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Zero Background™/Kan Cloning Kit

Zero Background™ Cloning Kit with Selection on Kanamycin

Catalog no. K2600-01

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Important Information

Shipping/Storage

The Zero Background™/Kan Cloning Kit is shipped on dry ice. Upon receipt, store at -20°C. The *E. coli* stab is shipped and should be stored at room temperature.

Kit Contents

The Zero Background™/Kan Cloning Kit contains the following reagents:

Item	Concentration	Storage
pZErO™-2 vector, supercoiled, 25 µg	1 µg/µl in TE Buffer, pH 7.5	-20°C
Sterile water, 1 ml	Nuclease-free water	-20°C
10X Ligation Buffer, 100 µl	60 mM Tris-HCl, pH 7.5 60 mM $MgCl_2$ 50 mM NaCl 1 mg/ml bovine serum albumin 70 mM β-mercaptoethanol 1 mM ATP 20 mM dithiothreitol 10 mM spermidine	-20°C
T4 DNA Ligase, 25 µl	4.0 Weiss units/µl	-20°C
TE Buffer, 2. ml	10 mM Tris-HCl, pH 7.5 1 mM EDTA	-20°C
Test Inserts, Blunt Ended ΦX174 *Hae* III DNA, 10 µl	20 ng/µl in TE buffer, pH 7.5	-20°C
TOP10 cells, 1 stab	--	Room Temperature

Genotype of TOP10

TOP10: F^- *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ*(ara-leu)*7697 *gal*U *gal*K *rps*L *end*A1 *nup*G

Introduction

Overview

Introduction

This kit is designed to clone cohesive or blunt-ended DNA fragments with a low background of non-recombinants. The technology described here is based on vectors containing the lethal *E. coli* gene, *ccd*B (Bernard, *et al.*, 1994). The cloning vector, pZErO™-2, contains the *ccd*B gene fused to the C-terminus of LacZα. Insertion of a DNA fragment disrupts expression of the *lacZα-ccd*B gene fusion permitting growth of only positive recombinants. Cells that contain non-recombinant vector are killed. The vector also contains the kanamycin resistance gene for selection in *E. coli*; the f1 origin of replication for single-strand rescue; a versatile multiple cloning site with 17 unique sites, flanking Sp6 and T7 promoter/priming sites for *in vitro* transcription and sequencing; and all of the M13 universal primer sites for sequencing.

Description of System

The pZErO™-2 vector allows direct selection of inserts via disruption of a lethal gene. Very high cloning efficiencies (~95%) are often achieved without the need for exotic strains, X-Gal, calf intestinal phosphatase (CIP), or other components. *ccd*B, which inhibits growth of transformed cells containing non-disrupted *ccd*B, is constitutively expressed in cells that do not carry the *lac*I^q gene.

Important

E. coli TOP10 is the recommended host strain for pZErO™-2. Because this strain does not contain a *lac*I^q gene, the *ccd*B gene will be constitutively expressed without the need for IPTG induction.

Strains that contain an F plasmid are not recommended for transformation and selection of recombinant clones. The F plasmid encodes the CcdA protein which acts as an inhibitor of the CcdB protein (see the next page for a more detailed explanation).

Any *E. coli* strain that contains the complete Tn*5* transposable element (i.e. DH5αF´IQ, SURE, SURE2) encodes the kanamycin resistance gene. For the most efficient selection it is highly recommended that you choose an *E. coli* strain that does not contain the Tn*5* gene (e.g. TOP10).

Do not use INVαF´ cells. The transformation efficiency of INVαF´ is very low using pZErO™-2 and selection on kanamycin.

Mechanism of Action of the CcdB Protein

The CcdB protein acts by poisoning bacterial DNA-gyrase (topoisomerase II), an essential enzyme that catalyzes the ATP-dependent negative supercoiling of DNA. DNA gyrase acts by creating a transient double-strand nick in the DNA substrate, passing the DNA helix through the break to decrease the linking number, and then resealing the nick. During the breaking-resealing reaction, the 5´ phosphate termini are covalently linked to a tyrosine residue in the A subunit of DNA gyrase (*gyr*A). This gyrase-DNA intermediate is called the cleavable complex. The CcdB protein has been shown both *in vivo* (Bernard and Couturier, 1992) and *in vitro* (Bernard, *et al.*, 1993) to poison the cleavable complex by inhibiting the resealing of the double-strand nick in the DNA. This causes DNA breakage, activation of the SOS response, and cell death.

Continued on next page

Overview, continued

The *ccd*B Gene

The *ccd*B gene is found in the *ccd* (control of cell death) locus on the F plasmid. This locus contains two genes, *ccd*A and *ccd*B, which encode proteins of 72 and 101 amino acids respectively (Karoui, *et al.*, 1983; Ogura and Hiraga, 1983; and Miki, *et al.*, 1984). The *ccd* locus participates in stable maintenance of F plasmid by post-segregational killing of cells that do not contain the F plasmid (Jaffé, *et al.*, 1985). The CcdB protein is a potent cell-killing protein when its action is not inhibited by the CcdA protein. The half-life of the CcdA protein is shorter than that of the CcdB protein. Persistence of the CcdB protein leads to death of bacterial segregants that do not contain the F plasmid. Overexpression of the CcdB protein causes cell death when its action is not prevented by sufficient CcdA protein (Van Melderen, *et al.*, 1994).

pZErO™-2 Vector

Description of pZErO™-2

pZErO™-2 is a 3297 bp cloning vector that allows direct selection of positive recombinants via disruption of the lethal gene, *ccd*B. Expression of *ccd*B results in the death of cells containing non-recombinant vector. The product of the *ccd*B gene is actually expressed as a fusion protein to the LacZα peptide fragment. This was done to exploit the proven disruption technology of LacZα and to include all of the universal M13 forward and reverse priming sites.

Features of pZErO™-2

The important elements of pZErO™-2 are described in the following table. The pZErO™-2 vector has been completely sequenced and all features have been functionally tested. If you suspect an error in the sequence, please contact our Technical Service Department (page 23).

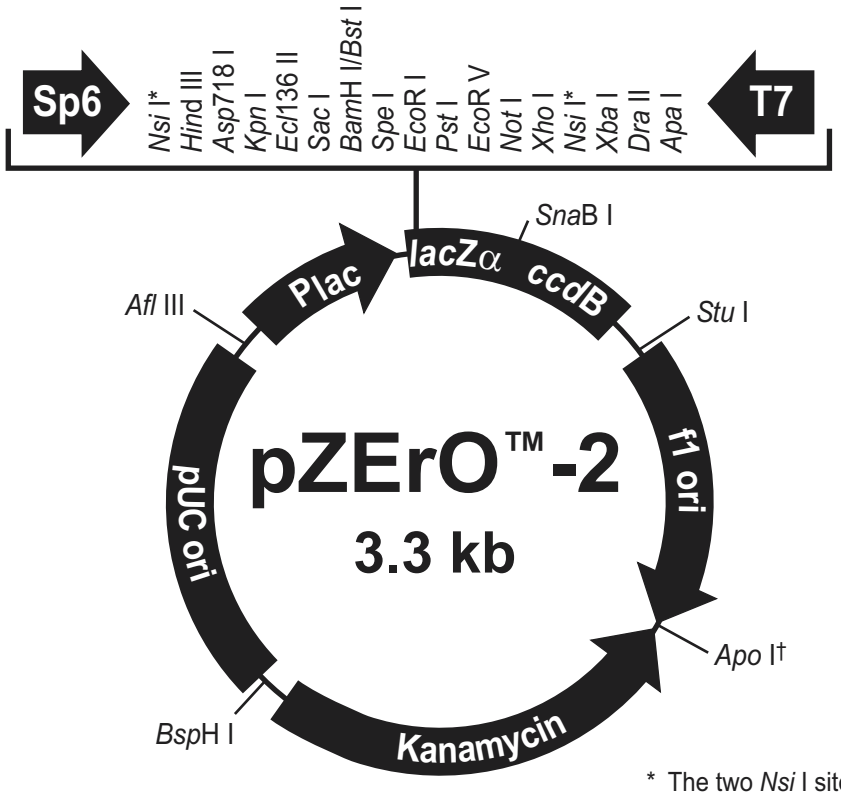
Features	Function
Promoter (P_{lac})	Allows inducible expression of *lac*Zα-*ccd*B gene fusion
M13 Reverse priming site	Permits sequencing of your insert
Sp6 promoter/priming site	Allows *in vitro* transcription of the sense strand and sequencing of your insert
Multiple Cloning Site (17 unique sites)	Permits insertion of desired gene to disrupt expression of the *ccd*B gene
*lac*Zα-*ccd*B fusion gene	Provides lethal selection against non-recombinant vector Utilizes the LacZα disruption technology to clone inserts Includes all the universal M13 forward and reverse priming sites for sequencing
T7 promoter/priming site	Allows *in vitro* transcription of the anti-sense strand and sequencing of your insert
M13 Forward (-20,-40) priming sites	Permit sequencing of your insert
f1 origin	Allows isolation of single-stranded DNA from *E. coli* strains containing the F´
Kanamycin resistance gene	Provides resistance to the antibiotic kanamycin. Derived from the bacterial transposon Tn*5*
pUC origin	Allows high copy replication and maintenance of the plasmid in *E. coli*.

Continued on next page

pZErO™-2 Vector, continued

Map of pZErO™-2

The figure below summarizes the features of the pZErO™-2 vector. The sequence is available for downloading from our Web site (http://www.invitrogen.com) or from Technical Service (page 23). Details of the multiple cloning site are shown on page 8.



Comments for pZErO™-2
3297 nucleotides

Lac Promoter/Operator Region: bases 95-216
M13 Reverse Priming Site: bases 205-221
*Lac*Zα ORF: bases 217-558
Sp6 Promoter/Priming Site: bases 239-256
Multiple Cloning Site: bases 269-381
T7 Promoter/Priming Site: bases 388-407
M13 (-20) Forward Priming Site: bases 415-430
M13 (-40) Forward Priming Site: bases 434-450
Fusion Joint: bases 559-567
*ccd*B Lethal Gene ORF: bases 568-870
f1 origin: bases 895-1307
Kanamycin Resistance ORF: bases 2116-1322 (C)
pUC origin: bases 2502-3175

* The two *Nsi* I sites in the MCS are the only sites in the vector.

† There are two tandem *Apo* I sites at this location. *Apo* I also recognizes the *Eco*R I site.

Methods

FastStart

Introduction

The procedure below is designed to get you quickly started with the Zero Background™/Kan Cloning Kit. Information is provided elsewhere in the manual if you need help with any of the steps.

Before Starting

1. Prepare LB plates containing 25-50 µg/ml kanamycin (see page 16). Store at +4°C.

   If you are using a cell line that contains the *lac*I^q gene (i. e. TOP10F´, DH5αF´), include 1 mM IPTG in the plating medium to achieve complete induction.
2. Prepare LB or SOB medium containing 25-50 µg/ml kanamycin liquid medium for DNA minipreps.
3. Prepare or purchase (see page 12) either chemically competent or electrocompetent TOP10 cells. See pages 19-22 for protocols to prepare competent cells. The minimal efficiencies required are 1 x 10^8 cfu/µg DNA for chemically competent cells and 1 x 10^9 cfu/µg DNA for electrocompetent cells.
4. Determine a cloning strategy for ligation into pZErO™-2. See the detail of the multiple cloning site (page 8) for help.

Ligation into pZErO™-2

Be sure to include a "no DNA", a "cells only", and linearized vector only controls.

1. Digest 1 µg each of pZErO™-2 supercoiled vector (1 µl) and your DNA in total volumes of 10 µl using the recommended buffer, temperature, and reaction conditions described by the manufacturer of the restriction enzyme. Optimal digestion time is 10 to 30 minutes using 10-20 units of enzyme. **DO NOT digest longer than 30 minutes.**
2. Inactivate the restriction enzyme(s) by heating the reaction at 65°C for 10-20 minutes or by phenol extraction. Dilute the cut vector to 10 ng/µl with TE buffer (provided). **If the enzyme is not heat inactivated** (i.e. *Eco*R V), you will need to phenol-extract the enzyme. Ethanol precipitate the DNA and resuspend it in 90 µl of TE buffer. We recommend that you use the linearized DNA immediately. The DNA can be stored at -20°C for 1 to 2 weeks but the cloning efficiency may decrease.
3. Set up the ligation reaction. You will need enough insert for a 2:1 insert:vector molar ratio if performing a cohesive-end ligation, or use a 10:1 insert:vector molar ratio if performing a blunt-end ligation. See page 10 to determine how much insert you need. Set up the following 10 µl ligation reaction:

Digested vector (~10 ng)	1 µl
Digested DNA insert	x µl
Sterile water	to 8.5 µl
10X Ligation Buffer (with ATP)	1 µl
T4 DNA Ligase (4 U/µl)	0.5 µl
Total Volume	10 µl

4. Incubate at 16°C for 30 minutes (if performing a cohesive-end ligation) or 60 minutes (if performing a blunt-end ligation). Do not ligate at room temperature or exceed 1 hour for ligation and do not add PEG to the ligation reaction. Increased background levels have been observed under these conditions. Proceed to **Transformation**, next page.

Continued on next page

FastStart, continued

Transformation

For more information on chemical transformation or electroporation, please see pages 13-14.

1. Add 2 µl of each ligation reaction to a separate tube of competent cells (40 to 50 µl) and transform using your method of choice.
2. Plate 10 to 100 µl of each transformation mix on LB-kanamycin plates. Let the liquid absorb, invert, and incubate at 37°C for 18 to 24 hours. Proceed to **Analysis of Transformants**, below.

Analysis of Transformants

1. Remove plates from the incubator. Pick at least 10 kanamycin resistant transformants and inoculate into 2 ml SOB medium containing 25-50 µg/ml kanamycin. Grow 6-8 hours or overnight at 37°C.
2. Isolate plasmid DNA by miniprep for restriction analysis or sequencing using the M13 forward and reverse primers. Remember to isolate a single colony and reconfirm the presence of insert before making a glycerol stock.
3. Prepare a glycerol stock of your desired clone for safekeeping by combining 0.85 ml of a mid-log bacterial culture with 0.15 ml of sterile 100% glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at -80°C.
4. Once the desired clone is isolated, you may proceed with further subcloning and/or analysis of your insert.

Cloning into pZErO™-2

Introduction

Selection of cloned inserts using the Zero Background™ technique is extremely powerful; however, because of the nature of selection, do not propagate the vector in common laboratory strains. We have developed ligation and transformation procedures to optimize the use of the pZErO™-2 vector provided in this kit. The kit contains enough vector for ~25 restriction digestions and ~2000 ligations.

Before Starting

Users should be familiar with basic molecular biology and microbiological techniques. For information on these topics, please see Ausubel, *et al.*, 1990 or Sambrook, *et al.*, 1989.

1. Prepare LB plates containing 25-50 µg/ml kanamycin (see page 16). Store at +4°C. If you are using cells that carry the *lac*I^q gene, include 1 mM IPTG in the plating medium to achieve complete induction.
2. Prepare LB or SOB medium containing 25-50 µg/ml kanamycin liquid medium for DNA minipreps.
3. Prepare or purchase (see page 12) either chemically competent or electrocompetent TOP10 cells. See pages 19-22 for protocols to prepare competent cells. The minimal efficiencies required are 1 x 10^8 cfu/µg DNA for chemically competent cells and 1 x 10^9 cfu/µg DNA for electrocompetent cells.
4. Determine a cloning strategy for ligation into pZErO™-2. See the detail of the multiple cloning site (next page) for help.

Important

The LacZ/CcdB fusion protein can be made nonlethal by minor changes in the peptide sequence within the multiple cloning site. Disruption of the LacZ/CcdB fusion protein can occur by frameshift, addition of stop codons, or by alterations in the fusion peptide sequence. We recommend the following precautions to avoid isolation of non-recombinants.

Dephosphorylation of pZErO™-2 is not required or recommended.

Do not overdigest (> 30 minutes) with restriction enzymes and take precautions to prevent nuclease contamination. Any problems with high background (self-ligation of the vector that produces colonies) are generally caused by low levels of exonuclease contamination in your restriction enzyme digest, ligation reaction, or the solution containing your insert. Use the buffers and water provided in the kit. If you need to use your own materials, all buffers and water used should be autoclaved extensively or boiled for 5-10 minutes in a microwave oven.

Exonuclease digestion of vector ends may cause a frameshift mutation resulting in disruption of the *lacZ*α-*ccd*B gene if the vector self-ligates. This results in a high background of non-recombinants. We recommend using the highest quality restriction enzymes for digestion of pZErO™-2.

Isolation and Purification of Inserts

For best results, phenol-extract PCR reactions and precipitate the DNA before ligating PCR products into pZErO™-2.

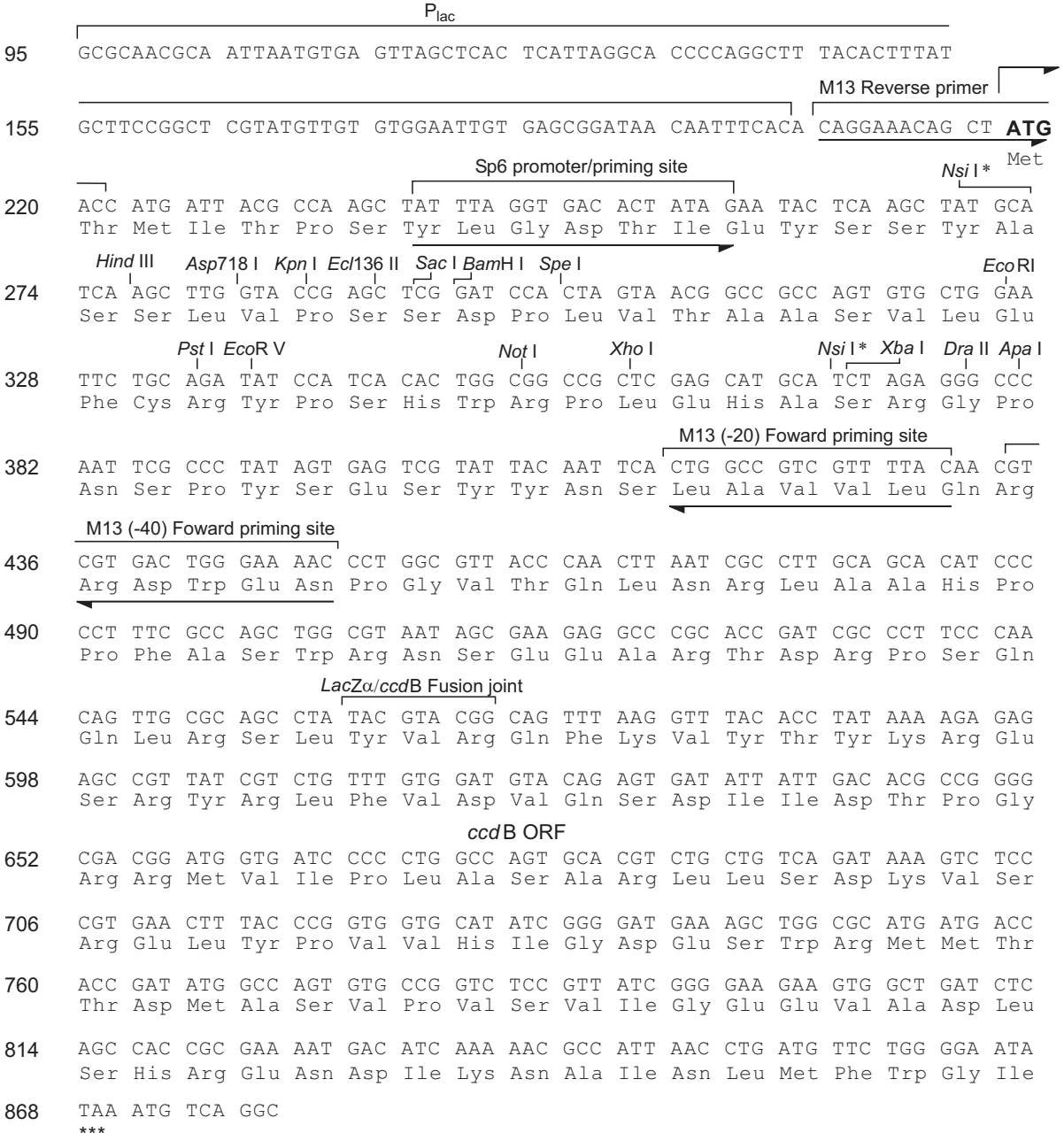
If you gel-purify your insert before ligating into pZErO™-2, you may see a higher background of colonies without insert because of nucleases contamination. To reduce nuclease contamination, do not use communal ethidium bromide baths, use solutions that are free of nucleases, and use high quality agarose. We have found that either electroeluting or using glassmilk works the best to isolate DNA fragments from gels.

Continued on next page

Cloning into pZErO™-2, continued

Detail of the Multiple Cloning Site

Below is a diagram of the multiple cloning site and surrounding sequences. Restriction sites are marked to indicate the cleavage site. Cloning into the multiple cloning site disrupts expression of the *ccd*B gene. The multiple cloning site has been sequenced.



*The two *Nsi* I sites in the polylinker are the only *Nsi* I sites in the vector.

Continued on next page

Cloning into pZErO™-2, continued

Ligation into pZErO™-2

Be sure to include a "no cells", a "cells only", and linearized vector controls.

1. Digest 1 µg each of pZErO™-2 supercoiled vector (1 µl) and your DNA in total volumes of 10 µl for 15-30 minutes using the recommended buffer, temperature, and reaction conditions described by the manufacturer of the restriction enzyme. **DO NOT OVERDIGEST.**
2. To ligate blunt fragments, digest pZErO™-2 with 10-20 units of *Eco*R V for 15-30 minutes. Ligations can be optimized by adjusting insert:vector quantities to a molar ratio of 2:1 for a cohesive-end ligation and 10:1 for a blunt-end ligation. Adjust digestion quantities accordingly.

   For enzymes that are inactivated by heat, add TE buffer to a final volume of 100 µl and heat reaction to 70°C for 10 minutes. Cool to room temperature, then place reaction on ice. The final concentration of the reaction will be 10 ng/µl. Allow the reaction to cool at room temperature.

   You may use the enzyme digestion directly in the ligation reaction (Step 5) unless the enzyme is not fully inactivated by heat (see Step 3).
3. **If the enzyme(s) are not fully inactivated by heat** (e.g. *Bam*H I, *Eco*R V, *Kpn* I, *Pst* I), extract the digest with 10 µl phenol/chloroform, precipitate the DNA with 1/10 volume 3 M sodium acetate, pH 5.6, and 2 volumes 100% ethanol, centrifuge, and carefully wash the pellet with 80% ethanol. Air dry the pellet and resuspend in 90 µl of TE buffer. Assuming 90% recovery, the DNA concentration will be 10 ng/µl.
4. To verify complete digestion and recovery, run a 20 µl aliquot of the digestion on a 1% agarose gel.
5. If you are ligating cohesive ends, you will need enough insert for a 2:1 insert:vector molar ratio. If you are ligating blunt ends, increase the insert:vector molar ratio to 10:1. See page 10 to determine how much insert you need. Set up the following 10 µl ligation reaction using the reagents supplied with the kit:

Digested vector (~10 ng)		1 µl
Digested DNA insert		x µl
Sterile water	to a final volume of	8.5 µl
10X Ligation Buffer (with ATP)		1 µl
T4 DNA Ligase (4 U/µl)		0.5 µl
Total Volume		10 µl

6. Incubate at 16°C for 30 minutes (cohesive-end ligations) or 60 minutes (blunt-end ligations). **Do not ligate at room temperature.** Place vials on ice. Proceed to **Transformation**, page 12.

Continued on next page

Cloning into pZErO™-2, continued

Calculation of Molar Ratios

To clone your insert into pZErO™-2, you will need to know the concentration of your insert DNA solution. This may be determined by OD_{260}, agarose gel electrophoresis, fluorescence, or using the DNA Dipstick™ Kit from Invitrogen (Catalog no. K5632-01). The concentration is needed to calculate the volume required to achieve a particular molar ratio of vector to insert.

1. Determine the concentration of insert in µg/ml.
2. Use the following formula to calculate the amount of insert needed to give a molar ratio of 2:1* between insert and linearized pZErO™-2. Note that the amount of pZErO™-2 is 10 ng.

$$\text{x ng insert} = \frac{(2)\ (\text{bp insert})\ (10\text{ ng linearized pZErO}^{\text{™}}\text{-2})}{(3297\text{ bp pZErO}^{\text{™}}\text{-2})}$$

   * If you are performing a blunt-ended ligation, calculate the insert:vector molar ratio to be 10:1 by replacing the 2 with a 10 in the above equation.

3. Based on the calculation above, calculate the volumes needed for the ligation reaction.

General Guidelines for Control Reactions

The table below gives some suggestions for possible control reactions for the experiments presented in this manual. It is useful to have control data to evaluate your experiments or if you need to contact Invitrogen Technical Service for assistance.

Experiment	Control	Reason
Ligation and Transformation	No DNA	Checks for contamination of ligation reagents.
	Linearized vector only	Checks for nuclease contamination in your reactions. A frameshift mutation will disrupt *ccdB* function, resulting in a high background of colonies that will not contain insert.
	Cells only	Checks for the presence of antibiotic in the plates and contamination of competent cells and SOC medium.
	Supercoiled vector	Checks the efficiency of the competent cells.
	Test Inserts (see next page)	Checks general ligation conditions. Confirms disruption of *ccd*B expression.

Continued on next page

Cloning into pZErO™-2, continued

Using the Test Inserts

The test inserts are provided to check the general ligation conditions and confirm the lack of *ccdB* function when its expression is disrupted. Briefly, pZErO™-2 is digested with *Eco*R V and the *Hae* III digested ΦX174 DNA ligated into the vector. Ligation mixture is transformed into competent TOP10 cells and plated onto LB-kanamycin plates.

1. Digest 1 µg of pZErO™-2 with *Eco*R V (10 units) for 15-30 minutes.
2. **Note: *Eco*R V is not heat inactivated**. Extract digested vector with phenol/chloroform and precipitate the DNA in the aqueous layer with 1/10 volume 3 M sodium acetate pH 5.6 and 2 volumes of ethanol. Carefully wash pellet with 80% ethanol and air dry.
3. Resuspend the DNA pellet in 90 µl TE buffer.
4. Set up the following 10 µl ligation reaction:

Digested vector (~10 ng)	1 µl
Test inserts, blunt-ended ΦX174 DNA (20 ng/µl)	1 µl
Sterile water	6.5 µl
10X ligation buffer	1 µl
T4 DNA Ligase (4 U/µl)	0.5 µl
Total Volume	10 µl

5. Set up a "vector only" ligation reaction:

Digested vector (~10 ng)	1 µl
Sterile water	7.5 µl
10X ligation buffer	1 µl
T4 DNA Ligase (4 U/µl)	0.5 µl
Total Volume	10 µl

6. Ligate at 16°C for 1 hour. Proceed to **Transformation**, pages 12-14. Use 2 µl of the ligation reaction to transform TOP10 cells and plate 10 µl of each transformation reaction.

The number of transformants per plate varies from 50 to 2000 colonies per 10 µl using competent TOP10 cells. The cloning efficiency should be ~95%. Since neither the *Hae* III or the *Eco*R V sites are regenerated, inserts may be released with *Nsi* I or a combination of other enzymes. Digestion of a recombinant plasmid will release one of 11 possible ΦX174 DNA fragments (bp): 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, or 1353. Note that tandem inserts may occur. This will not affect disruption of *ccd*B expression. ΦX174 DNA has no sites for *Apa* I, *Bam*H I, *Eco*R I, *Hin*d III, *Kpn* I, *Not* I, *Nsi* I, *Spe* I, or *Xba* I.

Determining Cloning Efficiency

To determine the cloning efficiency, compare the number of colonies produced in the test insert ligation (10 µl plate) to the total number of colonies seen on the 10 µl test insert ligation plate plus the number of colonies on the 10 µl vector only plate.

$$\text{cloning efficiency} = \frac{\text{(colonies on test insert plate)}}{\text{(colonies on test insert plate) + (colonies on vector only plate)}} \times 100\%$$

Transformation

Introduction

At this point you have ligation reactions which you will transform by chemical means or electroporation into competent TOP10. After transformation, the cells will be plated onto LB-kanamycin plates (see **Recipes**, page 16) and incubated for 24 hours. Kanamycin resistant colonies are then analyzed by DNA miniprep and restriction mapping to find the desired clones.

If you prepare your own competent cells, please follow the transformation procedures on page 13.

Chemically competent (One Shot® TOP10 competent cells) and electrocompetent (Electrocomp™) TOP10 cells are available from Invitrogen for your convenience.

Item	Amount	Efficiency	Catalog no.
One Shot® TOP10	21 x 50 µl	1 x 10^9	C4040-03
Electrocomp™ TOP10	5 x 80 µl	1 x 10^9	C664-55
Electrocomp™ TOP10	10 x 80 µl	1 x 10^9	C664-11

Controls

See the section on **Control Reactions** (page 10) to determine which controls you wish to include.

General Handling

Be extremely gentle when working with competent cells. Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Transformation should be started immediately following the thawing of the cells on ice, and all mixing should be done by stirring with a pipet tip, not by pipetting.

Important

If you are using cells that carry a *lac*I^q gene, IPTG is required to induce expression from the *lac* promoter. Be sure to include IPTG in the agar medium at a final concentration of 1 mM. **Do not spread IPTG on the plate.**

Continued on next page

Transformation, continued

Before Starting

- Equilibrate a water bath to 42°C
- Warm one vial of SOC medium to room temperature
- Place an appropriate number of 10 cm diameter LB-kanamycin agar plates in a 37°C incubator to remove excess moisture (use one plate for each transformation).
- Obtain a test tube rack (float) that will hold all transformation tubes so that they all can be put into a 42°C water bath at once.

Transformation Methods

1. If transforming by electroporation, you need an electroporation device; sterile, glass transfer pipettes or pipette tips; electroporation cuvettes; sterile, 15 ml, polypropylene snap-cap tubes; and SOC medium. Electroporation cuvettes are available from Invitrogen. Contact Technical Service for more information (page 23).
2. Determine the total number of transformations, including controls. You will need two LB-kanamycin plates per ligation/transformation.
3. If you wish to test the transformation efficiencies of your cells, prepare a stock solution (10 pg/µl) of any supercoiled plasmid (e.g. pUC19, pBR322) to use as a control for transformation. Be sure to have appropriate antibiotic plates on hand to test the transformation efficiency of your competent cells.
3. Make sure the SOC medium is at room temperature.

Chemical Transformation

You will need to prepare additional SOC. Please refer to the recipe on page 16.

1. Equilibrate a water bath or heat block to 42°C. Remove the appropriate number of tubes of frozen TOP10 chemically competent cells (50 µl each) and thaw on ice.
2. Add 2 µl of each ligation reaction to a separate tube of competent cells. Mix gently with the pipette tip. DO NOT PIPETTE UP AND DOWN. Repeat for all ligations.
3. For control reactions, add 10 pg of pUC19 plasmid to a separate tube of cells.
4. Incubate all tubes on ice 20 minutes.
5. Transfer all tubes to 42°C heat block or water bath and incubate for 30-45 seconds, then place on ice for 2 minutes.
6. Add 250 µl of room temperature SOC medium to each tube and shake at 225 rpm for 60 minutes at 37°C. **Note**: Place the tubes horizontally and secure with tape to maximize aeration.
7. Plate 10 and 100 µl of each transformation mix on LB-kanamycin plates. Let all the liquid absorb, invert, and incubate at 37°C 18-24 hours. Proceed to **Analysis of Transformants**, page 14.

Continued on next page

Transformation, continued

Electroporation Transformation

You will need to prepare additional SOC. Please refer to the recipe on page 16.

1. Remove the appropriate number of microcentrifuge tubes of TOP10 electrocompetent cells from the -80°C freezer and thaw on ice. Chill electroporation cuvettes on ice.
2. Set up your electroporation device for electroporation of bacteria using the manufacturer's instructions.
3. Dilute ligation reaction with 10 µl of sterile water and place at 65°C for 5 minutes.

   Ligation reactions are diluted to reduce the salt concentration. Excess salt may cause arcing during electroporation. Heating to 65°C inactivates the ligase.
4. Add 2 µl of the ligation reaction to each tube containing 80 µl competent cells. Repeat for all ligation reactions.
5. For the control reactions, add 10 pg of pUC19 plasmid to a separate tube of 40 µl competent cells.
6. Incubate all tubes on ice for 1 minute.
7. Take one sample at a time and transfer the cell/DNA mix to an electroporation cuvette. Be sure not to trap air bubbles in the sample. Place the cuvette in the chamber and discharge the electrical pulse.
8. Remove cuvette and **immediately** add 450 µl room temperature SOC medium and transfer to a 15 ml snap-cap polypropylene tube (Falcon 2059 or similar). Place on ice. Repeat Steps 7-8 until all samples have been transferred to 15 ml tubes.
9. Incubate all tubes with shaking (200-225 rpm) at 37°C for 60 minutes. Place the tubes on an angle to maximize aeration.
10. Plate 50 and 100 µl of the transformation mix onto LB-kanamycin plates. After the liquid is absorbed, invert and incubate at 37°C for 18-24 hours.

Analysis of Transformants

1. Remove plates from the incubator. Pick at least 10 kanamycin resistant transformants and inoculate into 2 ml LB or SOB medium containing 25-50 µg/ml kanamycin. Grow 6-8 hours or overnight at 37°C.
2. Isolate plasmid DNA by miniprep for restriction analysis. Miniprep DNA may need to be phenol-extracted to prevent smearing on agarose gels. Remember to isolate a single colony and reconfirm the presence of insert before making a glycerol stock.
3. Prepare a glycerol stock of your desired clone for safekeeping by combining 0.85 ml of a mid-log bacterial culture with 0.15 ml of sterile 100% glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at -80°C.
4. Once the desired clone is isolated, you may proceed with further subcloning and/or analysis of your insert.

Note

If you need pure plasmid DNA for automated sequencing or any other technique, the S.N.A.P.™ Miniprep Kit (Catalog no. K1900-01) is available. Using the S.N.A.P.™ Miniprep Kit, you can purify up to 10 µg of pure plasmid DNA from a 3 ml overnight culture.

Appendix

Troubleshooting

Troubleshooting Table

The table below provides solutions to possible problems you might encounter.

Problem	Reason	Solution
Very few or no transformants arise	Loss of DNA during precipitation	Use more DNA. Be careful not to lose the DNA pellet during precipitation/wash
	Insert not ligating properly	Check the subcloning strategy
	Molar ratio of insert to vector is incorrect	Determine the concentration of insert and calculate the correct molar ratio
	Low transformation efficiency of *E. coli* strain	Chemically competent cells should yield ~1 x 10^9 transformants/µg DNA. Electrocompetent cells should yield >1 x 10^9 transformants/µg DNA. Check transformation efficiency with a control vector, prepare competent cells following procedures listed on pages 19-22.
High background of transformants which do not contain inserts	Overdigestion of vector with restriction enzymes	Use the minimum amount of enzyme necessary to digest the vector. Limit digests to 15-30 minutes
	If using cells that carry the *lac*I^q gene, insufficient amount of IPTG in plate	IPTG must be in excess to achieve proper induction and cell death
	Nuclease contamination in reagents	Use the reagents supplied with the kit or autoclave all reagents used for cloning (especially water)
Thin "lawn" of cells on plate	Insufficient amount of IPTG in plate medium	Be sure to add the correct amount of IPTG to the plate medium and let medium cool sufficiently before adding. DO NOT spread IPTG onto plates
	Insufficient amount of antibiotic in medium	Be sure that the correct amount of antibiotic has been added to the medium
DNA migrates anomalously on agarose gels (bands run at a larger molecular weight than expected and seem slightly smeared)	Protein bound to DNA	Extract the DNA with phenol/chloroform during plasmid preparation or use the S.N.A.P.™ Miniprep Kit (Catalog no. K1900-01)

Recipes

Low Salt LB Agar Plates with Kanamycin

Low Salt LB Medium (per liter)
1% Tryptone
0.5% Yeast Extract
0.5% NaCl
1.5% Agar
pH 7.5

Note: As the salt concentration of the medium decreases, the activity of aminoglycoside antibiotics (e.g. streptomycin, kanamycin) increases. You may find that 25 μg/ml kanamycin is sufficient to select transformants. Please test your host strain for sensitivity to 25 μg/ml kanamycin before selecting transformants.

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.5 with 5 M NaOH, add 15 g agar, and bring the volume to 1 liter.
3. Autoclave for 20 minutes on liquid cycle.
4. Let agar cool to ~55°C. Add kanamycin to a final concentration of 25-50 μg/ml.

   If using a cell line that carries the *lac*I^{q} gene, add IPTG to a final concentration of 1 mM (1 ml/liter).
5. Pour into 10 cm petri plates. Let the plates harden, then invert and store at +4°C. Plates containing kanamycin and IPTG are stable for 1-2 weeks.

Low Salt LB-Kanamycin Medium

Low Salt LB Medium (per liter)
1% Tryptone
0.5% Yeast Extract
0.5% NaCl
pH 7.5

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.5 with 5 M NaOH and bring the volume to 1 liter.
3. Autoclave for 20 minutes on liquid cycle.
4. Let solution cool to ~55°C. Add kanamycin to a final concentration of 25-50 μg/ml. Store the medium at +4°C.

Continued on next page

Recipes, continued

SOB Medium

SOB (per liter)
2% Tryptone
0.5% Yeast Extract
0.05% NaCl
2.5 mM KCl
10 mM $MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.5 with 5 M NaOH and add deionized water to 1 liter.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$ and kanamycin, if desired.
5. Store at room temperature or +4°C. **Do not add IPTG.**

SOC Medium

SOB Medium
20 mM glucose

1. Prepare and autoclave the SOB medium as described above.
2. After autoclaving, cool the solution to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$ and 7.2 ml of 50% glucose.
3. Store at room temperature or +4°C.

Continued on next page

Recipes, continued

FSB Transformation Solution

10 mM potassium acetate, pH 7.5
45 mM $MnCl_2$-$4H_2O$
10 mM $CaCl_2$-$2H_2O$
100 mM KCl
3 mM hexaamminecobalt chloride (Sigma-Aldrich #20309-2; 1-800-558-9160 to order)
10% glycerol

1. Make 100 ml of 1 M potassium acetate by dissolving 9.82 g in 90 ml deionized water. Adjust pH to 7.5 with 2 M acetic acid. Bring the volume up to 100 ml.
2. For 100 ml of FSB transformation solution combine the following ingredients:
   1 ml 1 M potassium acetate, pH 7.5
   890 mg $MnCl_2$-$4H_2O$
   150 mg $CaCl_2$-$2H_2O$
   750 mg KCl
   80 mg hexaamminecobalt chloride
   10 ml 100% glycerol
   80 ml deionized water
3. Carefully adjust pH to 6.4 with 0.1 N HCl. If you go past the correct pH, remake solution. Do not readjust pH with base.
4. Adjust the final volume to 100 ml with deionized water and filter sterilize. Store at +4°C.

DMSO

It is very important to use fresh, analytical grade DMSO. If you routinely transform cells by chemical means using the method of Hanahan, 1983, you probably have frozen aliquots of DMSO in your laboratory; if not, then follow this procedure:

1. Order the smallest amount of analytical grade DMSO.
2. When the DMSO arrives, take 5-10 ml and aliquot 200-500 µl per microcentrifuge tube. You may use the rest of the DMSO for other applications or you may aliquot the remainder for competent cells. It depends on whether you plan to use the method described in this manual on a routine basis.
3. Freeze these tubes at -20°C and use one tube per preparation of competent cells. Discard any remaining DMSO in the tube. **Use a fresh tube for every preparation of competent cells.**

Protocol for Chemically Competent Cells

Introduction

This protocol is used to make chemically competent cells for transformation with plasmid DNA (Hanahan, 1983). These cells will not substitute for electrocompetent cells for electroporation. The cells are grown to mid-log phase, then washed with FSB solution, and treated with DMSO. The cells are frozen in a dry ice/ethanol bath and stored at -80°C.

Yield

This protocol will yield enough cells for about 60 transformations. The expected efficiency of chemically competent TOP10 cells is 1 x 10^8 cfu/μg supercoiled DNA. This is also the minimum efficiency needed to obtain 100-200 colonies per 100 μl transformation mix.

Important

Sterile technique is absolutely essential to avoid contamination of the competent cells. Remember to use sterile solutions, medium, and supplies.

Preparation

For each preparation, prepare the following solutions (see **Recipes**, pages 16-18):

- 5 ml SOB medium in a sterile culture tube
- 250 ml SOB in a sterile 500 ml or 1 liter culture flask
- FSB solution (~25 ml)
- Fresh, reagent grade DMSO

Growth of Cells: Day 1

Streak TOP10 on an LB plate, invert the plate, and incubate at 37°C overnight.

Growth of Cells: Day 2

- Inoculate 5 ml of SOB medium in a sterile culture tube with one colony from the LB plate.
- Grow overnight (12-16 hours) in a shaking incubator (200-225 rpm) at 37°C.

Growth of Log-phase Cells: Day 3

1. For each preparation, place the following items on ice or at +4°C.
   - Two 250 ml sterile centrifuge bottles
   - Two 50 ml sterile centrifuge tubes
   - Two 5 ml sterile pipettes
2. Inoculate 250 ml of fresh SOB medium in a 500 ml or 1 liter culture flask with 2.5 ml of the overnight culture.
3. Grow the culture at 37°C at 200-225 rpm in a shaking incubator until the OD_{550} reaches between 0.55-0.65 (2-3 hours).
4. Divide the culture between the two cold (0-4°C), sterile 250 ml centrifuge bottles and place on ice for 30 minutes.

Continued on next page

Protocol for Chemically Competent Cells, continued

Preparing the Cells: Day 3

1. Centrifuge the 250 ml bottles at 2000 x g for 10-15 minutes at 0-4°C.
2. Decant the medium and resuspend each pellet in 10 ml cold (0-4°C) FSB solution and transfer to two cold, sterile, 50 ml centrifuge tubes. Incubate on ice for 15 minutes.
3. Centrifuge the tubes at 2000 x g for 10-15 minutes at 0-4°C.
4. Decant the buffer and resuspend each pellet in 1.8 ml cold FSB solution using a sterile 5 ml pipette.
5. While gently swirling the tubes, slowly add 65 µl of DMSO drop by drop to each tube. Incubate on ice for 15 minutes.
6. While gently swirling the tubes, slowly add an additional 65 µl of DMSO drop by drop to each tube.
7. Combine the cell suspensions from both tubes into one and incubate on ice for 15 minutes. Keep on ice.

Aliquoting and Storage of Cells Day 3

1. Prepare a dry ice/ethanol bath.
2. For each preparation, place approximately sixty 1.5 ml microcentrifuge tubes on ice. Keep cell suspension on ice.
3. Pipette 50 µl of cell suspension into each tube.
4. As soon as all of the cell suspension is aliquoted, quick-freeze the tubes in the dry ice/ethanol bath and store at -80°C.

Protocol for Electrocompetent Cells

Introduction

The purpose of this procedure is to prepare cells for transformation with plasmid DNA by electroporation. The procedure describes the growth of cells and subsequent washing and concentrating steps. The washing is necessary to ensure that salts are removed to reduce the conductivity of the cell solution. High conductivity may result in arcing during electroporation.

These cells are only to be used for electroporation. Do not use them for any other transformation protocol.

Yield

The following procedure will yield enough electrocompetent cells for about 30 transformations. Remember to use **sterile** solutions, medium, and supplies.

Note

The expected efficiency of the electrocompetent TOP10 cells is 1 x 10^9 cfu/µg supercoiled DNA. This is the minimum efficiency needed to obtain 100-200 colonies per 100 µl of the transformation reaction.

Important

Sterile technique is absolutely essential to avoid contamination of the electrocompetent cells.

Growing the Cells: Day 1

Streak TOP10 on an LB plate, invert the plate, and incubate at 37°C overnight.
Prepare the following:

- 50 ml LB medium in a 250 ml sterile culture flask
- 1 liter of LB medium in a 2 liter or 4 liter sterile culture flask (Store at room temperature)
- 50 ml of sterile 10% glycerol
- 1.5 liter of sterile water

  Store at +4°C

Continued on next page

Electrocompetent Cells, continued

Growing the Cells: Day 2

Inoculate the 50 ml of LB medium in a 250 ml culture flask with a single colony from the LB plate and incubate at 37°C with shaking (200-225 rpm) for 12-16 hours (overnight).

Growing the Cells: Day 3

1. For each preparation, pre-chill on ice or at +4°C:
   - Two sterile 500 ml centrifuge bottles
   - Two sterile 50 ml centrifuge tubes
   - Two sterile 25 ml pipettes
   - One sterile 5 ml pipette
2. Inoculate 1 liter of LB medium in a 2 liter or 4 liter flask with the 50 ml overnight culture. Grow the 1 liter culture in shaking incubator (200-225 rpm) at 37°C until the OD_{550} is between 0.5 and 0.6 (approximately 2-3 hours).
3. Transfer the 1 liter culture to the two chilled, sterile 500 ml centrifuge bottles and incubate on ice for 30 minutes.

Harvesting and Washing the Cells: Day 3

1. Centrifuge the cultures at 2000 x g for 15 minutes at 0-4°C. Keep the cell pellet and decant the broth. Place bottles back on ice.
2. Resuspend the cell pellet in each bottle in approximately 500 ml of cold (0-4°C), sterile water.
3. Centrifuge cells at 2000 x g for 15 minutes at 0-4°C. Keep the pellet and decant the water. Place bottles back on ice.
4. Resuspend the cells in each bottle in approximately 250 ml of cold (0-4°C), sterile water.
5. Centrifuge cells at 2000 x g for 15 minutes at 0-4°C. Decant the water and place bottles back on ice.
6. Using a pre-chilled, sterile 25 ml pipette, resuspend cells in each bottle in 20 ml cold (0-4°C), sterile, 10% glycerol and transfer each cell suspension to a chilled, sterile, 50 ml centrifuge tube.
7. Centrifuge cells at 4000 x g for 15 minutes at 0-4°C. Decant the 10% glycerol and place tubes on ice.
8. Resuspend each cell pellet in 1 ml cold (0-4°C), sterile, 10% glycerol. Using a pre-chilled 5 ml pipette, pool the cells into one of the 50 ml tubes. Keep on ice.

Aliquoting and Storage of Cells: Day 3

1. Prepare a dry ice/ethanol bath.
2. For each preparation, place thirty-five to forty 1.5 ml microcentrifuge tubes on ice and pipette 40 µl of the cell suspension into each tube. Keep cell suspension and tubes on ice until all of the cell solution is aliquoted.
3. After all of the cell suspension is aliquoted, quick-freeze tubes in the dry ice/ethanol bath and store at -80°C until ready for use.

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Product Qualification

pZErO™-2

The pZeO™-2 vector is qualified by restriction enzyme digestion with the restriction enzymes listed below. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

Vector	Restriction Enzyme	Expected Fragments(bp)
pZErO™-2	*Eco*R V	3.3 kb
	Nco I	3.3 kb
	Pvu II	1, 381, 1111, 445, 360

*ccd*B gene

To verify lethality of the *ccd*B gene, competent cells are transformed with 1 μg of supercoiled pZErO™-2 vector and spread on LB-Kanamycin plates. Less than 100 colonies must be obtained.

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