24 hours of induction with mifepristone. Use the protocol below to lyse cells. Other protocols are suitable.

1. Wash cell monolayers (~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 the **Appendix**, page 23 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 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.

**Note**

The C-terminal peptide containing the V5 epitope and the polyhistidine (6xHis) tag will add approximately 5 kDa to the size of your protein.

### Purifying Your Recombinant Protein

You will need approximately 5 x $10^6$ to 1 x $10^7$ transfected cells to purify your protein on a 2 ml ProBond™ or Ni-NTA column. Refer to the procedure on page 21 to prepare cells for lysis.

### Optimizing Expression

You may vary the concentration of mifepristone (1 x $10^{-7}$ M to 1 x $10^{-10}$ M) and the time of exposure to mifepristone (12 to 72 hours) to optimize or modulate expression for your cell line.

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# Creating Stable Cell Lines

## Introduction

Once you have established that your construct can be inducibly expressed, you may create a stable cell line that inducibly expresses your gene of interest. The pGene/V5-His and the pSwitch vectors contain the Zeocin™ resistance gene and the hygromycin resistance gene, respectively, to allow selection of stable cell lines using the selection agents, Zeocin™ and hygromycin B. We recommend that you first generate a stable cell line that expresses only the GAL4-DBD/hPR-LBD/p65-AD regulatory fusion protein, then use that cell line to create a second cell line which will express your gene of interest from the inducible expression plasmid (see **Note** below). Alternatively, you can cotransfect with both plasmids (pSwitch and pGene/V5-His) and dual-select with hygromycin B and Zeocin™ to isolate a single stable cell line expressing both the regulatory fusion protein and your gene of interest. The following section provides guidelines and instructions to generate stable cell lines by transfection.

**Note**

Your gene of interest should not be expressed if you transfect your pGene/V5-His construct into mammalian host cells prior to transfecting the pSwitch plasmid. Mammalian cells do not contain endogenous GAL4 protein; therefore, transcription of the gene of interest should not be induced unless the GAL4-DBD/hPR-LBD/p65-AD regulatory fusion protein is present. While the E1b TATA sequence may not be completely silent, the basal transcription from the pGene/V5-His plasmid is generally undetectable.

**Reminder:** When generating a stable cell line expressing the GAL4-DBD/hPR-LBD/p65-AD regulatory fusion protein from pSwitch, select for clones that express the lowest levels of the GeneSwitch™ protein in the absence of mifepristone and the highest levels after induction with mifepristone. You may screen stable pSwitch clones for low basal expression and high inducibility by transient transfection with the pGene/V5-His/*lacZ* control plasmid.

## Zeocin™

The pGene/V5-His plasmid contains the Zeocin™ resistance gene to allow selection of stable transfectants using Zeocin™. For more information about preparing and handling Zeocin™, refer to the **Appendix**, pages 25-26.

## Hygromycin B

The pSwitch vector contains the *E. coli* hygromycin resistance gene (*HPH*) (Gritz and Davies, 1983) for selection of transfectants using hygromycin B (Palmer *et al.*, 1987). When added to cultured mammalian cells, hygromycin B acts as an aminocyclitol to inhibit protein synthesis by disrupting translocation and promoting mistranslation. Hygromycin B is supplied with the GeneSwitch™ Complete kit and is also available separately from Invitrogen (see page vii for ordering information).

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# Creating Stable Cell Lines, continued


- Hygromycin B 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 B and hygromycin B-containing solutions.

### Preparing and Storing Hygromycin B

The hygromycin B included with the GeneSwitch™ Complete kit is supplied as a 100 mg/ml stock solution in autoclaved, deionized water and is filter-sterilized. The solution is brown in color. The stability of hygromycin B is guaranteed for six months, if stored at +4°C. Medium containing hygromycin B is stable for up to six weeks.

### Determining Antibiotic Sensitivity

To successfully generate a stable cell line expressing the GAL4-DBD/hPR-LBD/p65-AD regulatory protein and your recombinant protein of interest, you need to determine the minimum concentration of each selection agent (Zeocin™ and hygromycin B) required to kill your untransfected host cell line. For each selection agent, test a range of concentrations (see below) to ensure that you determine the minimum concentration necessary for your cell line. Use the protocol below to determine the minimal concentrations of Zeocin™ and hygromycin B required to kill the parental cell line. Refer to the **Appendix**, pages 25-26 for instructions on how to prepare and store Zeocin™. See above for instructions to prepare and store hygromycin B.

**Note:** Typically, concentrations ranging from 50 to 1000 µg/ml Zeocin™ and 10 to 400 µg/ml hygromycin B are sufficient to kill most untransfected mammalian cell lines.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. For each selection agent, prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, add the following concentrations of antibiotic to each plate in a set:
   - For Zeocin™ selection, test 0, 50, 125, 250, 500, 750, and 1000 µg/ml Zeocin™
   - For hygromycin selection, test 0, 10, 50, 100, 200, 400, 600 µg/ml hygromycin B
3. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of each selection agent that kills the cells within 1-2 weeks after addition of the antibiotic.

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# Creating Stable Cell Lines, continued

### Effect of Zeocin™ on Sensitive and Resistant Cells

Zeocin™'s method of killing is quite different from other antibiotics including hygromycin B, Geneticin®, and blasticidin. **Cells do not round up and detach from the plate.** Sensitive cells may exhibit the following morphological changes upon exposure to Zeocin™:

- Vast increase in size (similar to the effects of cytomegalovirus infecting permissive cells) and abnormal cell shape
- Presence of large empty vesicles in the cytoplasm (breakdown of the endoplasmic reticulum and Golgi apparatus, or other scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes in these membranes)

Eventually, these "cells" will completely break down and only "strings" of protein remain.

Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to cells not under selection with Zeocin™. For more information about Zeocin™ and its mechanism of action, refer to the **Appendix**, pages 25-26.

### Possible Sites to Linearize pSwitch

To obtain stable transfectants, we recommend that you linearize the pSwitch plasmid 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 the *GAL4-DBD/hPR-LBD/p65-AD* gene fusion or other elements necessary for expression in mammalian cells. The table below lists unique sites that may be used to linearize the pSwitch plasmid prior to transfection. **Other restriction sites are possible.**

| Enzyme | Restriction Site (bp) | Location | Supplier |
|---|---|---|---|
| *Bst*1107 I | 5128 | End of SV40 polyA | AGS*, Fermentas, Takara, Roche Molecular Biochemicals |
| *Sap* I | 5391 | Backbone | New England Biolabs |
| *Bsp*LU11 I | 5507 | Backbone | Roche Molecular Biochemicals |
| *Fsp* I | 6622 | Ampicillin gene | Many |

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# Creating Stable Cell Lines, continued

### Possible Sites to Linearize pGene/V5-His A, B, and C

We recommend that you also linearize your pGene/V5-His construct before transfection. The table below lists unique sites that may be used to linearize the plasmid prior to transfection. Other restriction sites are possible. **Note that the cleavage site is indicated for versions A, B, and C of pGene/V5-His. Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.**

| Enzyme | Restriction Site (bp) (A,B,C) | Location | Supplier |
|---|---|---|---|
| *Bst*1107 I | 2439, 2443, 2435 | End of SV40 polyA | AGS*, Fermentas, Takara, Roche Molecular Biochemicals |
| *Sap* I | 2702, 2706, 2698 | Backbone | New England Biolabs |
| *Bsp*LU11 I | 2818, 2822, 2814 | Backbone | Roche Molecular Biochemicals |
| *Eam*1105 I | 3708, 3712, 3704 | Ampicillin gene | AGS*, Fermentas, Takara |
| *Fsp* I | 3930, 3934, 3926 | Ampicillin gene | Many |
| *Pvu* I | 4078, 4082, 4074 | Ampicillin gene | Many |
| *Ssp* I | 4512, 4516, 4508 | *bla* promoter | Many |

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# Creating Stable Cell Lines, continued

## Selecting Stable Integrants

Once you have determined the appropriate Zeocin™ and hygromycin B concentrations to use for selection, you can generate a stable cell line expressing pSwitch and your pGene/V5-His construct. We recommend that you first generate a stable cell line expressing pSwitch, and then use this cell line as the host for your pGene/V5-His construct. If you wish to perform cotransfection and dual selection of stable integrants, see below.

1. Transfect the mammalian cell line of choice with pSwitch using the method of choice. Include a plate of untransfected cells as a negative 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. Split the cells such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells.
4. Incubate the cells at 37°C for at least 2-3 hours until they have attached to the culture dish. Remove the medium and add fresh medium containing hygromycin B at the pre-determined concentration required for your cell line.
5. Feed the cells with selective medium every 3-4 days until foci can be identified.
6. Pick at least 20 hygromycin-resistant foci and expand each clone to test for mifepristone-inducible gene expression by transiently transfecting with the pGene/V5-His/*lacZ* positive control plasmid. Screen for those clones which exhibit the lowest levels of basal transcription and the highest levels of β-galactosidase expression after addition of mifepristone.
7. Once you have obtained a stable cell line expressing the GAL4-DBD/hPR-LBD/p65-AD fusion protein, you can use this cell line to isolate a stable cell line expressing your gene of interest from the pGene/V5-His construct. Repeat Steps 1 through 6, above, using your pGene/V5-His construct and Zeocin™ to select foci. Remember to maintain your cells in medium containing hygromycin as well.
8. Pick and expand at least 20 foci to test for mifepristone-regulated gene expression.

## Dual Selection of Stable Integrants

We recommend using the protocol above to generate double stable cell lines. However, if you wish to perform dual selection, you may cotransfect both pSwitch and your pGene/V5-His expression plasmid into the cell line of choice at a 1:1 ratio, and select for double stable transfectants using hygromycin B and Zeocin™ (see **Note** below). Screen at least 40 foci for mifepristone-regulated expression of your gene of interest using an appropriate assay for your protein (*e.g.* western blot, enzymatic assay).

**Note**

Some cells may be more sensitive to dual selection than selection with a single antibiotic; therefore, you may find that you can use lower concentrations of hygromycin B and Zeocin™ for dual selection of transfectants.

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Once you have isolated stable cell lines containing both pSwitch and your pGene/V5-His construct and have tested for mifepristone-inducible expression of your gene of interest, we recommend that you perform a time course of mifepristone induction to optimize expression of your protein of interest (*e.g.* 0, 12, 24, 48, 72 hours, etc.). Use the appropriate concentration of mifepristone for your cell line as previously determined (see page 14). We have observed as much as 50-200-fold induction of β-galactosidase in a double stable cell line (NIH3T3 cells transfected with pSwitch and pGene/V5-His/*lacZ*) after 24 hours of treatment with 1 x $10^{-8}$ M mifepristone. Note that your induction levels may vary depending on the nature of your gene of interest and the particular clone that you choose.

**Note**

After 24 hours of induction with 1 x $10^{-8}$ M mifepristone, we have observed continuous expression of β-galactosidase in a double stable cell line (NIH3T3 cells transfected with pSwitch and pGene/V5-His/*lacZ*) even after removal of mifepristone from the tissue culture medium. High levels of β-galactosidase protein are still detectable after culturing cells for 144 hours in the absence of mifepristone. If you wish to perform repetitive induction experiments with your gene of interest, the GeneSwitch™ System may not be appropriate for this use. Empirical experimentation will be necessary as results may vary depending on the nature of the gene of interest and the host cell line.

### Preparing Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™ or Ni-NTA. You will need approximately 5 x $10^6$ to 1 x $10^7$ **stably** transfected cells to purify your protein on a 2 ml ProBond™ or Ni-NTA column.

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 approximately 50% confluent.
3. Add the appropriate concentration of mifepristone and induce expression of your protein of interest to the desired level.
4. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
5. Inactivate the trypsin by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
6. Centrifuge the cells at 1500 rpm for 5 minutes. Resuspend the cell pellet in PBS.
7. 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. Refer to the ProBond™ or Ni-NTA manual for guidelines about sample preparation.

# Appendix

## Recipes

### LB (Luria-Bertani) Medium and Plates

**Composition:**

10 g Tryptone
10 g NaCl
5 g Yeast Extract
pH 7.0

1. Combine the reagents above and add deionized, distilled water to 950 ml.
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 (*i.e.* 50-100 μg/ml of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

### Low Salt LB Medium with Zeocin™

10 g Tryptone
**5 g NaCl**
5 g Yeast Extract
pH 7.5

1. Combine the reagents above and add deionized, distilled water to 950 ml.
2. 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.
3. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
4. Thaw Zeocin™ on ice and vortex before removing an aliquot.
5. Allow the medium to cool to at least 55°C before adding Zeocin™ to a 25 μg/ml final concentration.
6. Store plates at +4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

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# 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.

**Note:** Protease inhibitors may be added at the following concentrations:

1 mM PMSF

1 μg/ml pepstatin

1 μg/ml leupeptin

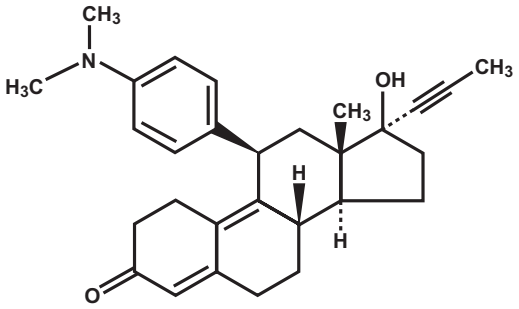
# Mifepristone

## Mifepristone

Mifepristone (11β-[4-dimethylamino]phenyl-17β-hydroxy-17-[1-propynyl]estra-4,9-dien-3-one) is a synthetic 19-norsteroid that binds with high affinity to the human progesterone receptor ($K_d$ <1 x $10^{-9}$ M) and the glucocorticoid receptor ($K_d$ <1 x $10^{-9}$ M) and acts as both a progesterone and glucocorticoid antagonist (Baulieu, 1989). Mifepristone is also known as RU 486 and has been used as an abortifacient at high doses (3-10 mg/kg) (Baulieu, 1989; Philibert *et al.*, 1985). In the GeneSwitch™ System, mifepristone acts as an agonist to activate gene transcription by binding to a truncated human progesterone receptor (Wang *et al.*, 1997). The concentrations of mifepristone used to induce gene expression in the GeneSwitch™ System are extremely low (1 x $10^{-8}$ M), non-toxic, and exert no known pleiotrophic effects on mammalian cells that lack endogenous progesterone and glucocorticoid receptors (Wang *et al.*, 1994).

## Molecular Weight, Formula, and Structure

The formula for mifepristone is $C_{29}H_{35}NO_2$ and the molecular weight is 429.6. The diagram below shows the structure of mifepristone.




## Handling Mifepristone

- Mifepristone is toxic. Do not ingest or inhale the powder or solutions containing the drug.
- Use caution when handling large quantities of mifepristone (> 100 mg). At high doses (see above), mifepristone may impair fertility and may cause harm to the unborn child.
- Always wear gloves, a laboratory coat, and safety glasses or goggles when handling mifepristone and mifepristone-containing solutions.

## Preparing and Storing Stock Solutions

Mifepristone is supplied with the GeneSwitch™ Complete and Core Systems, but may also be obtained separately from Invitrogen (Catalog no. H110-01) in 100 µg aliquots. To prepare mifepristone:

1. Resuspend the 100 µg aliquot of mifepristone in 233 µl of 100% ethanol to make a 1 mM (1 x $10^{-3}$ M) stock solution of mifepristone that is clear. Do not heat.
2. Dilute 100 µl of the 1 mM mifepristone stock solution in 10 ml of 100% ethanol to make a 10 µM (1 x $10^{-5}$ M) working solution of mifepristone. Use this working solution when adding mifepristone to your cells.
3. Store the 1 mM stock solution and the 10 µM working solution of mifepristone at -20°C to prevent evaporation. The solutions are stable indefinitely.

# Zeocin™

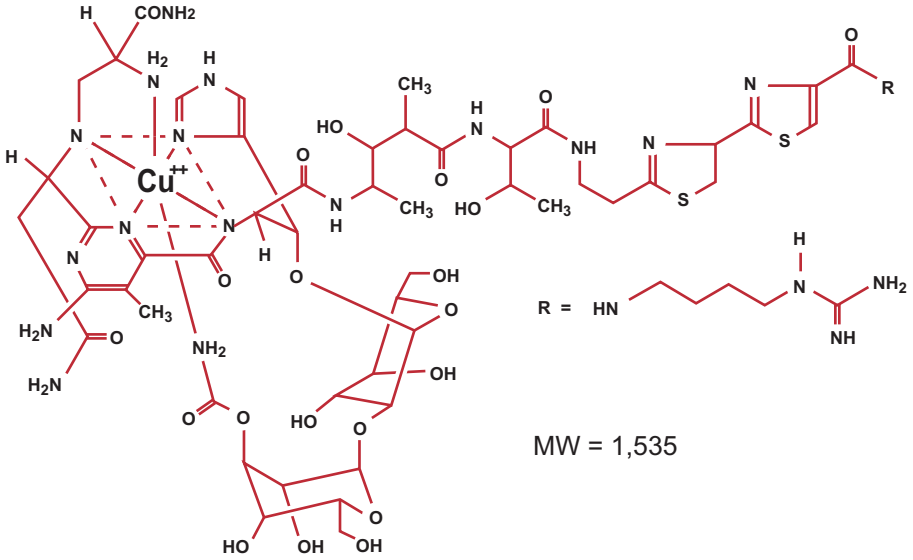
## Zeocin™

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong anti-bacterial and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron *et al.*, 1992; Drocourt *et al.*, 1990; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

## Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.




## Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant *et al.*, 1988); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in mammalian cell lines and *E. coli* are listed below:

| Organism | Zeocin™ Concentration and Selective Medium |
|---|---|
| *E. coli* | 25-50 µg/ml in **Low Salt LB** medium* (see page 22 for a recipe) |
| Mammalian Cells | 50-1000 µg/ml (varies with cell line) |

*Efficient selection requires that the concentration of NaCl be no more than 5 g/L (< 90 mM)

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# Zeocin™, continued

## Handling Zeocin™

- High salt and acidity or basicity inactivate Zeocin™; therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see Low Salt LB Medium, page 22). Note that the salt concentration and pH do not need to be adjusted when preparing tissue culture medium containing Zeocin™.
- Store Zeocin™ at -20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store drug, plates, and medium containing drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling solutions containing Zeocin™.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.
- Store tissue culture medium containing Zeocin™ at +4°C in the dark. Medium containing Zeocin™ is stable for 1-2 months.

## Ordering Information

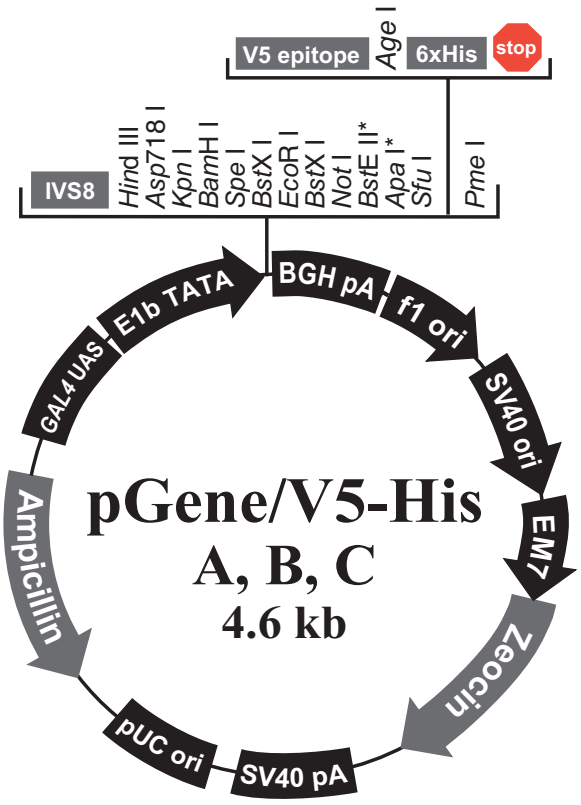
Zeocin™ can be purchased from Invitrogen. For your convenience, the drug is prepared in autoclaved, deionized water and available in 1.25 ml aliquots at a concentration of 100 mg/ml. The Zeocin™ solution is blue in color. The stability of Zeocin™ is guaranteed for six months, if stored at -20°C.

| Amount | Catalog no. |
|---|---|
| 1 gram | R250-01 |
| 5 grams | R250-05 |

# pGene/V5-His Vector

## Map of pGene/V5-His

The pGene/V5-His A, B, and C vectors allow cloning of your gene of interest in frame with a C-terminal peptide. Expression of the gene of interest is controlled by a GAL4 UAS and an E1b TATA box. The vector also contains a synthetic intron to enhance expression of your gene. The figure below summarizes the features of the pGene/V5-His vectors. **The complete nucleotide sequences for pGene/V5-His A, B, and C are available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see page 33).**




* Not unique in all versions

**Comments for pGene/V5-His A:**
**4650 nucleotides**

*GAL4* Upstream Activating Sequences (UAS)
*GAL4* binding site: bases 24-40
*GAL4* binding site: bases 43-59
*GAL4* binding site: bases 62-78
*GAL4* binding site: bases 94-110
*GAL4* binding site: bases 113-129
*GAL4* binding site: bases 132-148
Adenovirus E1b TATA sequence: bases 161-173
Synthetic intron IVS8: bases 329-446
pGene forward priming site: bases 357-375
Multiple cloning site: bases 407-550
V5 epitope: bases 551-592
Polyhistidine (6xHis) region: bases 602-619
BGH polyadenylation sequence: bases 645-872
BGH reverse priming site: bases 642-659
f1 origin: bases 918-1346
SV40 early promoter and origin: bases 1354-1694
EM7 promoter: bases 1736-1802
*Zeocin*™ resistance gene: bases 1803-2177
SV40 early polyadenylation sequence: bases 2307-2437
pUC origin: bases 2820-3490 (complementary strand)
*bla* promoter: bases 4496-4594 (complementary strand)
Ampicillin (*bla*) resistance gene: 3635-4495 (complementary strand)

*continued on next page*

# pGene/V5-His Vector, continued

## Features of pGene/V5-His

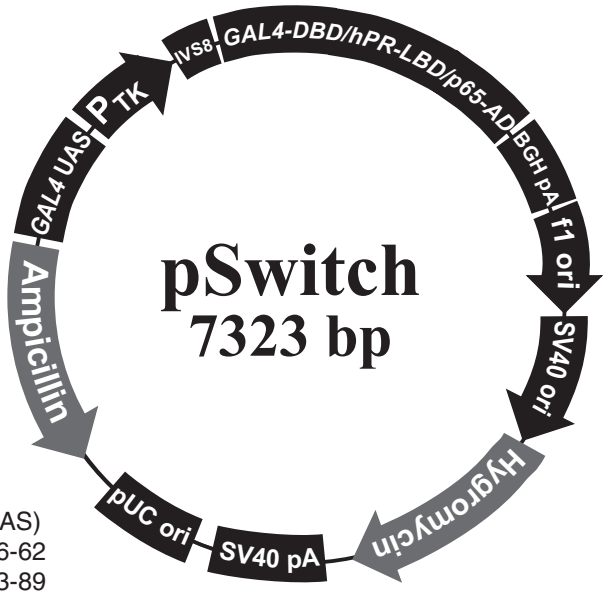
The table below describes the relevant features of pGene/V5-His. All features have been functionally tested.

| Feature | Benefit |
|---|---|
| *GAL4* Upstream Activating Sequences (UAS) | Contains six copies of a 17 bp sequence that allows binding and transcriptional activation of the gene of interest by the GAL4 DBD/hPR-LBD/p65 AD fusion protein (Giniger *et al.*, 1985; Wang *et al.*, 1994) |
| Adenovirus E1b TATA sequence | Permits transcriptional initiation of the gene of interest (Lillie and Green, 1989) |
| Synthetic intron IVS8 | Enhances expression of the gene of interest |
| pGene forward priming site | Allows sequencing in the sense orientation |
| Multiple cloning site | Allows insertion of your gene of interest |
| V5 epitope<br>(Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) | Allows detection of your recombinant protein with the Anti-V5 antibodies (Southern *et al.*, 1991) |
| C-terminal polyhistidine (6xHis) tag | Permits purification of your recombinant protein on metal-chelating resin such as ProBond™ or Ni-NTA<br>In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) antibodies (Lindner *et al.*, 1997) |
| Bovine growth hormone (BGH) polyadenylation sequence | Permits efficient polyadenylation of mRNA (Goodwin and Rottman, 1992) |
| BGH reverse priming site | Permits sequencing of the non-coding strand |
| f1 origin | Allows rescue of single-stranded DNA |
| SV40 early promoter and origin | Allows efficient, high-level expression of the Zeocin™ resistance gene in mammalian cells and episomal replication in cells expressing SV40 large T antigen |
| EM7 promoter | Synthetic prokaryotic promoter for expression of the Zeocin™ resistance gene in *E. coli* |
| Zeocin™ resistance (*Sh ble*) gene | Permits selection of stable transfectants in mammalian cells (Mulsant *et al.*, 1988) and transformants in *E. coli* (Drocourt *et al.*, 1990) |
| SV40 early polyadenylation signal | Allows polyadenylation of mRNA |
| pUC origin | Permits high-copy number replication and maintenance in *E. coli* |
| *bla* promoter | Allows expression of the ampicillin (*bla*) resistance gene |
| Ampicillin (*bla*) resistance gene (β-lactamase) | Allows selection of transformants in *E. coli* |

# pSwitch Vector

## Map of pSwitch

pSwitch is a 7323 bp vector that expresses a 73 kDa fusion protein consisting of the yeast GAL4 DNA binding domain (DBD), a truncated human progesterone receptor ligand binding domain (hPR-LBD), and the human NF-κB p65 activation domain (AD) under the control of *GAL4* upstream activating sequences (UAS) and a Herpes Simplex Virus thymidine kinase (TK) minimal promoter. The vector also includes a synthetic intron to enhance expression of the fusion gene. For more information about the individual components of the fusion gene, refer to page 31. The figure below summarizes the features of the pSwitch vector. **The complete sequence for pSwitch is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 33).**




**Comments for pSwitch:**
**7323 nucleotides**

*GAL4* Upstream Activating Sequences (UAS)
*GAL4* consensus binding site: bases 46-62
*GAL4* consensus binding site: bases 73-89
*GAL4* consensus binding site: bases 100-116
*GAL4* consensus binding site: bases 127-143
Herpes Simplex Virus thymidine kinase (TK) minimal promoter: bases 157-262
Synthetic intron IVS8: bases 379-496
*GAL4-DBD/hPR-LBD/p65-AD* fusion gene
ATG initiation codon: bases 519-521
GAL4 DNA binding domain (DBD): bases 540-818
Human progesterone receptor ligand binding domain (hPR-LBD): bases 840-1664
Human p65 activation domain (AD): bases 1674-2483
BGH polyadenylation sequence: bases 2747-2975
f1 origin: bases 3021-3449
SV40 early promoter and origin: bases 3457-3797
Hygromycin B resistance gene: bases 3841-4864
SV40 early polyadenylation sequence: bases 4996-5126
pUC origin: bases 5509-6182 (complementary strand)
*bla* promoter: bases 7188-7286 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 6327-7187 (complementary strand)

*continued on next page*

# pSwitch Vector, continued

## Features of pSwitch

The table below describes the relevant features of pSwitch. All features have been functionally tested and the vector has been fully sequenced.

| Feature | Benefit |
|---|---|
| *GAL4* Upstream Activating Sequences (UAS) | Contains four copies of a 17 bp sequence that allows binding and transcriptional activation of the gene of interest by the GAL4-DBD/hPR-LBD/p65-AD fusion protein (Giniger *et al.*, 1985; Wang *et al.*, 1994) |
| Herpes Simplex Virus thymidine kinase (TK) minimal promoter | Allows expression of the *GAL4-DBD/hPR-LBD/p65-AD* fusion gene (McKnight, 1980) |
| Synthetic intron IVS8 | Enhances expression of the *GAL4-DBD/hPR-LBD/p65-AD* fusion gene |
| *GAL4-DBD/hPR-LBD/p65-AD* fusion gene | Encodes a 73 kDa fusion protein containing the yeast GAL4 DNA binding domain (Laughon and Gesteland, 1984; Marmorstein *et al.*, 1992), a truncated human progesterone receptor ligand binding domain (Kastner *et al.*, 1990; Misrahi *et al.*, 1987; Wang *et al.*, 1994), and the human NF-κB p65 activation domain (Burcin *et al.*, 1999; Deloukas and Loon, 1993; Ruben *et al.*, 1991) to allow mifepristone-regulated expression of the gene of interest |
| Bovine growth hormone (BGH) polyadenylation sequence | Permits efficient polyadenylation of mRNA (Goodwin and Rottman, 1992) |
| BGH reverse priming site | Permits sequencing of the non-coding strand |
| f1 origin | Allows rescue of single-stranded DNA |
| SV40 early promoter and origin | Allows efficient, high-level expression of the hygromycin resistance gene in mammalian cells and episomal replication in cells expressing SV40 large T antigen |
| Hygromycin (*HPH*) resistance gene | Permits selection of stable transfectants in mammalian cells (Gritz and Davies, 1983) |
| SV40 early polyadenylation signal | Allows polyadenylation of mRNA |
| pUC origin | Permits high-copy number replication and maintenance in *E. coli* |
| *bla* promoter | Allows expression of the ampicillin (*bla*) resistance gene |
| Ampicillin (*bla*) resistance gene (β-lactamase) | Allows selection of transformants in *E. coli* |

*continued on next page*

# pSwitch Vector, continued

## GAL4 DNA Binding Domain

The *Saccharomyces cerevisiae GAL4* gene encodes a transcription factor that activates expression of genes required for galactose catabolism in yeast (Laughon and Gesteland, 1984). The GAL4 protein regulates transcription of target genes by binding as a homodimer to specific 17 nucleotide GAL4 binding sites contained within the upstream activating sequences (UAS) of these genes (Giniger *et al.*, 1985; Marmorstein *et al.*, 1992). The DNA binding function of GAL4 has been mapped to the N-terminal portion of the protein (Carey *et al.*, 1989).

The *GAL4-DBD/hPR-LBD/p65-AD* fusion gene contains a DNA fragment encoding amino acids 1-93 of the GAL4 gene. This portion of the GAL4 protein includes regions that have been shown to be responsible for DNA binding (amino acids 1-65) (Keegan *et al.*, 1986), dimerization (amino acids 65-93) (Carey *et al.*, 1989), and nuclear localization (amino acids 1-29) (Nelson and Silver, 1989).

## Human Progesterone Receptor Ligand-Binding Domain

The human progesterone receptor (Kastner *et al.*, 1990; Misrahi *et al.*, 1987) is a member of the steroid and thyroid hormone receptor superfamily (Evans, 1988; Truss and Beato, 1993). In the absence of ligand, the progesterone receptor is localized in the nucleus in an inactive form (Guiochon-Mantel *et al.*, 1989; Perrot-Applanat *et al.*, 1985). In the presence of its cognate ligand, progesterone and other progesterone agonists, the progesterone receptor undergoes a conformational change to an active form. The ligand-bound receptor then homodimerizes and functions as a transcription factor to regulate expression of genes involved in cellular proliferation and differentiation (Evans, 1988; Simons, 1998). The ligand-binding functions of the progesterone receptor reside in the C-terminal portion of the protein (amino acids 640-933) (Vegeto *et al.*, 1992). Synthetic progesterone antagonists including mifepristone also bind to the progesterone receptor in this region (Vegeto *et al.*, 1992).

The portion of the ligand-binding domain included in the *GAL4-DBD/hPR-LBD/p65-AD* fusion gene encodes amino acids 640-914 of the human progesterone receptor. This hPR-LBD fragment contains a deletion of 19 amino acids from the C-terminal end of the native hPR-LBD, which allows the truncated hPR-LBD to bind with high affinity to mifepristone and other synthetic progesterone antagonists, but abolishes its ability to bind to progesterone or other endogenous steroid hormones (Wang *et al.*, 1994; Wang *et al.*, 1997).

## Human p65 Activation Domain

The human p65 protein, originally identified as a relative of the *rel* oncogene, encodes a subunit of the second messenger, NF-κB (Ruben *et al.*, 1991). NF-κB consists of a heterodimer between two proteins, p65 and p50, and functions as a pleiotrophic transcriptional activator in eukaryotes (Baeuerle, 1991). In its inactive form, NF-κB is localized in the cytoplasm as a complex with the inhibitor, IκB (Baeuerle and Baltimore, 1988). Binding of IκB to NF-κB occurs via the p65 protein. In addition to its interaction with IκB, the p65 protein is also responsible for the transcription activation function of NF-κB (Schmitz and Baeuerle, 1991).

The portion of p65 included in the GAL4-DBD/hPR-LBD/p65-AD fusion gene encodes amino acids 283-551 of the protein. Two distinct transactivation domains have been identified within this region of the protein (Schmitz and Baeuerle, 1991).

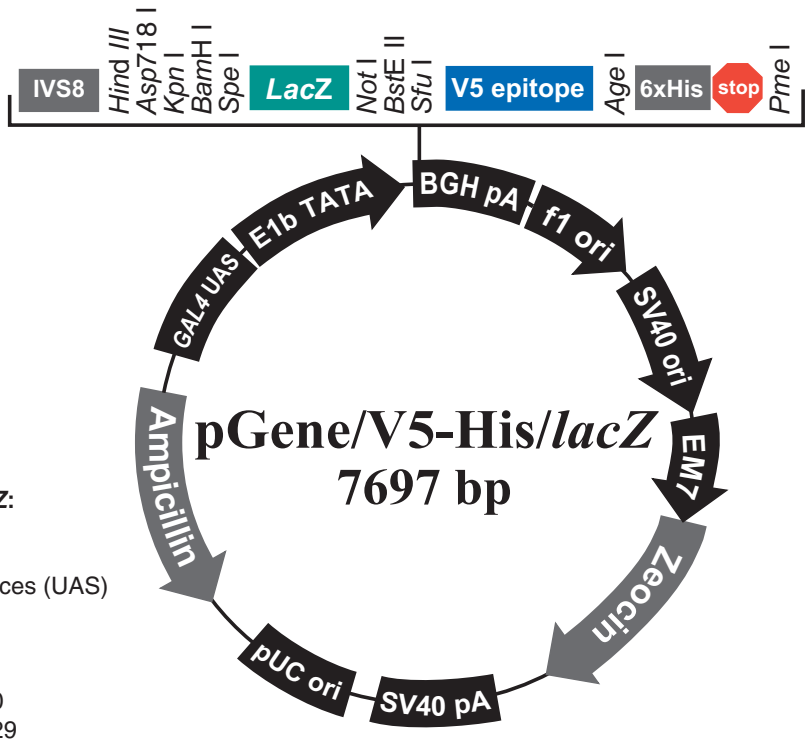
# pGene/V5-His/*lacZ* Vector

## Description

pGene/V5-His/*lacZ* is a 7697 bp control vector containing the gene for β-galacto-sidase. This vector, also known as pBG1352, was derived from pcDNA4/V5-His/*lacZ* and modified by Valentis, Inc.

## Map of pGene/V5-His/*lacZ*

The figure below summarizes the features of the pGene/V5-His/*lacZ* vector. **The complete nucleotide sequence for pGene/V5-His/*lacZ* is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (see the next page).**




**Comments for pGene/V5-His/*lacZ*:**
**7697 nucleotides**

*GAL4* Upstream Activating Sequences (UAS)
*GAL4* binding site: bases 24-40
*GAL4* binding site: bases 43-59
*GAL4* binding site: bases 62-78
*GAL4* binding site: bases 94-110
*GAL4* binding site: bases 113-129
*GAL4* binding site: bases 132-148
Adenovirus E1b TATA sequence: bases 161-173
Synthetic intron IVS8: bases 329-446
pGene forward priming site: bases 357-375
*LacZ* ORF: bases 514-3570
V5 epitope: bases 3598-3639
Polyhistidine (6xHis) region: bases 3649-3666
BGH polyadenylation sequence: bases 3692-3919
BGH reverse priming site: bases 3689-3706
f1 origin: bases 3965-4393
SV40 early promoter and origin: bases 4401-4741
EM7 promoter: bases 4783-4849
*Zeocin*™ resistance gene: bases 4850-5224
SV40 early polyadenylation sequence: bases 5354-5484
pUC origin: bases 5867-6537 (complementary strand)
*bla* promoter: bases 7543-7641 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 6682-7542 (complementary strand)

# Technical Service

## Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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## Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

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## Introduction

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# Product Qualification

## Introduction

This section describes the criteria used to qualify the components of the GeneSwitch™ System.

## Vectors

Each vector is qualified by restriction enzyme digestion with specified restriction enzymes as listed below. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel (see below).

| Vector | Restriction Enzymes | Expected Results (bp) |
|---|---|---|
| pSwitch | *Eco*R I | 3421, 1577, 861, 827, 637 |
| | *Eco*R V | 7323 |
| | *Hin*d III | 7323 |
| pGene/V5-His A | *Apa* I | 4650 |
| | *Not* I | 4650 |
| | *Sac* II | 4650 |
| | *Xba* I | 4273, 377 |
| pGene/V5-His B | *Apa* I | 4654 |
| | *Not* I | 4654 |
| | *Sac* II | 4388, 266 |
| | *Xba* I | 4277, 377 |
| pGene/V5-His C | *Apa* I | no site |
| | *Not* I | 4646 |
| | *Sac* II | 4646 |
| | *Xba* I | 4646 |
| pGene/V5-His/*lacZ* | *Apa* I | no site |
| | *Not* I | 7697 |
| | *Sac* II | 7697 |
| | *Xba* I | 7697 |

## Primers

Sequencing primers are lot tested by automated DNA sequencing experiments.

## Mifepristone

Mifepristone is lot qualified by functional testing using NIH3T3 cells that have been stably transfected with pSwitch and pGene/V5-His/*lacZ*. At least 50% of the cells must exhibit β-galactosidase expression as assayed by staining with the β-Gal Staining Kit from Invitrogen (Catalog no. K1465-01) after 24 hours of induction with 1 x $10^{-8}$ M mifepristone.

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# Product Qualification, continued

| | |
|---|---|
| **Hygromycin B** | Hygromycin B is lot qualified by performing a kill curve experiment with S2 insect cells in *Drosophila* Expression System (DES™) Expression Medium. For information about the lot specific activity of hygromycin B, refer to the label on the bottle. |
| **Zeocin™** | Zeocin™ is lot qualified by demonstrating that Low Salt LB media containing 25 µg/ml Zeocin™ prevents growth of the *E. coli* strain, TOP10. |

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