# pZeoSV2(+) or pZeoSV2(-)

## A Constitutive Mammalian Expression Vector with Zeocin™ Selection

**Catalog nos. V850-01, V855-01**

**Version G**
111210
*25-0140*

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# Kit Contents and Storage

## Storage

Store kit at -20°C. Store Zeocin™ in the dark.

## Kit Contents

| Item | Amount |
|---|---|
| pZeoSV2 (+) or pZeoSV2 (-) | 20 µg, supercoiled, lyophilized |
| pZeoSV2/*lac*Z | 20 µg, supercoiled, lyophilized |
| Zeocin™ | 3 x 1.25 ml<br>100 mg/ml in sterile, deionized water |

# Introduction

## Overview

### Introduction

The plasmids pZeoSV2 (+) and pZeoSV2 (-) are constitutive mammalian expression vectors containing a gene that confers resistance to the novel antibiotic Zeocin™. Zeocin™ can be used to select for the pZeoSV2 vectors in bacterial and mammalian cells.

### Experimental Process

This kit includes either pZeoSV2 (+) or pZeoSV2 (-) which allow constitutive expression of the desired gene in mammalian cells. Zeocin™ is included and used to select for this vector in *E. coli* and mammalian cells. A positive control, pZeoSV2/*lac*Z, is also included to test for proper transfection and expression in your cell line. The following table describes the overall experimental process.

| Step | Action |
|---|---|
| 1 | Propagate pZeoSV2 and pZeoSV2/*lac*Z by transforming them into a *rec*A, *end*A1 *E. coli* strain such as TOP10, DH5α, or JM109. |
| 2 | Develop a cloning strategy and ligate your gene into pZeoSV2. |
| 3 | Transform into *E. coli* and select transformants on Zeocin™-containing solid medium. |
| 4 | Analyze 10-20 transformants by restriction mapping or sequencing to determine the presence and orientation of your gene. |
| 5 | Prepare recombinant plasmid for transfection into your mammalian cell line. |
| 6 | Transfect your mammalian cell line and assay for transient expression. Zeocin™ selection is not needed for transient expression. |
| 7 | If you wish to isolate stable transfectants, determine the concentration of Zeocin™ necessary to kill mammalian cells without pZeoSV2. This concentration will be used for the selection of stable cell lines. |
| 8 | Select for stable transfectants using the conditions determined in Step 7. |

Zeocin™ is a trademark of CAYLA.

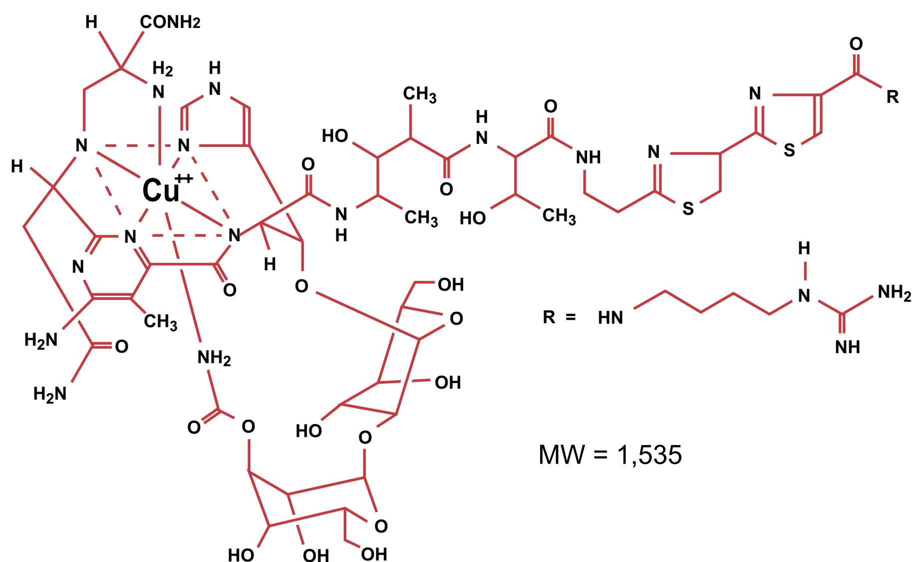
# Zeocin™

## Description

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and antitumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells. Zeocin™ is not as toxic as bleomycin on fungi. As a broad-spectrum antibiotic Zeocin™ is particularly useful, allowing selection in a number of cell types containing vectors with a Zeocin™ resistance gene.

## Chemical Properties

Zeocin™ is a basic, water-soluble compound isolated from *Streptomyces verticillus* as a copper-chelated glycopeptide. The presence of copper gives the solution its blue color. The chemical formula for Zeocin™ is $C_{55}H_{83}N_{19}O_{21}S_2Cu$. It contains several unique amino acids, sugars, and aliphatic amines. For general information about the family of bleomycin antibiotics, please see Berdy, 1980 (**Reference** section). The general structure of Zeocin™ is shown below.




## Mechanism of Action

The exact mechanism of action of Zeocin™ is not known; however, it is thought to be the same as bleomycin and phleomycin due to its similarity to these drugs and its inhibition by the *Sh ble* resistance protein (see next section). The copper/glycopeptide complex is selective and involves chelation of copper ($Cu^{2+}$) by the amino group of the α-carbox-amide, single nitrogen atoms of both the pyrimidine chromophore and the imidazole moiety, and the carbamoyl group of mannose. The copper-chelated form is inactive. When the antibiotic enters the cell, the copper cation is reduced from $Cu^{2+}$ to $Cu^{1+}$ and removed by sulfhydryl compounds in the cell. Upon removal of the copper, Zeocin™ is activated to bind DNA and cleave it causing cell death (Berdy, 1980). **High salt concentrations and acidity or basicity inactivate Zeocin™; therefore, it is necessary to reduce the salt in bacterial medium to 90 mM (5 g/liter) or less and adjust the pH to 7.5 to make sure the drug remains active.**

*Continued on next page*

## Resistance to Zeocin™

A Zeocin™ resistance protein has been isolated and characterized (Gatignol, *et al.*, 1988; Drocourt, *et al.*, 1990; and Calmels, *et al.*, 1991). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13,665 Da protein that binds Zeocin™ in a stoichiometric manner. The binding of Zeocin™ inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™. The nucleic acid and protein sequence is given below:

```
   Nco I
ACC ATG GCC AAG TTG ACC AGT GCC GTT CCG GTG CTC
     M   A   K   L   T   S   A   V   P   V   L

ACC GCG CGC GAC GTC GCC GGA GCG GTC GAG TTC TGG
 T   A   R   D   V   A   G   A   V   E   F   W
                           Sma I
ACC GAC CGG CTC GGG TTC TCC CGG GAC TTC GTG GAG
 T   D   R   L   G   F   S   R   D   F   V   E
               SgrA I
GAC GAC TTC GCC GGT GTG GTC CGG GAC GAC GTG ACC
 D   D   F   A   G   V   V   R   D   D   V   T

CTG TTC ATC AGC GCG GTC CAG GAC CAG GTG GTG CCG
 L   F   I   S   A   V   Q   D   Q   V   V   P

GAC AAC ACC CTG GCC TGG GTG TGG GTG CGC GGC CTG
 D   N   T   L   A   W   V   W   V   R   G   L

GAC GAG CTG TAC GCC GAG TGG TCG GAG GTC GTG TCC
 D   E   L   Y   A   E   W   S   E   V   V   S

ACG AAC TTC CGG GAC GCC TCC GGG CCG GCC ATG ACC
 T   N   F   R   D   A   S   G   P   A   M   T

GAG ATC GGC GAG CAG CCG TGG GGG CGG GAG TTC GCC
 E   I   G   E   Q   P   W   G   R   E   F   A

CTG CGC GAC CCG GCC GGC AAC TGC GTG CAC TTC GTG
 L   R   D   P   A   G   N   C   V   H   F   V

GCC GAG GAG CAG GAC TGA
 A   E   E   Q   D   STOP
```

**Note**: Both the *Sgr*A I and *Sma* I sites are unique, but the *Nco* I site is not.

*Continued on next page*

### Applications of Zeocin™

Zeocin™ and the *Sh ble* gene are 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 tissue culture cells and *E. coli* are listed below:

| Organism | Zeocin™ Concentration and Selective Medium |
|---|---|
| *E. coli* | 25-50 µg/ml in **low salt** LB medium* |
| Mammalian cells | 50-1000 µg/ml (depends on cell line) |

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

### 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 it is highly recommended that you choose an *E. coli* strain that does not contain the Tn*5* gene (i.e. TOP10, DH5, DH10, etc.).

It is always important to test the sensitivity of your host cell to Zeocin™ as natural resistance varies among cell lines. See page 17 for a protocol on mammalian cell selection.

### Purchase of Zeocin™

375 mg of Zeocin™ has been provided with the pZeoSV2 vector to allow you to begin your experiments. Additional Zeocin™ can be purchased from Invitrogen in 1 gram (Catalog no. R250-01) and 5 gram (Catalog no. R250-05) quantities. For your convenience, the drug is prepared in autoclaved, deionized water and aliquoted into 1.25 ml aliquots at 100 mg/ml. The stability of Zeocin™ is guaranteed for six months, if stored at –20°C.

### Handling Zeocin™

- **High salt and acidity or basicity inactivate Zeocin™.** Reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see **Low Salt LB Medium,** page 13).
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug, and plates or medium containing drug, in the dark.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Zeocin™-containing solutions.
- Do not ingest or inhale solutions containing the drug.
- Be sure to bandage any cuts on your fingers to avoid exposure to the drug.

# pZeoSV2 (+) and (-) Properties

### Description of pZeoSV2 (+) and pZeoSV2 (-)

pZeoSV2 (+) and pZeoSV2 (-) are 3.45 kb vectors containing the *Sh ble* resistance gene to Zeocin™ that allows for selection in both prokaryotic and eukaryotic cells. Expression of the *Sh ble* gene in *E. coli* is controlled by the synthetic EM-7 promoter, while eukaryotic expression of the gene is under control of the cytomegalovirus (CMV) immediate-early promoter. Genes cloned into pZeoSV2 (+) or (-) are expressed from the Simian Virus 40 (SV40) early enhancer/promoter for high level transient and stable expression in mammalian cell lines.

### Features of pZeoSV2 (+) and pZeoSV2 (-)

The important elements of pZeoSV2 (+) and (-) are described in the following table. All features have been functionally tested. The multiple cloning site has been tested by restriction analysis.

| Features | Function |
|---|---|
| *Sh ble* gene (expressed from the CMV immediate-early promoter or the EM-7 promoter) | Stable selection in the presence of Zeocin™ in mammalian cells and selection in *E. coli* |
| SV40 polyadenylation signal | mRNA stability and transcription termination |
| Simian Virus (SV40) early enhancer/promoter | Provides high-level expression of the gene of interest |
| Multiple cloning site (MCS) in positive or negative orientations | Permits insertion of gene of interest |
| Bovine growth hormone (BGH) polyadenylation signal | mRNA stability and efficient transcription termination |
| EM-7 promoter | Synthetic prokaryotic promoter for expression of the *Sh ble* gene in *E. coli* |
| SV40 origin | Allows replication in cells expressing large T antigen |
| f1 origin | Rescue of single-strand DNA for mutagenesis and sequencing |
| pUC origin | Replication, maintenance, and high copy number in *E. coli* |
| T7 promoter priming site | Allows sequencing of insert |

*Continued on next page*

### Map of pZeoSV2 (+) and (-)

The figure below summarizes the features of the pZeoSV2 (+) and (-) vectors. The complete nucleotide sequence of each vector can be obtained by downloading from our World Wide Web site or by calling Technical Service (see page 21).

**Comments for pZeoSV2**
**3515 nucleotides**

SV40 promoter: bases 10-356
T7 promoter/primer: bases 401-420
Multiple Cloning Site: bases 433-548
BGH reverse priming site: bases 560-577
BGH polyA: bases 560-773
pUC origin: bases 814-1487
f1 origin: bases 1780-2290
CMV promoter: bases 2304-2930
EM-7 promoter: bases 2931-2997
Zeocin resistance gene: 2998-3372
SV40 polyA: bases 3380-3515

# pZeoSV2/*lac*Z Properties

## Description of pZeoSV2/*lac*Z

pZeoSV2/*lac*Z is a 6.6 kb vector encoding the dominant selectable *Sh ble* protein, allowing selection with Zeocin™ in both prokaryotic and eukaryotic cells. It expresses β-galactosidase from the SV40 early enhancer-promoter. From this promoter, high levels of β-galactosidase are constitutively expressed in a variety of cell lines. pZeoSV2/*lac*Z is included in this kit as a positive control for expression in mammalian cell lines.

## Features of pZeoSV2/*lac*Z

The elements of pZeoSV2/*lac*Z are described in the following table. All features have been functionally tested.

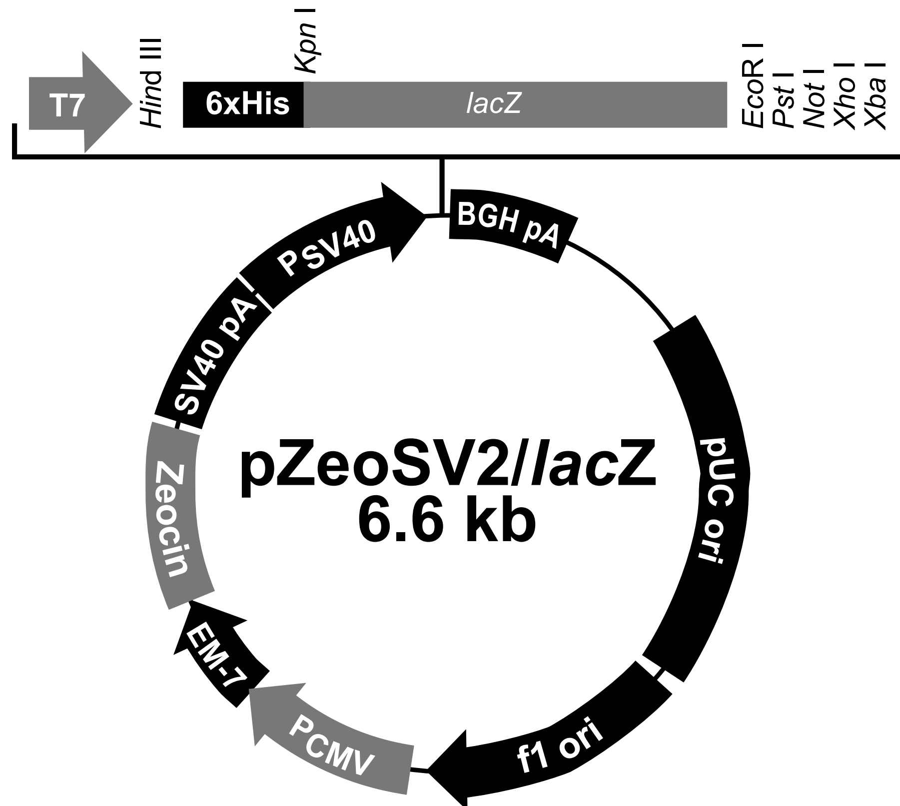
| Features | Function |
|---|---|
| *Sh ble* gene (expressed from the CMV immediate-early promoter or the EM-7 promoter) | Stable selection in the presence of Zeocin™ in mammalian cells and selection in *E. coli* |
| SV40 polyadenylation signal | mRNA stability and transcription termination |
| Simian Virus (SV40) early enhancer/ promoter | Promotes high levels of expression of β-galactosidase from the *lac*Z gene |
| Bovine growth hormone (BGH) polyadenylation signal | mRNA stability and efficient transcription termination |
| EM-7 promoter | Synthetic prokaryotic promoter for expression of the *Sh ble* gene in *E. coli* |
| SV40 origin | Allows replication in cells expressing large T antigen |
| f1 origin | Rescue of single-strand DNA for mutagenesis and sequencing |
| pUC origin | Replication, maintenance, and high copy number in *E. coli* |
| *Lac*Z | β-galactosidase gene as a positive control for expression in mammalian cells |

*Continued on next page*

# pZeoSV2/*lac*Z Properties, continued

**Map of pZeoSV2/*lac*Z**

The figure below summarizes the features of the pZeoSV2/*lac*Z vector. To construct pZeoSV2/*lac*Z, a 3.1 kb fragment containing the *lac*Z gene fused to a six histidine N-terminal tag was cloned into *Hin*d III–*Eco*R I digested pZeoSV2 (+). The sequence of pZeoSV2/*lac*Z can be obtained by downloading from our World Wide Web site or by calling Technical Service (see page 21).




**Comments for pZeoSV2/lacZ**
**6661 nucleotides**

SV40 promoter: bases 10-356
T7 promoter/priming site: 401-420
LacZ ORF: bases 458-3626
BGH polyA: bases 3706-3919
pUC origin: bases 3959-4683
f1 origin: bases 4926-5436
CMV promoter: bases 5450-6076
EM-7 promoter: bases 6077-6143
Zeocin resistance gene ORF: bases 6144-6518
SV40 polyA: bases 6526-6661

# Methods

## Cloning into pZeoSV2 (+) or (-)

### Maintenance of pZeoSV2 (+), pZeoSV2 (-), and pZeoSV2/*lacZ*

In order to propagate and maintain pZeoSV2 (+) or (-) and pZeoSV2/*lac*Z, we recommend that you transform them into a *rec*A, *end*A1 strain such as TOP10, TOP10F′, DH5α, JM109, or equivalent. For the most efficient selection choose an *E. coli* strain that does not contain the Tn*5* gene (i.e. TOP10, DH5, DH10, etc.).

To propagate the plasmids:

- Resuspend the lyophilized DNA in 20 µl sterile water to prepare a 1 µg/µl stock solution. This stock solution may be stored at –20°C.
- Transform the *E. coli* strain of choice with 10 pg to 10 ng of plasmid. You may need to serially dilute the stock solution.
- Select on Low Salt LB plates containing 25 µg/ml Zeocin™ (see page 13 for recipe).

**Important**

pZeoSV2 (+) and pZeoSV2 (-) are nonfusion vectors allowing constitutive mammalian expression of the gene of interest. Your insert should contain an initiation codon, a Kozak sequence (see **References**), and a stop codon for proper expression of your protein in mammalian cells.

*Continued on next page*

## Cloning into pZeoSV2 (+)

The graphic below shows the SV40 early enhancer/promoter and the multiple cloning site of pZeoSV2 (+). The underlined regions at the 5´ end of the fragment containing the SV40 promoter are the two 72 bp enhancer repeats.

*Continued on next page*

## Cloning into pZeoSV2 (-)

The graphic below shows the SV40 early enhancer/promoter and the multiple cloning site of pZeoSV2 (-). The underlined regions at the 5´ end of the fragment containing the SV40 promoter are the two 72 bp enhancer repeats.

```
      5´ SV40 promoter                  AP3          AP2
    GCTG TGGAATGTGT GTCAGTTAGG GTGTGGAAAG TCCCCAGGCT

 AP2                                  OCT1        AP1
CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC

AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA TGCAAAGCAT

                                            GC Boxes
GCATCTCAAT TAGTCAGCAA CCATAGTCCC GCCCCTAACT CCGCCCATCC

CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA TGGCTGACTA

           TATA Box           Transcriptional Start      3´ SV40
ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT CTGAGCTATT

CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG CCTAGGCTTT TGCAAAAAGC

TCTCTGGCTA ACTAGAGAAC CCACTGCTTA CTGGCTTATC GAAATTAATA

T7 promoter primer binding site        Nhe I      Pme I        Apa I
CGACTCACTA TAGGGAGACC CAAGCTGGCT AGCGTTTAAA CGGGCCCTCT

   Xho I     Not I            BstX I   EcoR V  Pst I  EcoR I     BstX
AGACTCGAGC GGCCGCCACT GTGCTGGATA TCTGCAGAAT TCCACCACAC

   Spe I    BamH I       Asp718 I Kpn I Hind III  Afl I  Pme I
TGGACTAGTG GATCCGAGCT CGGTACCAAG CTTAAGTTTA AACCGCTGAT

     BGH reverse priming site
CAGCCTCGAC TGTGCCTTCT AGTTGCCAGC CATCTGTTGT TTGCCCCTCC

CCCGTGCCTT CCTTGACCCT GGAAGGTGCC ACTCCCACTG TCCTTTCCTA

BGH poly (A) site
ATAAAATGAG GAAATTGCAT
```

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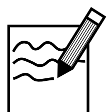
# Cloning into pZeoSV2 (+) or (-), continued



**Note**

1. Using greater than 100 units of *Eco*R I per µg DNA will cause this enzyme to cut at sites other than its particular recognition site ("star activity"). Be sure to use as few units as possible to complete the digestion.
2. pZeoSV2 does not encode the LacZα fragment; therefore, blue/white screening for recombinants in *E. coli* cannot be done with this vector.
3. pZeoSV2 does not encode the ampicillin resistance gene; therefore selection for recombinants can be done only with Zeocin™.

**General Molecular Biology Techniques**

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, please see *Molecular Cloning: a Laboratory Manual* or *Current Protocols in Molecular Biology* (**Reference** section).

# Transformation into *E. coli*

## Introduction

Ligation mixtures may be transformed into *E. coli* strains such as TOP10 (Catalog no. C610-00), TOP10F´ (Catalog no. C615-00), DH5α, or JM109 (Catalog no. C666-00) and selected on **Low Salt** LB medium (see below) with Zeocin™. Genotypes for these strains are on page 14. Transformants are isolated and analyzed for the presence and orientation of insert. There is no blue/white screening for the presence of insert with pZeoSV2 (+) or (-). After obtaining the desired recombinant plasmid, proceed to transfection of your mammalian cell line.

## IMPORTANT! Low Salt LB Medium with Zeocin™

For Zeocin™ to be active, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.5. Prepare LB broth and plates using the following recipe. Please note the lower salt content of this medium.

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

**Low Salt LB Medium:**

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 1 N 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. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/ml final concentration.
4. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

**Note:** Pre-mixed Low Salt LB Medium is available from Invitrogen in convenient pouches or in bulk. Please contact Technical Service for more information (see page 21).

## Transformation

Your construct, pZeoSV2 (+) or (-) without insert, and the pZeoSV2/*lac*Z control vector should be transformed into TOP10 or similar *E. coli* strain. Since pZeoSV2 has an f1 origin, the plasmid can be transformed into TOP10F´ for single-stranded DNA rescue. Guidelines are as follows:

- Transformation may be done by either electroporation or chemical methods.
- Add either Low Salt LB or LB medium to the cells after heat shock or electroporation to allow them to recover.
- Plate on **Low Salt LB medium** with 25 µg/ml Zeocin™.

If you wish, electrocompetent and chemically competent cells are available from Invitrogen. We recommend TOP10F´ or similar as a general strain for transformation.

| Catalog no. | Description | Efficiency | Aliquots |
| --- | --- | --- | --- |
| C3030-03 | One Shot ® Chemically competent TOP10F´ | $1 \times 10^9$ | 20 x 50 µl |
| C665-55 | Electrocompetent TOP10F´ | $1 \times 10^9$ | 5 x 80 µl |
| C665-11 | Electrocompetent TOP10F´ | $1 \times 10^9$ | 10 x 80 µl |

After transformation into the *E. coli* strain of choice, the transformation mixtures are plated on **Low Salt LB medium** with Zeocin™ plates and incubated overnight at 37°C.

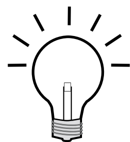
*Continued on next page*

### Analysis of Transformants

Plasmid DNA should be isolated from Zeocin™-resistant transformants and analyzed to confirm the presence and orientation of the desired DNA fragment (see Ausubel, *et al.*, 1990 or Sambrook, *et al.*, 1989).

- Select 10-20 transformants and isolate plasmid DNA.
- Analyze the DNA by restriction mapping or sequencing.

Once you have your insert cloned in the correct orientation, you may test for expression in mammalian cell lines.



Sequencing is recommended to confirm the presence of an initiation codon, a Kozak sequence, and/or a stop codon. The T7 primer and BGH reverse primer (Catalog no. N560-02 and N575-02) can be used to sequence across the multiple cloning site.

### Genotypes of Selected *E. coli* Strains

**DH5α**: $F^-$ *end*A1 *rec*A1 *hsd*R17 ($r_k^-$, $m_k^+$) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169

**JM109**: {F´: *tra*D36 *pro*$AB^+$ *lac*$I^q$ *lac*ZΔM15}*end*A1 *rec*A1 *hsd*R17 ($r_k^-$, $m_k^+$) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 Δ(*lac*-proAB)

**TOP10**: $F^-$, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

**TOP10F´**: F'{*lacIq Tn10*($Tet^R$)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

# Transfection and Transient Expression

## Introduction

At this point, you should have a positive clone with your fragment inserted in the correct orientation for expression in pZeoSV2 (+) or (-). The next step is to isolate very clean DNA and transfect your cell line (see below). After mammalian transfection, the cells may be harvested and assayed for transient expression, or split and selected for stable transformants using the appropriate concentration of Zeocin™ (see page 17). Selection with Zeocin™ is not necessary for transient expression. It is only necessary when selecting for stable transfectants.

## Plasmid Preparation for Transfection

Plasmid DNA must be of high quality and free of contaminants. Contaminated DNA can be toxic to many cell lines. You may already have a suitable protocol in your laboratory for large-scale plasmid purification, if not, refer to *Current Protocols in Molecular Biology*, pp. 9.1.5-9.1.6.

## Methods of Transfection

You may already have a protocol for transfecting your cell line. For established cell lines (e.g. HeLa), please consult original references or the supplier of your cell line for the optimal method of transfection. It is recommended 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* (**Reference** section).

There are a variety of methods available for mammalian cell transfection. Invitrogen offers the Calcium Phosphate Transfection Kit for mammalian transfection and Lipofectamine 2000 Reagent for lipid-mediated transfection.

| Catalog No. | Description | Quantity |
|---|---|---|
| K2780-01 | Calcium Phosphate Transfection Kit | 75 reactions |
| 11668-019 | Lipofectamine 2000 Reagent | 1.5 ml |

## Positive Control

pZeoSV2/*lac*Z is provided as a positive control vector for mammalian transfection and expression. It may be used to optimize transfection conditions for your cell line. The *E. coli* gene encoding β-galactosidase is expressed in mammalian cells under the broad host range early enhancer/promoter from SV40. A successful transfection will result in positive β-galactosidase expression and can be easily monitored with a colorimetric β-galactosidase assay (see page 16).

## Transient Expression

Twenty-four hours after transfection, the cells may need to be washed and then fed with complete medium. Cells are allowed to attach and grow for 2-3 doublings before assaying for transient expression. It is recommended that a time course be performed to determine the optimal time to assay for transient expression. Optimal times may vary from 24 to 96 hours from the time of transfection depending on cell line.

- Harvest $10^6$-$10^7$ cells (one 100 mm plate at 80% confluence), pellet, and transfer to micro-centrifuge tubes. Be sure to include untransfected cells as a control for background activity.
- The pellet of cells may be stored at –80°C if the assay cannot be performed immediately.
- To lyse the cell pellet, freeze-thaw 3 times in 250 mM Tris-HCl, pH 7.5.
- The crude lysate is centrifuged, and the supernatant transferred to clean tubes to assay for expression of the gene of interest or β-galactosidase. The lysate may be stored at –80°C.

# β-Galactosidase Assay

## Introduction

In the β-galactosidase assay, the lactose analog orthonitrophenyl-β-D-galactoside or ONPG, is added to the cell lysate (Miller, 1972, p. 403). ONPG is cleaved by β-galactosidase to produce the ONP anion which produces a yellow color under basic conditions. The assay is monitored by observing the change in absorbance at 420 nm using a UV-VIS spectrophotometer. A protein assay is also performed for each sample in order to normalize the number of β-galactosidase units per mg total protein (Bollag and Edelstein, 1991).

Two kits for β-galactosidase detection are available from Invitrogen, the β-Gal Assay Kit (Catalog No. K1455-01) and the β-Gal Staining Kit (Catalog No. K1465-01).

## Before Starting

The solutions for the assay are found in the **Recipe** section (page 20). You will need to make the following solutions:

- 4 mg/ml ONPG
- "Z" Buffer
- 1 M Sodium carbonate

## Assay for β-Galactosidase Protein

1. For each lysate (see **Transient Expression**, page 15), take 1-10 µl and transfer to a fresh microcentrifuge tube.
2. Add distilled, deionized water to a final volume of 30 µl.
3. Add 66 µl of 4 mg/ml ONPG and 204 µl "Z" buffer. Mix by gently flicking the tube and centrifuging briefly.
4. Incubate the tube at 37°C for 30 minutes. You should see a faint yellow color develop if β-galactosidase is present.
5. To stop reaction, add 500 µl of 1 M sodium carbonate.
6. Read the absorbance at 420 nm against a blank without lysate. Be sure to assay sample of the untransfected cell lysate as a control.
7. Be sure to assay at least three different volumes of lysate. Changes in absorbance should be linear with respect to the amount of lysate assayed. If it is not, you will not get an accurate determination of expression. The most common error is using too much lysate which will cause you to overestimate the expression levels. Decrease the amount of lysate until absorbance is linear with the amount of lysate.
8. Once you have determined you are assaying the lysate accurately, calculate the amount of ONPG hydrolyzed using the following formula, assuming a 1 cm path length:

   $$\text{ONPG hydrolyzed (nmoles)} = \frac{(\text{OD420})\ (\text{volume assayed (nl)})}{(4500\ \text{nl/nmole-cm})\ (1\ \text{cm})}$$

   where 4500 is the extinction coefficient of the ONP anion.
9. The specific activity of β-galactosidase is defined as nmoles of ONPG hydrolyzed per minute per mg of protein. Determine the protein concentration of the lysate and determine the specific activity as follows:

   Specific activity = nmoles of ONPG hydrolyzed/t/mg protein

   where t = the time of incubation in minutes at 37°C and mg protein is the amount of protein assayed. Be sure to subtract the background activity of the untransfected cell lysate.

# Isolation of Stable Transfectants

## Introduction

Once your gene is expressed in your cell line, you may wish to generate a stable cell line expressing your protein. To do this, you need to determine the minimum concentration of Zeocin™ needed to prevent growth of untransfected cells. This concentration of drug will be used to select for stable transfectants. In general, it takes 2 to 6 weeks to select foci with Zeocin™, depending on the cell line. You want to be able to isolate several foci to expand into stable cell lines. Be sure to use buffered medium as Zeocin™ is sensitive to changes in pH.

## Determination of Zeocin™ Sensitivity

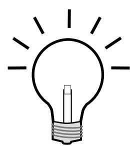
To obtain a stable integrant, you must first determine if the cell line in question can grow as an isolated colony. You may already know this for your cell line. If you do not, seed ~100 cells in a 60 mm plate and feed every 4 days for 10-12 days. Count the number of colonies. Growing in soft agar can help cells to grow when they are diluted; however, some cell lines (e.g. NIH3T3) require plating at a certain density in order to grow properly (see Ausubel, *et al.*, 1990).

Next, determine the minimal concentration of Zeocin™ required to prevent growth of the parental cell line using the protocol below:

1. Plate or split a confluent plate so there are approximately 2.5 x $10^5$ cells per 60-100 mm dish. Prepare 8 plates and add varying concentrations of Zeocin™ (0, 50, 250, 500, 750, and 1000 µg/ml) to each plate.
2. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
3. Count the number of viable cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth.

## Linearizing Vector for Stable Integration

To obtain stable transfectants, you may choose to linearize your vector before transfection. While linearizing your vector may not improve your chances of obtaining stable transfectants, it ensures that the vector does not integrate in a way that disrupts the gene of interest. If you decide to linearize your vector, perform the digestion with a restriction enzyme that is located in the non-eukaryotic DNA sequences of pZeoSV2. Transfect your cell line as you would for transient expression. We have successfully obtained stable transfectants using either electroporation or calcium phosphate transfection techniques.



It is recommended that you include the positive control vector pZeoSV2/*lac*Z in order to test for stable expression in your cell line.

*Continued on next page*

### Selection of Stable Integrants

Once you have determined the appropriate Zeocin™ concentration to use (see page 17), you can generate a stable cell line with your construct.

1. Transfect $10^6$ cells with 20 µg of vector using the desired protocol and plate onto 100 mm culture plates. Remember to include a plate of untransformed cells as a negative control.
2. 24 hours after transfection, wash the cells one time with 1X PBS and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Zeocin™ at the pre-determined concentration required for your cell line. Split the cells into four to eight 100 mm plates such that the cells are no more than 25% confluent.
4. Feed the cells with selective medium every 3-4 days until foci can be identified.
5. When transfected cell foci are formed, the colonies may be picked using either cloning rings (if the colonies are isolated and large enough) or a pipette tip (if the colonies are small) and transferred to either 96- or 48-well plates. Grow cells to near confluence before expanding to larger wells or plates.
6. Test clones for expression of your protein. Positive clones can be expanded further into large microtiter wells and then into flasks or plates as desired and re-tested to confirm expression.

# Appendix

## Troubleshooting Guidelines

### Introduction

The following is designed to provide basic guidelines for expression from the pZeoSV2 (+) and (-) vectors.

### No Transient Expression

1. Make sure there is an initiation codon in a proper Kozak consensus sequence (see **References**) for eukaryotic expression. Be sure there is also a stop codon.
2. Be sure to use the pZeoSV2/*lac*Z control to test your cell line for optimal transfection and expression.
3. Depending on your experiment, you may wish to go ahead and isolate stable transfectants even though you do not detect transient expression. This might be relevant if you are studying a mutant protein in a particular cell line.

### No Stable Expression

1. Use the pZeoSV2/*lac*Z vector to generate stable transfectants expressing β-galactosidase as control.
2. Confirm integration of your construct by either isolating genomic DNA and performing a Southern blot or PCR to see if your gene is present.
3. Confirm transcription by isolating mRNA and performing a Northern or RT-PCR to test for the expression of your gene.

   **Note**: Be sure plasmid has been eliminated from your cell line.
4. Be sure and isolate at least 50 independent foci as the location of integration may affect expression.

# Recipes

### Z Buffer

60 mM $Na_2HPO_4$-$7H_2O$
40 mM $NaH_2PO_4$-$H_2O$
10 mM KCl
1 mM $MgSO_4$-$7H_2O$
50 mM β-mercaptoethanol
pH 7.0

1. Dissolve the following in 950 ml deionized water:

   16.1 g $Na_2HPO_4$-$7H_2O$
   5.5 g $NaH_2PO_4$-$H_2O$
   0.75 g KCl
   0.246 g $MgSO_4$-$7H_2O$
   2.7 ml β-mercaptoethanol

2. Adjust pH to 7.0 with either NaOH or HCl and bring the volume up to 1 liter with water.
3. Do not autoclave! Store at +4°C.

### ONPG Solution

4 mg/ml in 100 mM phosphate buffer, pH 7.0

1. Dissolve the following in 90 ml deionized water

   1.61 g $Na_2HPO_4$-$7H_2O$
   0.55 g $NaH_2PO_4$-$H_2O$

2. Adjust pH to 7.0 with either NaOH or HCl and add 400 mg of ONPG. Stir to dissolve and bring the volume up to 100 ml with water.
3. Store at –20°C away from light.

### 1 M Sodium Carbonate

Dissolve 12.4 g sodium carbonate in 100 ml of deionized water. Store at room temperature.

# Technical Service

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5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

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# Technical Service, continued

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# Purchaser Notification

## Introduction

Use of pZeoSV2 (+) or pZeoSV2 (-) is covered under a number of different licenses as described below.

## Limited Use Label License No. 22: Vectors and Clones Encoding Histidine Hexamer

This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

# Product Quality Control

## Product Qualification

Zeocin™ is lot qualified by demonstrating that LB medium containing 25 µg/ml Zeocin™ prevents growth of the *E. coli* strain, TOP10.

Each of the pZeoSV2 (+/-) vectors is qualified by restriction enzyme digestion with specific restriction enzymes as listed below. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel. The table below lists the restriction enzymes and the expected fragments.

| Vector | Restriction Enzyme | Expected Fragments (bp) |
|---|---|---|
| pZeoSV2 (+) | *Nhe* I | 3515 |
| | *Pst* I | 3515 |
| | *Sac* I | 2349, 1166 |
| pZeoSV2 (-) | *Nhe* I | 3514 |
| | *Pst* I | 3514 |
| | *Sac* I | 2288, 1226 |
| pZeoSV2/*lacZ* | *Nhe* I | 6600, 61 |
| | *Pst* I | 6661 |
| | *Sac* I | 3452, 3209 |

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