USER GUIDE

invitrogen
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ZeoCassette™ Vectors

Catalog numbers V500-20, V501-20, V504-20, V503-20

Revision date 19 January 2012
Publication Part number 28-0173

MAN0000657

For Research Use Only. Not for diagnostic procedures.

life technologies™

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Important Information

Contents

This manual is supplied with all of the ZeoCassette™ vectors listed below. Each vector is supplied with 1.25 mL of Zeocin™ antibiotic (100 mg/ml).

Vector	Amount	Catalog no.
pEM7/Zeo	20 µg, lyophilized in TE, pH 8	V500-20
pTEF1/Zeo	20 µg, lyophilized in TE, pH 8	V503-20
pCMV/Zeo	20 µg, lyophilized in TE, pH 8	V501-20
pSV40/Zeo2	20 µg, lyophilized in TE, pH 8	V504-20

Shipping/Storage

The ZeoCassette™ vectors and Zeocin™ are shipped on dry ice. Upon receipt, store the vector and Zeocin™ at –20°C. Zeocin™ should be stored protected from light.

Zeocin™ Antibiotic

Additional Zeocin™* is available from Life Technologies. It is supplied as a 100 mg/mL solution, 1.25 mL per tube. The antibiotic is stored at –20°C away from the light and is guaranteed for six months. Please refer to the table below for ordering information.

Item	Amount	Catalog no.
Zeocin™	1 gram	R250-01
	5 gram	R250-05

Intended Use

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

Methods

Overview

Introduction

ZeoCassette™ vectors are small DNA plasmids (2.8 to 3.6 kb) that contain a Zeocin™ expression cassette (ZeoCassette™) flanked by 5´ and 3´ polylinkers. The ZeoCassette™ encodes one (or more) promoter(s) and the resistance factor to the antibiotic Zeocin™. In all vectors, expression of the resistance factor is driven by a bacterial promoter (EM7) for expression in *E. coli*. In addition, one vector contains the *Saccharomyces cerevisiae TEF1* promoter upstream of the EM7 promoter for expression in yeast. Two other vectors contain one of two viral promoters (either the SV40 promoter or the CMV promoter) upstream of the EM7 promoter for expression in mammalian cells.

Applications

The ZeoCassette™ vectors can be used for the following applications:

- The ZeoCassette™ vectors can be excised and transferred to the vector of choice to confer resistance to Zeocin™.
- A eukaryotic promoter of choice can be cloned into the 5´ polylinker of pEM7/Zeo to drive expression of the Zeocin™ resistance factor in other systems.
- Any of the vectors can be used as a backbone to develop your own vector of choice.

The table below summarizes the vectors, promoters, and applications. **Note**: Other uses may be possible.

Vector	Prokaryotic Promoter	Eukaryotic Promoter	Application
pEM7/Zeo 2.8 kb	EM7	None	Excise the ZeoCassette™ for use in another vector Clone a eukaryotic promoter of choice upstream of the ZeoCassette™ Use backbone for vector development
pTEF1/Zeo 3.6 kb	EM7	*TEF1*	Excise the ZeoCassette™ for use in another vector Use backbone for vector development
pCMV/Zeo 3.6 kb	EM7	CMV	Excise the ZeoCassette™ for use in another vector Use backbone for vector development
pSV40/Zeo2 3.4 kb	EM7	SV40	Excise the ZeoCassette™ for use in another vector Use backbone for vector development

continued on next page

Zeocin™

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. It is produced as a copper/glycopeptide complex which 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). Zeocin™ shows strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells. As a broad-spectrum antibiotic Zeocin™ is particularly useful, allowing selection in a number of cell types containing vectors with a Zeocin™ resistance gene. For more information on Zeocin™, please call Technical Support (see page 19).

Zeocin™ Resistance

A Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990; Gatignol *et al.*, 1988). 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 both eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

ZeoCassette™ Vectors

Features

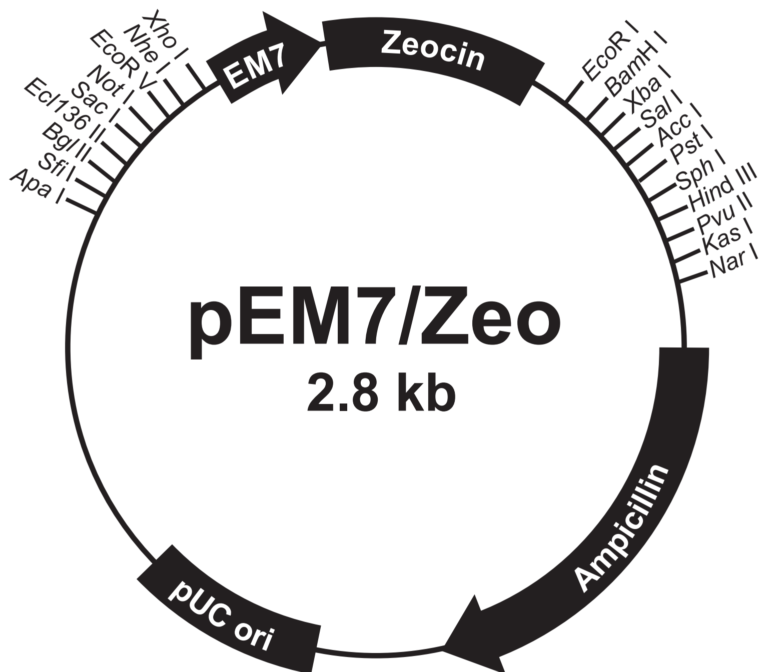
The table below describes the features of the ZeoCassette™ vectors. Features unique to individual vectors are noted.

Features	Function
5´ Polylinker	Allows excision of the ZeoCassette™ or subcloning of a heterologous promoter into pEM7/Zeo.
TEF1 promoter (pTEF1/Zeo only)	Allows expression of the Zeocin™ resistance gene in yeast cells. This promoter drives expression of the *S. cerevisiae* α subunit of translation elongation factor 1 (Cottrelle *et al.*, 1985).
CMV promoter (pCMV/Zeo only)	Allows expression of the Zeocin™ resistance gene in mammalian cells . This is the human CMV immediate-early promoter (Towne strain) without intron A (Stenberg *et al.*, 1984; Thomsen *et al.*, 1984).
SV40 promoter (pSV40/Zeo2 only)	Allows expression of the Zeocin™ resistance gene in mammalian cells. This is the SV40 early enhancer/promoter (Moreau *et al.*, 1981; Southern and Berg, 1982).
EM7 promoter	Synthetic promoter based on the bacteriophage T7 promoter for expression of the Zeocin™ resistance factor in *E. coli*.
Zeocin™ resistance factor (*Sh ble* gene from *Streptoalloteichus hindustanus*)	Allows selection on Zeocin™ in *E. coli*, yeast, plants, and mammalian hosts (see page 8).
SV40 late polyadenylation region (pSV40/Zeo2 and pCMV/Zeo)	Allows efficient mRNA processing and polyadenylation to stabilize message in mammalian cells (Fitzgerald and Shenk, 1981).
3´ Polylinker	Allows s excision of the ZeoCassette™ or subcloning of additional elements.
β-lactamase promoter	Native promoter for expression of β-lactamase.
β-lactamase gene	Confers resistance to the antibiotic ampicillin.
pUC origin	Allows replication, maintenance, and high copy number in *E. coli*.

Map of pEM7/Zeo

Description

pEM7/Zeo is a 2829 bp plasmid that expresses the Zeocin™ resistance factor using the bacterial EM7 promoter. The map below summarizes the features of this vector including the polylinkers which can be used to excise the ZeoCassette™ or clone in additional elements. The sequence of this vector can be obtained by downloading it from our website (**www.lifetechnologies.com**) or by calling Technical Support (see page 19).



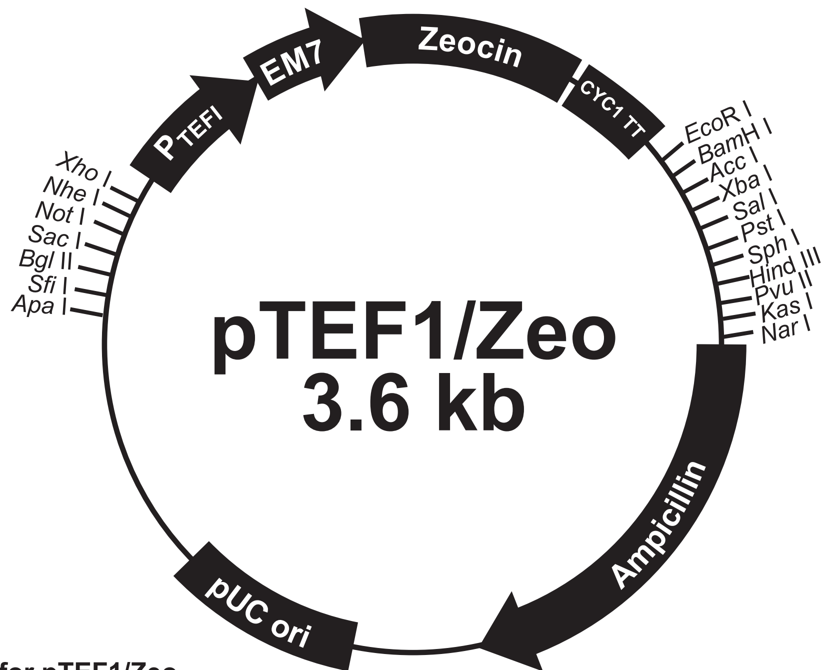
Comments for pEM7/Zeo
2829 nucleotides

bla promoter (-10 region): bases 160-166
Ribosome binding site: bases 189-193
Beta-lactamase ORF: bases 201-1061
pUC origin: bases 1206-1879
5´ Polylinker: bases 1887-1937
EM7 promoter: bases 1942-2008
Sh ble ORF: bases 2009-2383
3´ Polylinker: bases 2384-2593

Map of pTEF1/Zeo

Description

pTEF1/Zeo is a 3556 bp plasmid that expresses the Zeocin™ resistance factor using either the bacterial EM7 promoter or the *S. cerevisiae TEF1* promoter. The map below summarizes the features of this vector including the polylinkers which can be used to excise the ZeoCassette™ or clone in additional elements. The sequence of this vector can be obtained by downloading it from our website (**www.lifetechnologies.com**) or by calling Technical Support (see page 19).



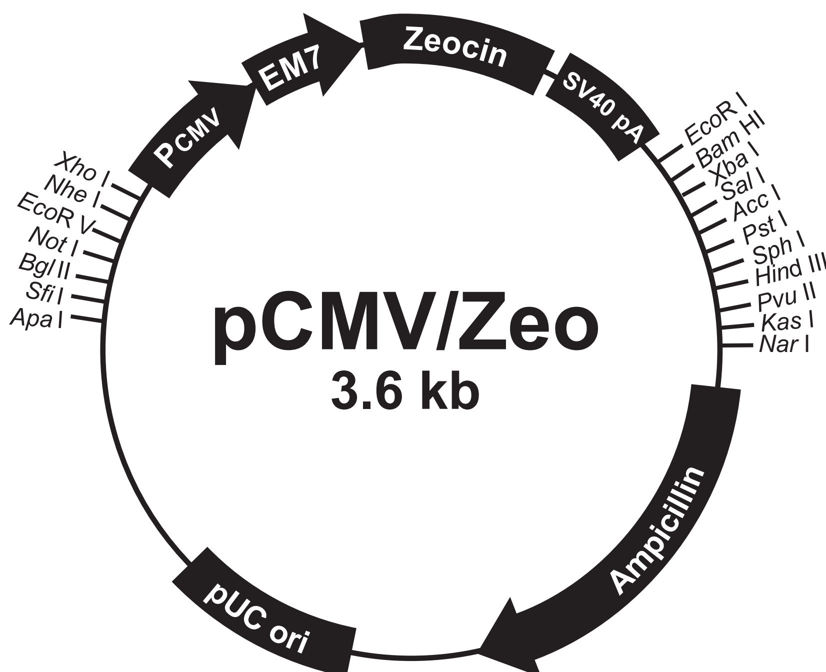
Comments for pTEF1/Zeo
3556 nucleotides

bla promoter: bases 160-166
RBS: bases 189-193
Beta-lactamase ORF: bases 201-1061
pUC origin: bases 1206-1879
5´ Polylinker: bases 1887-1937
TEF1 promoter: bases 1938-2346
EM-7 promoter: bases 2351-2417
Sh Ble ORF: bases 2418-2792
CYC1 transcription termination sequence: bases 2793-3110
3´ Polylinker: bases 3111-3320

Map of pCMV/Zeo

Description

pCMV/Zeo is a 3595 bp plasmid that expresses the Zeocin™ resistance factor using either the bacterial EM7 promoter or the CMV immediate-early promoter. The map below summarizes the features of this vector including the polylinkers which can be used to excise the ZeoCassette™ or clone in additional elements. The sequence of this vector can be obtained by downloading it from our website (**www.lifetechnologies.com**) or by calling Technical Support (see page 19).



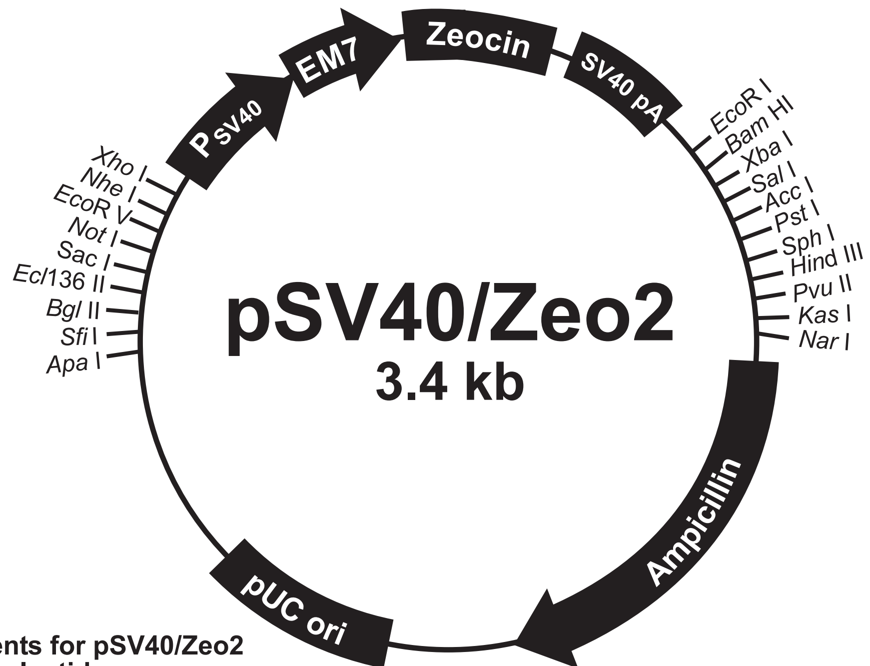
Comments for pCMV/Zeo
3595 nucleotides

bla promoter (-10 region): bases 160-166
Ribosome binding site: bases 189-193
Beta-lactamase ORF: bases 201-1061
pUC origin: bases 1206-1879
5´ Polylinker: bases 1887-1937
CMV promoter: bases 1941-2449
EM7 promoter: bases 2565-2631
Sh ble ORF: bases 2632-3006
SV40 polyadenylation sequence: bases 3020-3101
3´ Polylinker: bases 3150-3359

Map of pSV40/Zeo2

Description

pSV40/Zeo2 is a 3443 bp plasmid that expresses the Zeocin™ resistance factor using either the bacterial EM7 promoter or the SV40 early promoter. The map below summarizes the features of this vector including the polylinkers which can be used to excise the ZeoCassette™ or clone in additional elements. The sequence of this vector can be obtained by downloading it from our website (**www.lifetechnologies.com**) or by calling Technical Support (see page 19)
.



Comments for pSV40/Zeo2
3443 nucleotides

bla promoter (-10 region): bases 160-166
Ribosome binding site: bases 189-193
Beta-lactamase ORF: bases 201-1061
pUC origin: bases 1206-1879
5´ Polylinker: bases 1887-1937
SV40 promoter: bases 1932-2255
EM7 promoter: bases 2290-2345
Sh ble ORF: bases 2364-2738
SV40 polyadenylation sequence: bases 2868-2998
3´ Polylinker: bases 2998-3207

Cloning into the 5´ Polylinker

5´ Polylinker

If you wish to use the ZeoCassette™ vectors as a backbone for developing your own vector, use the diagram below to clone additional elements into the 5´ polylinker. For example, the 5´ polylinker in pEM7/Zeo can be used to clone in a eukaryotic promoter of choice. The sequence and numbering system is the same in all vectors from nucleotide 1 to nucleotide 1880. Differences after nucleotide 1880 are indicated for each vector. Restriction sites are labeled to indicate the cleavage site.

```
1831  ACGCGGCCTT TTTACGGTTC CTGGCCTTTT GCTGGCCTTT TGCTCACATG

                         Apa I  Sfi I      Bgl II    Ecl136 II  Sac I  Not I     EcoR V  Nhe I
pEM7/Zeo:   TGTGCTGGGC CCAGCCGGCC AGATCTGAGC TCGCGGCCGC GATATCGCTA
             Xho I              5´ end of EM7 promoter
            GCTCGAGCAC GTGTTGACAA TTAATCATCG GCATAGTATA TCGGCATAGT

                         Apa I  Sfi I      Bgl II               Sac I  Not I             Nhe I
pTEF1/Zeo:  TGTGCTGGGC CCAGCCGGCC AGATCTGAGC TCGCGGCCGC GATATCGCTA
             Xho I     5´ end of TEF1 promoter
            GCTCGAGCCC ACACACCATA GCTTCAAAAT GTTTCTACTC CTTTTTTACT

                         Apa I  Sfi I      Bgl II                      Not I     EcoR V  Nhe I
pCMV/Zeo:   TGTGCTGGGC CCAGCCGGCC AGATCTGAGC TCGCGGCCGC GATATCGCTA
             Xho I             5´ end of CMV promoter
            GCTCGAGGTC CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG

                    Apa I   Sfi I     Bgl II    Ecl136 II Sac I  Not I     EcoR V   Nhe I  Xho I
pSV40/Zeo2: CTGGGCCCAG CCGGCCAGAT CTGAGCTCGC GGCCGCGATA TCGCTAGCTC
             5´ end of SV40 promoter
            GAGGGTGTGG AAAGTCCCCA GGCTCCCCAG CAGGCAGAAG TATGCAAAGC
```

Cloning into the 3´ Polylinker

3´ Polylinker

If you wish to clone additional elements into the 3´ polylinker, use the diagram below. The sequence of the 3´ polylinker is the same in all vectors, but the numbering is different. Sequences upstream of the 3´ polylinker may be different for each vector and are shown below. Please refer to the sequence for exact numbering. Sequences can be downloaded from our website (**www.lifetechnologies.com**) or by calling Technical Support (see page 19). Restriction sites are labeled to indicate the cleavage site.

pEM7/Zeo:

Sh ble ORF

```
TGC GTG CAC TTC GTG GCC GAG GAG CAG GAC TGA....
Cys Val His Phe Val Ala Glu Glu Gln Asp ***
```

pTEF1/Zeo:

CYC1 transcription termination sequence

```
AGAAGGTTTT GGGACGCTCG AAGGCTTTAA TTTGCAAGCT...
```

pCMV/Zeo:

SV40 polyadenylation sequence

```
GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCT...
```

pSV40/Zeo2:

SV40 polyadenylation sequence

```
TTTGTCCAAA CTCATCAATG TATCTTATCA TGTCT...
```

3´ Polylinker of all ZeoCassette™ vectors:

```
   EcoR I              BamH I    Xba I   Sal I  Acc I        Pst I BspM I Sph I Hind III
...GAATTCC CGGGGATCCT CTAGAGTCGA CCTGCAGGCA TGCAAGCTTG

GCACTGGCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC

                                                Pvu II
CCAACTTAAT CGCCTTGCAG CACATCCCCC TTTCGCCAGC TGGCGTAATA

GCGAAGAGGC CCGCACCGAT CGCCCTTCCC AACAGTTGCG CAGCCTGAAT
      Kas I  Nar I
GGCGAATGGC GCCTGATGCG GTATTTTCTC CTTACGCATC TGTGCGGTAT...
```

Using Zeocin™

Suggested Concentrations 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 *E. coli*, yeast, and mammalian tissue culture cells are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25–50 µg/mL in **low salt** LB medium*
Yeast	25–300 µg/mL depending on species, strain, and 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/L (<90 mM).**

Handling Zeocin™

- **High salt and acidity or basicity reduce the activity of Zeocin™.** Please read the following guidelines for recommendations for your particular organism.

  Bacteria: Salt concentration in bacterial medium can be reduced and the pH adjusted to 7.5 to increase the activity of the drug (see **Low Salt LB Medium,** page 11).

  Yeast: We recommend that you perform a kill curve to determine the concentration of Zeocin™ required to select resistant cells. For selection on YPD, increase the pH from 7.0 to 7.5. By adjusting the pH, significantly less Zeocin™ may be used. Be sure to include a positive and a negative control to ensure selection of resistant cells.

  Mammalian Cell Culture: Perform a kill curve to determine the minimum amount of Zeocin™ necessary to prevent growth. **Note**: For many cell lines, a particular salt concentration is required for normal growth. While salt does not inactivate Zeocin™, it will reduce its activity.
- 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 solutions containing Zeocin™.
- Do not ingest or inhale solutions containing the drug.
- Be sure to bandage any cuts on your fingers to avoid exposure to the drug.

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, do not choose an *E. coli* strain that does contains the Tn*5* gene (i.e. TOP10, DH5, DH10, etc.).

Selection in *E. coli*

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 LB broth and plates using the recipe below.

Failure to lower the salt content of your LB medium will result in non-selection due to inactivation of the drug unless a higher concentration of Zeocin™ is used.

Low Salt LB Medium

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/liter 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 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.

E. coli Transformation

Vectors containing any ZeoCassette™ may be transformed into *E. coli* as follows.

- Transformation may be done by either electroporation or chemical methods. Use your method of choice.
- 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™ and incubate overnight at 37°C.

Note: Electrocompetent and chemically competent cells are available from Life Technologies. We recommend TOP10F´ or similar strain as a general strain for transformation.

Catalog no.	Description	Efficiency	Amount
C3030-03	One Shot® Competent Cells	1×10^8	20 × 50 µl
C665-55	Electrocomp™ TOP10F´	1×10^9	5 × 80 µl

Analysis of Transformants

Plasmid DNA should be isolated from Zeocin™ resistant transformants and analyzed to confirm the resulting construct.

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

Important

After transformation, if you observe the formation of a haze of cells or satellite colonies, increase the Zeocin™ concentration to 50 µg/mL in selective plates.

Selection in Yeast

Introduction

The concentration of Zeocin™ required for selection in yeast varies dramatically depending on the salt **and especially on the pH of the selective medium**. We recommend that you perform a kill curve for each strain and medium combination to find the appropriate concentration of Zeocin™ for selection of resistant cells. Concentrations can range from 25–300 µg/ml.

Note: Increase the pH of YPD from 7.0 to 7.5 to reduce the concentration of Zeocin™ required for selection of resistant cells. Be sure to include a positive and a negative control to evaluate your kill curve.

Note

The *Saccharomyces TEF1* promoter is active in the methylotrophic yeast *Pichia pastoris*.

Important

We do not recommend spheroplasting for transformation of yeast with plasmids containing the Zeocin™ resistance marker. Spheroplasting involves removal of the cell wall to allow DNA to enter the cell. Cells must first regenerate the cell wall before they are able to express the Zeocin™ resistance gene. For this reason, plating spheroplasts directly onto selective medium containing Zeocin™ does not yield any transformants.

Frozen, Competent Yeast

If you wish to prepare frozen, competent yeast cells, two kits are available from Life Technologies.

Kit	Amount	Catalog no.
S.c. EasyComp™ Transoformation Kit	6 reactions	K5050-01
Pichia EasyComp™ Transformation Kit	6 reactions	K1730-01

Lithium Transformation of *Saccharomyces*

Introduction

A protocol to transform *Saccharomyces cerevisiae* using lithium acetate is provided below for your convenience. Other protocols may be suitable.

Materials Needed

Be sure to have the following reagents on hand before starting.

- YPD
- 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5)
- 1X LiAc/0.5X TE (100 mM lithium acetate, 5 mM Tris-HCl, 0.5 mM EDTA, pH 7.5)
- Denatured sheared salmon sperm DNA (Sigma, Catalog no. D9156)
- Plasmid DNA to be transformed
- 1X LiAc/40% PEG-3350/1X TE
- DMSO
- Selective plates containing Zeocin™

Protocol

1. Inoculate 10 mL of YPD with a yeast colony and shake overnight at 30°C.
2. Determine the OD_{600} of your overnight culture. Dilute culture to an OD_{600} of 0.4 in 50 mL of YPD and grow an additional 2–4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 mL 1X TE.
4. Pellet the cells at 2500 rpm and resuspend pellet in 2 mL of 1X LiAc/0.5X TE.
5. Incubate the cells at room temperature for 10 minutes.
6. For each transformation, mix together 1 μg plasmid DNA and 100 μg denatured sheared salmon sperm DNA with 100 μL of the yeast suspension from Step 5.
7. Add 700 μL of 1X LiAc/40% PEG-3350/1X TE and mix well.
8. Incubate solution at 30°C for 30 minutes.
9. Add 88 μL DMSO, mix well, and heat shock at 42°C for 7 minutes.
10. Centrifuge in a microcentrifuge for 10 seconds and remove supernatant.
11. Resuspend the cell pellet in 1 mL YPD and incubate in a 30°C shaker for 1 hour.
12. Centrifuge in a microcentrifuge for 10 seconds and remove supernatant.
13. Resuspend the cell pellet in 1 mL 1X TE and re-pellet.
14. Resuspend the pellet in 50–100 μL TE and plate on the selective plate containing the appropriate amount of Zeocin™.

Electroporation of *Pichia*

Before Starting

You will need the following reagents for transforming *Pichia* and selecting transformants on Zeocin™. **Note**: Inclusion of sorbitol in YPD plates stabilizes electroporated cells as they appear to be osmotically sensitive.

- 5–10 µg linearized vector containing the ZeoCassette™
- YPD Medium
- 50 mL conical polypropylene tubes
- 1 liter cold (+4°C) sterile water (place on ice the day of the experiment)
- 25 mL cold (+4°C) sterile 1 M sorbitol (place on ice the day of the experiment)
- 30°C incubator
- Electroporation device and 0.2 cm cuvettes
- YPDS plates containing 100 µg/mL Zeocin™ (see recipe below)

YPD

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract
2% peptone
2% dextrose (glucose)
1 M sorbitol
2% agar
100 µg/mL Zeocin™

1. Dissolve: 10 g yeast extract
   182.2 g sorbitol
   20 g of peptone

   in 900 mL of water.
2. Add 20 g of agar.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 100 mL of 20% dextrose (filter-sterilize dextrose before use).
5. Cool solution to ~60°C and add 1.0 mL of 100 mg/mL Zeocin™.

Store YPD slants or plates containing Zeocin™ at +4°C in the dark. The shelf life is one to two weeks.

Preparation of *Pichia* for Electroporation

1. Grow 5 mL of your yeast strain in YPD in a 50 mL conical tube at 30°C overnight.
2. Inoculate 500 mL of fresh medium in a 2 liter flask with 0.1–0.5 mL of the overnight culture. Grow overnight again to an OD_{600} = 1.3–1.5.
3. Centrifuge the cells at 1500 × *g* for 5 minutes at +4°C. Resuspend the pellet with 500 mL of ice-cold (0°C), sterile water.
4. Centrifuge the cells as in Step 3, then resuspend the pellet with 250 mL of ice-cold (0°C), sterile water.
5. Centrifuge the cells as in Step 3, then resuspend the pellet in 20 mL of ice-cold (0°C) 1 M sorbitol.
6. Centrifuge the cells as in Step 3, then resuspend the pellet in 1 mL of ice-cold (0°C) 1 M sorbitol for a final volume of approximately 1.5 ml. Keep the cells on ice and use that day. Do not store cells.

continued on next page

Electroporation of *Pichia*, continued

Transformation by Electroporation

1. Mix 80 µL of the cells from Step 6 (previous page) with 5–10 µg of linearized DNA (in 5–10 µL sterile water) and transfer them to an ice-cold (0°C) 0.2 cm electroporation cuvette.
2. Incubate the cuvette with the cells on ice for 5 minutes.
3. Pulse the cells according to the recommended parameters for *Pichia* as suggested by the manufacturer of the specific electroporation device being used.
4. Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transfer the cuvette contents to a sterile 15 mL tube.
5. Let the tube incubate at 30°C without shaking for 1–2 hours.
6. Spread 10, 25, 50, 100, and 200 µL each on separate, labeled YPDS plates containing 100 µg/mL Zeocin™.
7. Incubate plates for 2–3 days at 30°C until colonies form.
8. Pick 10–20 colonies and purify (streak for single colonies) on fresh YPDS plates containing 100 µg/mL Zeocin™.
9. Analyze the colonies for the desired phenotype or by PCR to confirm the presence of the insert.

Lithium Transformation of *Pichia*

Introduction

This is a modified version of the procedure described for *S. cerevisiae* (Gietz and Schiestl, 1996). This protocol is provided as an alternative to transformation by electroporation. Transformation efficiency is between 10^2 to 10^3 cfu/µg of linearized DNA.

Preparation of Solutions

Lithium acetate does not work with *Pichia pastoris*. Use only lithium chloride.

1 M LiCl in distilled, deionized water. Filter sterilize. Dilute as needed with sterile water.

50% polyethylene glycol (PEG-3350) in distilled, deionized water. Filter sterilize. Store in a tightly capped bottle.

2 mg/mL denatured, sheared salmon sperm DNA in TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA). Store at –20°C.

Preparation of Cells

1. Grow a 50 mL culture of *Pichia pastoris* in YPD at 30°C with shaking to an OD_{600} of 0.8–1.0 (approximately 10^8 cells/ml).
2. Harvest the cells and wash with 25 mL of sterile water and centrifuge at 1500 × *g* for 10 minutes at room temperature.
3. Decant the water and resuspend the cells in 1 mL of 100 mM LiCl.
4. Transfer the cell suspension to a 1.5 mL microcentrifuge tube.
5. Pellet the cells at maximum speed for 15 seconds and remove the LiCl with a pipet.
6. Resuspend the cells in 400 µL of 100 mM LiCl.
7. Dispense 50 µL of the cell suspension into a 1.5 mL microcentrifuge tube for each transformation and use immediately. **Do not store on ice or freeze at –20°C.**

Transformation

1. Boil a 1 mL sample of denatured salmon sperm DNA for five minutes, then quickly chill in ice water. Keep on ice. **Note**: It is not necessary nor desirable to boil the carrier DNA prior to each use. Store a small aliquot at –20°C and boil every 3–4 times the DNA is thawed.
2. Centrifuge the LiCl cell solution from Step 7, above. Remove the LiCl with a pipet.
3. For each transformation sample, add the following reagents to the cells IN THE ORDER GIVEN. PEG shields the cells from the detrimental effects of the high concentration of LiCl.

   240 µL 50% PEG
   36 µL 1 M LiCl
   25 µL 2 mg/mL denatured salmon sperm DNA
   Plasmid DNA (5–10 µg) in 50 µL sterile water
4. Vortex each tube vigorously until the cell pellet is completely mixed (~1 minute).
5. Incubate the tube at 30°C for 30 minutes without shaking.
6. Heat shock in a water bath at 42°C for 20–25 minutes.
7. Centrifuge the tubes at 6000–8000 rpm and use a pipet to remove the supernatant.
8. Resuspend the pellet in 1 mL of YPD and incubate at 30°C with shaking.
9. After 1 hour and 4 hours of incubation, plate 25–100 µL of culture on YPD plates containing 100 µg/mL Zeocin™. Incubate the plates for 2–3 days at 30°C.

Selection in Mammalian Cells

Introduction

It is always important to test the sensitivity of your host cell to Zeocin™ as natural resistance varies among cell lines. We recommend performing a 'kill curve' to identify the minimal amount of Zeocin™ that will prevent growth of your host.

Determination of Zeocin™ Sensitivity

To obtain a stable integrant, you need to determine the minimal concentration of Zeocin™ required to prevent growth of the parental cell line in the desired cell culture medium. Use the guidelines below to perform a 'kill curve'.

1. Plate or split a confluent plate so there are approximately 2.5×10^5 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. **Note**: Other culture vessels may be used. Remember to adjust the number of cells as necessary.
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. Selection will take about 10–14 days. **Note:** The killing action of Zeocin™ is not the same as G418. See note on page 18.

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 your vector.

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* (Ausubel *et al.*, 1994).

There are a variety of methods available for mammalian cell transfection. Life Technologies offers the Calcium Phosphate Transfection Kit (Catalog no. K2780-01) for mammalian transfection and a large selection of cationic lipid reagents for lipid-mediated transfection. For more information, visit our website (**www.lifetechnologies.com**) or contact Technical Support (see page 19).

continued on next page

Selection in Mammalian Cells, continued

Selection of Stable Integrants

Once you have determined the appropriate Zeocin™ concentration to use, you can generate a stable cell line with your construct. Transfection method and conditions will vary from cell line to cell line. The guidelines below are for transfection in a 100 mm plate. Other culture vessels may be used.

1. Transfect 10^6 cells with 20 µg of vector using the desired protocol. 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, add fresh medium containing Zeocin™ at the pre-determined concentration required for your cell line.
4. Feed the cells with selective medium every 3–4 days until foci can be identified.
5. When Zeocin™ resistant foci are formed, select 40–50 foci using either cloning rings (if the foci are isolated and large enough) or a pipette tip (if the foci are small) and transfer 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.

Troubleshooting

No Stable Transfectants Selected

Try the ideas below if you are unsuccessful isolating stable transfectants.

1. If you excised the SV40 ZeoCassette™ or the CMV ZeoCassette™ and subcloned it into another vector, try transfecting your cell line with the original parent vectors pSV40/Zeo2 or pCMV/Zeo as a positive control.
2. If the parent vectors fail to confer Zeocin™ resistance to your cell line, it may be that the particular promoter is not active in your cell line. You may try subcloning the Zeocin™ resistance gene behind another eukaryotic promoter that you know works in your cell line and testing for Zeocin™ resistance.
3. Check transfection efficiencies. Perform a transient transfection with a reporter construct to verify the transfection efficiency of your cell line.
4. Linearize construct before transfection. Integration may be occurring at the *Sh ble* gene.

Note

For some cell lines, the killing action of Zeocin™ will not appear to be like G418. The cells may not come up off the plate and it may appear as if the cells have stopped dividing. Cells can be treated with trypsin/EDTA, re-plated in non-selective medium, and incubated for a few hours. During this time, only the resistant cells will reattach. Selective medium can then be added to resume selection.

Appendix

Technical Support

Obtaining support

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

At the website, you can:

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

Safety Sata Sheets (SDS)

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

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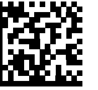

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