USER GUIDE

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Zero Blunt® PCR Cloning Kit

A high efficiency system for cloning blunt-ended PCR products

Catalog numbers K2700-20, K2700-40, K2750-20, and K2750-40

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For research use only. Not for use in diagnostic procedures.

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Contents

Kit Contents and Storage

Type of kits

This manual is supplied with the following kits.

Kit	Quantity	Cat. no.
Zero Blunt® PCR Cloning Kit	20 reactions	K2750-20
	40 reactions	K2750-40
Zero Blunt® PCR Cloning Kit with One Shot® TOP10 Chemically Competent *E. coli*	20 reactions	K2700-20
	40 reactions	K2700-40

Shipping and storage

The Zero Blunt® PCR Cloning Kits are shipped on dry ice.

Store the Zero Blunt® PCR Cloning reagents at **–30°C to –10°C** and the One Shot® TOP10 Competent Cells (supplied with Cat. nos. K2700-20 and K2700-40) at **–85°C to –68°C.**

Product use

For research use only. Not for use in diagnostic procedures.

Continued on next page

Kit Contents and Storage, Continued

Zero Blunt® PCR cloning reagents

Zero Blunt® PCR Cloning reagents (Box 1) are listed in the following table. Forty reaction kits are supplied as two 20-reaction kits.**Store reagents at –30°C to –10°C.**

Item	Concentration	Amount
pCR®-Blunt vector, linearized with blunt ends	25 ng/µL in TE Buffer, pH 8	3 × 10 µL, 750 ng
ExpressLink™ T4 DNA Ligase†	5.0 Weiss units/µL	25 µL
5X Express Link™ T4 DNA Ligase Buffer**	5X T4 DNA Ligase Buffer (50 mM Tris-HCl, pH 7.6 , 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 25 % (w/v) polyethylene glycol-8000)	200 µL
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP Neutralized to pH 8.0	10 µL
Control DNA Template	0.1 µg/µL in TE Buffer, pH 8	10 µL
Sterile Water	Deionized, autoclaved	1 mL
M13 Forward (–20) Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL
M13 Reverse Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL

Sequence of primers

The following table lists the sequence of the M13 Sequencing Primers supplied with the kit

Primer	Sequence	pMoles Supplied
M13 Forward (–20)	5´-GTAAAACGACGGCCAG-3´	407
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´	385

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One Shot® reagents

One Shot® TOP10 Chemically Competent *E. coli* are supplied with Cat. nos. K2700-20 and K2700-40 and contain the following reagents. Forty reaction kits are supplied as two 20-reaction kits.

The transformation efficiency for TOP10 cells is 1×10^9 cfu/µg DNA.

Store competent cells at −85°C to −68°C.

Item	Composition	Amount
S.O.C. Medium (may be stored at 2°C to 8°C or 15°C to 30°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL
TOP10 cells	—	21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL

Genotype of TOP10

TOP10: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Introduction

Product Overview

Description of the system

The Zero Blunt® PCR Cloning Kit is designed to clone blunt PCR fragments (or any blunt DNA fragment) with a low background of non-recombinants. The pCR®-Blunt vector contains the lethal *E. coli ccd*B gene fused to the C-terminus of LacZα (Bernard *et al.*, 1994). Ligation of a blunt PCR fragment disrupts expression of the *lac*Zα-*ccd*B gene fusion permitting growth of only positive recombinants upon transformation. Cells that contain non-recombinant vector are killed when the transformation mixture is plated.

ccdB 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; Miki *et al.*, 1984; Ogura and Hiraga, 1983). The *ccd* locus participates in stable maintenance of F plasmid by post-segregational killing of cells that do not contain the F plasmid (Jaffe *et al.*, 1985).

The CcdA protein acts as an inhibitor of CcdB protein. In the absence of CcdA protein, the CcdB protein becomes toxic as it interferes with bacterial DNA gyrase (topoisomerase II), an essential enzyme that catalyzes the ATP-dependent negative supercoiling of DNA. Inhibiting DNA gyrase results in DNA breakage and cell death.

Experimental outline

To clone your gene of interest into pCR®-Blunt, you must first generate a blunt PCR product. The blunt PCR product is ligated into pCR®-Blunt and transformed into competent cells. Since the PCR product can ligate into the vector in either orientation, individual recombinant plasmids need to be analyzed by restriction mapping for orientation.

The table below describes the major steps necessary to clone your gene of interest into pCR®-Blunt.

Step	Action	Page
1	Amplify your PCR product using a thermostable, proofreading polymerase and your own primers and parameters.	8
2	Ligate your PCR product into pCR®-Blunt.	9–10
3	Transform your ligation into competent *E. coli*.	11–12
4	Select colonies and isolate plasmid DNA. Analyze plasmid DNA for the presence and orientation of the PCR product by restriction enzyme digestion or sequencing.	13

Methods

Produce Blunt-End PCR Products

Guidelines for PCR

Generally 10–100 ng of DNA is sufficient to use as a template for PCR. If amplifying a pool of cDNA, the amount needed will depend on the relative abundance of the message of interest in your mRNA population. For optimal ligation efficiencies, we recommend using no more than 30 cycles of amplification.

Important

Do not use *Taq* polymerase to produce your PCR product as it will not produce blunt fragments. If you use *Taq* polymerase or an enzyme mix containing *Taq* polymerase, refer to page 15 for a protocol to blunt-end your PCR product before cloning into pCR®-Blunt.

Required materials

Components required but not supplied:

- Thermostable proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- Thermocycler
- DNA template and primers for PCR product

Components supplied with the kit:

- dNTPs (adjusted to pH 8)

Produce PCR products

Perform the PCR in a 50 µL volume containing:

DNA Template	10–100 ng
10X PCR Buffer	5 µL
100 mM dNTPs	0.5 µL
Primers	1 µM each
Sterile water	to a total volume of 49 µL
Proofreading Polymerase (2 to 2.5 U/µL)	1 µL
Total Volume	50 µL

Gel purification

Remove 5–10 µL from each PCR reaction and use agarose gel electrophoresis to verify the quality and quantity of your PCR product. If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before proceeding. Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (see page 26) can help you optimize your PCR. Contact Technical Support for more information (page 27).

Clone into pCR®-Blunt

Introduction

After producing the desired PCR product, you are ready to clone it into pCR®-Blunt. Follow the following guidelines.

If this is the first time you are using the Zero Blunt® PCR Cloning Kit, we recommend performing the control reactions to help you evaluate your results (see pages 18–20).

We recommend using a 10:1 molar ratio of insert:vector as a starting point. You may need to increase this ratio to optimize cloning efficiency.

If you are using the PCR product directly from the PCR reaction mixture, do not use more than 5 µL of the reaction since salts in the PCR sample may inhibit the T4 DNA Ligase.

Calculating amount of PCR product to use

A range from 10:1 to 100:1 molar ratio of insert:vector is recommended to optimize blunt-ended PCR ligation efficiency. If necessary, the amount of vector in the ligation reaction can be reduced to as little as 5 ng. Use the following formula below to estimate the amount of PCR product needed for a 10:1 molar ratio of insert to 25 ng of pCR™-Blunt vector.

$$x \text{ ng insert} = \frac{(10)\,(y \text{ bp PCR product})\,(25 \text{ ng linearized pCR®-Blunt})}{(3500 \text{ bp pCR®-Blunt})}$$

where x ng is the amount of PCR product of y base pairs to be ligated for a 10:1 insert:vector molar ratio.

Purify and concentrate PCR products

In most cases, the PCR reaction may be used directly in the ligation reaction; however, it may be necessary on occasion to purify and concentrate the PCR product. Follow the protocol below to purify your PCR product.

1. Extract the PCR reaction with an equal volume of phenol/chloroform. Centrifuge 1 minute at 10,000 × *g* and transfer the upper layer to a fresh, sterile tube.
2. Add 1/10 volume of 3 M sodium acetate, pH 5.6 and add 2 volumes of 100% ethanol at room temperature. You may also add 5 µg of carrier (e.g. glycogen) to help precipitate the DNA.
3. Flick the tube to mix and centrifuge at 10,000 × *g* for 5 minutes.
4. Remove the ethanol and rinse the pellet with 70% ethanol.
5. Centrifuge and remove all the ethanol. Air-dry the pellet for ~5 minutes.
6. Resuspend the pellet in 5–10µL of sterile water or TE buffer.
7. Estimate the DNA concentration by electrophoresis or absorbance at 260 nm.

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Ligate into pCR®-Blunt

Follow the following protocol to ligate your PCR product into pCR®-Blunt.

1. Determine the volume of PCR sample needed to reach the required amount of PCR product (see page 9).
2. Set up the following 10µL ligation reaction:

pCR®-Blunt (25 ng)	1 µL
Blunt PCR product	1–5 µL
5X ExpressLink™ T4 DNA Ligase Buffer	2 µL
Sterile water	to a total volume of 9 µL
ExpressLink™ T4 DNA Ligase (5 U/ µL)	1 µL
Total Volume	10 µL

1 Incubate the ligation reaction at room temperature for a minimum of 5 minutes. Longer ligation times increase the cloning efficiency, see the Control Ligation Reaction section below. Proceed to **Transform Competent Cells**, page 11. Proceed to **Transform Competent Cells**, on the following page.

Note: You may store your ligation reaction at −20ºC until you are ready for transformation

Transform Competent Cells

Introduction

After ligating your insert into pCR®-Blunt, you are ready to transform the construct into competent *E. coli*. One Shot® TOP10 cells are provided with Cat. nos. K2700-20 and K2700-40 to facilitate transformation. A protocol to transform One Shot® cells is provided in this section. To transform another competent strain, refer to the manufacturer's instructions.

E. coli host strain

We recommend using One Shot® TOP10 *E. coli* for transformation, however other host strains are suitable (see the following **Important** note). TOP10 does **not** contain *lac*I^q; therefore, expression from the *lac* promoter is constitutive. **There is no need to use IPTG or X-gal to identify possible recombinants.** If you use a strain that contains *lac*I^q, you will need to include IPTG to induce expression from the *lac* promoter since expression is required to produce the lethal phenotype.

Important

Do not use any *E. coli* strain that contains the complete Tn*5* transposon (e.g. DH5 F'IQ). This transposon contains resistance markers to both Kanamycin and Zeocin™ selective antibiotic and will not allow selection of positive recombinants.

Do not use any *E. coli* strain that contains the F´ episome (e.g. TOP10F´). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Zeocin™ selection

The Zeocin™ resistance gene in pCR®-Blunt allows you to select *E. coli* transformants using Zeocin™ antibiotic. For selection, use Low Salt LB agar plates containing 25 µg/mL Zeocin™ selective antibiotic (see page 21 for a recipe). Note that for Zeocin™ selective antibiotic to be active, the salt concentration of the bacterial medium must remain low (less than 90 mM) and the pH must be 7.5. We recommend growing positive recombinants in SOB medium containing 25 µg/mL Zeocin™ selective antibiotic for better growth and plasmid yields (see page 23 for a recipe). For more information on storing and handling Zeocin™ selective antibiotic, refer to page 23.

Continued on next page

Transform Competent Cells, Continued

Required materials

Components required but not supplied:

- LB plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin™ selective antibiotic (see page 21 for a recipe) (two for each transformation)
- 42°C water bath
- 37°C shaking and non-shaking incubator
- General microbiological supplies (e.g. plates, spreaders)
- SOB Medium, optional (warmed to room temperature; see page 22 for recipe)

Components supplied with the kit:

- S.O.C. medium (warmed to room temperature)
- Chemically competent *E. coli* suitable for transformation (One Shot® TOP10 Competent Cells (supplied with Cat. nos. K2700-20 and K2700-40)
- Positive control, optional (*e.g.* pUC19)

Prepare for transformation

- Equilibrate a water bath to 42°C.
- Warm the vial of S.O.C. medium or SOB medium to room temperature.
- Warm selective plates at 37°C for 30 minutes

One Shot® transformation protocol

Follow the protocol below to transform One Shot® TOP10 Competent Cells. To transform another strain, refer to the manufacturer's instructions.

1. Thaw on ice one 50-µL vial of One Shot® TOP10 cells for each transformation.
2. Pipet 2 µL of each ligation reaction directly into the vial of competent cells and mix by stirring gently with a pipette tip. **Do not mix cells by pipetting.**
3. Incubate the vials on ice for 30 minutes. Store the remaining ligation mixtures at –20°C.
4. Heat shock the cells for 45 seconds at 42°C without shaking. Immediately place the vials on ice for 2 minutes.
5. Add 250 µL of room temperature S.O.C. medium to each vial.
6. Shake the vials horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
7. Spread 10–100 µL from each transformation vial on LB plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin™ selective antibiotic. We recommend plating two different volumes to ensure well-spaced colonies. For plating smaller volumes, add 20 µL S.O.C. to ensure even spreading.
8. Incubate plates overnight at 37°C. Proceed to **Analyze Transformants** on page 13

Expected results

For an insert size of 400–800 bp, you should obtain 50–2000 colonies per plate depending on the volume plated and of these, greater than 80% should contain inserts. Note that ligation and transformation efficiency depend on insert size. As insert size increases, the efficiency will decrease.

Analyze Transformants

Analyze positive clones

1. Pick at least 10 transformants for analysis.
2. Grow colonies overnight in 2 mL LB or SOB medium containing 50 µg/mL kanamycin or Low Salt LB containing 25 µg/mL Zeocin™ selective antibiotic.
3. Isolate plasmid and analyze by restriction digestion or sequencing to verify the correct insert and orientation. We recommend using the Pure Link™ HQ Mini Plasmid Purification Kit for purifying your plasmid DNA (see page 26 for ordering information).

Sequence

You may sequence your pCR®-Blunt construct to confirm the presence and correct orientation of the insert. The M13 Forward and Reverse sequencing primers are included in the kit to help you sequence the insert. Refer to the diagram on page 24 for the location of the primer binding sites.

Analyze transformants by PCR

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use either the M13 Forward (–20) or the M13 Reverse primer and a primer that hybridizes within your insert. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol is provided below for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (see page 26)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µL of PCR SuperMix High Fidelity into a 0.5-mL microcentrifuge tube. Add 1 µL each of the forward and reverse PCR primer.
2. Pick 10 colonies and resuspend them individually in 50 µL of the PCR cocktail from step 1 of this procedure. Don't forget to make a patch plate to preserve the colonies for further analysis.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20–30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Visualize by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 18–20. These reactions will help you troubleshoot your experiment. Refer to **Troubleshoot** on page 16 for additional tips.

Continued on next page

Long-Term storage

After identifying the correct clone, be sure purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at –20°C.

1. Streak the original colony on LB plates containing 50µg/mL kanamycin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 100 µg/mL ampicillin or 50µg/mL kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Blunt-Ending PCR Fragments

Introduction

If you used *Taq* polymerase or an enzyme mix containing *Taq* polymerase to generate your PCR product, you will need to blunt-end the PCR product (i.e. remove the 3´ A-overhangs) before cloning into pCR®-Blunt

To clone PCR products generated using *Taq* polymerase, we recommend the TOPO TA Cloning® Kit (see page 26). The cloning vector supplied with this kit, pCR®-Blunt, is provided linearized with 3´ T-overhangs which will pair with the 3´ A-overhangs on *Taq*-generated PCR products.

Before starting

1. Prepare a 10-mM dNTP solution by diluting 1 µL of a 100-mM dNTP solution into 9 µL sterile water. Place on ice.
2. Equilibrate at water bath or heat block to 72°C.

Procedure

1. Purify and concentrate the PCR product as described on page 9. *Taq* polymerase must be removed to prevent 3´ extension from competing with blunt-ending.
2. Set up the following ~10 µL reaction:

PCR product (or any DNA fragment)	8 µL
10 mM dNTPs	1 µL
10X PCR Buffer (user supplied)	1 µL
Thermostable, proofreading polymerase (0.5 unit)	0.25 µL

3. Mix by flicking the tube and add 2 drops of mineral oil. Centrifuge briefly.
4. Incubate at 72°C for 20 to 30 minutes.
5. Use 1–5 µL directly in the ligation reaction, page 10.

Using other enzymes

If you do not have a thermostable, proofreading polymerase, you may try T4 DNA Polymerase to blunt end your fragment. Follow the manufacturer's instructions to blunt-end your PCR product, then proceed to the ligation reaction on page 10.

Troubleshoot

Introduction

If you do not obtain the results you expect, use the following table to troubleshoot your experiment. We recommend performing the control reactions (pages 18–20) to help you evaluate your results.

Observation	Cause	Solution
No PCR product	Either the thermostable polymerase is inactive or the conditions for your PCR are not optimal.	Perform the control reactions on pages 18–20 to test the activity of the polymerase. If the polymerase is active, you may need to optimize the conditions of your PCR reaction.
Few or no transformants obtained	Loss of DNA during precipitation	Be careful not to lose the DNA pellet when precipitating and washing. You may wish to add carrier to help precipitate the DNA (e.g. glycogen).
	Molar ratio of insert to vector is incorrect	Determine the concentration of insert and use a molar ratio of at least 10:1 (insert:vector). Increase the molar ratio up to 100:1 if necessary.
	Low transformation efficiency of *E. coli* strain	Check transformation efficiency with the pUC19 control vector included with the One Shot® Kit. One Shot® TOP10 cells should yield ~1 × 10^9 transformants/µg DNA.
	A polymerase that adds 3´ A-overhangs was used (e.g. *Taq* polymerase)	If you used an enzyme mix containing *Taq* polymerase, you will need to blunt-end your PCR product (see page 15).
	Too much of the amplification reaction was added to the ligation.	The high salt content of PCR reactions can inhibit ligation. Do not use more than 5 µL of the PCR mixture in the ligation reaction.
High background of transformants that do not contain inserts	Nuclease contamination in reagents	Autoclave all reagents used for cloning (especially water).
	Absence of insert allows vector to religate	Increase the molar ratio of insert to vector (see page 9).
	Insert too large for efficient transformation	Try electroporation with electrocompetent cells. Do not use One Shot® Chemically Competent Cells for electroporation.
	Difficult insert to clone by the usual methods	Some DNA sequences may be difficult to replicate and propagate (i.e. inverted repeats, AT- or GC- rich).

Continued on next page

Troubleshoot, Continued

Observation	Cause	Solution
Thin "lawn" of cells on plate	Insufficient amount of antibiotic in plate medium	Use LB plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin™ selective antibiotic. Let medium cool sufficiently (~55°C) before adding antibiotics.
	High salt in LB medium when selecting on Zeocin™ selective antibiotic	Make sure you make LB medium with 5 g/liter NaCl (see page 21 for a recipe).
	Too many cells plated	Plate a smaller volume of the transformation mixture or dilute 10-fold with S.O.C. before plating.
DNA bands run at a larger molecular weight than expected and seem slightly smeared	Protein bound to DNA	Perform phenol/chloroform extraction during plasmid preparation of after restriction digest. You may also use the PureLink™ HQ Mini Plasmid Purification Kit (see page 26).
Low plasmid yield	Medium is not sufficiently rich enough for optimal growth.	Use SOB medium containing antibiotic (see page 22 for recipe).

Appendix

Perform the Control Reactions

Introduction

We recommend performing the control reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involve producing a control PCR product using the reagents included in the kit and using the control PCR product in a ligation reaction.

Produce the control PCR product

1. Set up a 50µL PCR as follows:

Control DNA Template (100 ng)	1 µL
10X PCR Buffer	5 µL
100 mM dNTPs	0.5 µL
M13 Reverse Primer (200 ng)	2 µL
M13 Forward (–20) Primer (200 ng)	2 µL
Sterile Water	38.5 µL
Proofreading polymerase (2.5 units)	1 µL
Total Volume	50 µL

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1
Denature	1 minute	94°C	25
Anneal	1 minute	55°C	
Extend	1 minute	72°C	
Final Extension	7 minutes	72°C	1

3. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 800-bp band should be visible.
4. Estimate the amount of DNA by measuring against a known standard run on the same gel. You should get a concentration of about 20–30 ng/µL for your Control PCR Product. Proceed to the **Control Ligation Reaction** on page 19.

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Perform the Control Reactions, Continued

Control ligation reaction

Using the control PCR product produced on the previous page, set up the following ligation reaction.

1. Set up the 10 µL Control Ligation Reaction as follows. Note that the insert:vector molar ratio is 10:1.

5X ExpressLink™ T4 DNA Ligase Buffer	2 µL
pCR®-Blunt vector (25 ng/µL)	1 µL
Control PCR Product (~60 ng)	3 µL
Sterile water	3 µL
ExpressLink™ T4 DNA Ligase (5 U/ µL)	1µL
Total Volume	10 µL

1 Incubate the Control Ligation Reaction at room temperature for 1 hour. (See Note about incubation times on next page)

2 Transform 1 µL of the Control Ligation Reaction into One Shot® TOP10 Competent Cells or into another suitable competent *E. coli* strain.

3 Plate 10–50 µL of each transformation mix on LB agar plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin™ selective antibiotic.

2. Incubate plates overnight at 37ºC.

Transformation control

Zero Blunt® PCR Cloning Kits supplied with One Shot® TOP10 Competent Cells will also be supplied with pUC19 plasmid for use as a transformation control. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC 19 using the protocol on page 12. Plate 10–50 µL of the transformation mixture on LB plates containing 100 µg/mL ampicillin. Transformation efficiency should be greater than 1×10^9 cfu/µg DNA.

Continued on the next page

Perform the Control Reactions, Continued

Note about incubation times

The total number of transformants was reduced with shorter ligation incubation times. In the tables below, ligation reactions were performed using either Accuprime® PFx-generated blunt insert **(Table 1)** or Platinum® PFx-generated blunt insert **(Table 2)**.

Following the control ligation procedure using a 1:10 vector to insert ratio. Ligation reactions were incubated at room temperature for 5 minutes, 10 minutes, 15 minutes, 30 minutes and 1 hour, transformed into One Shot® Top10 cells and 50ul plated for colony formation. The data at each time point shows the total colony number for 3 replicates.

Table 1 - Accuprime® PFx

Time	Colonies
5 min	40 ± 12
10 min	77 ± 17
15 min	85 ± 14
30 min	148 ± 50
60 min	166 ± 63

Table 2 - Platinum® PFx

Time	Colonies
5 min	30 ± 19
10 min	73 ± 14
15 min	120 ± 60
30 min	192 ± 49
60 min	339 ± 44

Expected results

Pick 10 colonies and isolate plasmid DNA. Analyze the plasmids for the presence of insert by digesting the DNA with *Eco*R I to release the insert. Greater than 95% of the colonies should contain plasmid with the 800 bp insert.

Recipes

LB (Luria-Bertani) medium and plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow the solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at 4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/mL kanamycin or 50 µg/mL ampicillin), and pour into 10-cm plates.
4. Let the plates harden, then invert and store at 4°C in the dark.

Low salt LB medium

Composition:

1.0% 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. For plates, add 15 g/L agar.
2. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add Zeocin™ selective antibiotic to a final concentration of 25 µg/mL.
4. Store at room temperature or at 4°C.

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SOB medium with antibiotic

Composition: 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. Prepare 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 of this procedure.
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 $MgCl_2$.
5. If desired, add kanamycin to a final concentration of 50 µg/mL or Zeocin™ selective antibiotic to a concentration of 25 µg/mL.
6. Store at room temperature or 4°C.

Zeocin™ Selective Antibiotic

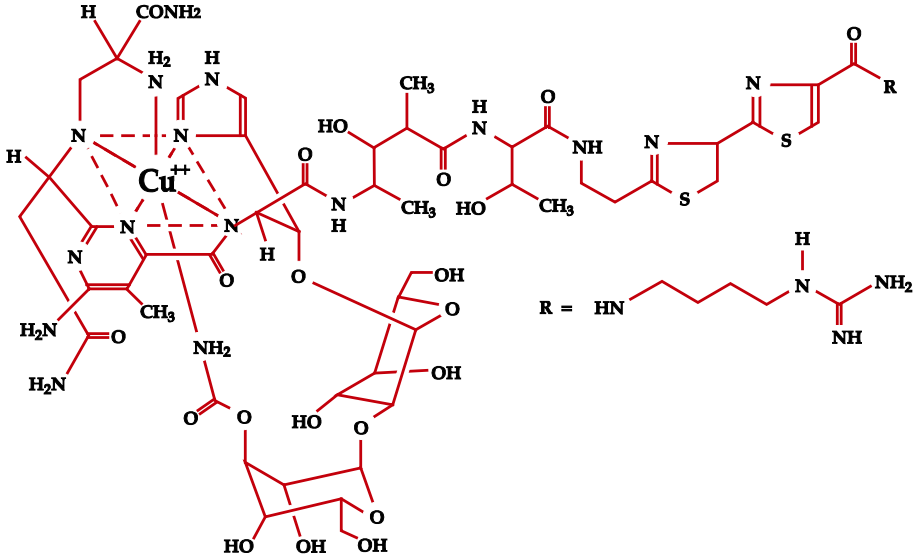
Introduction

Zeocin™ selective antibiotic is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. It shows strong toxicity against bacteria, fungi, plants and mammalian cell lines (Calmels *et al.*, 1991; Drocourt *et al.*, 1990; Gatignol *et al.*, 1987; Mulsant *et al.*, 1988; Perez *et al.*, 1989).

A Zeocin™ resistance protein has been isolated and characterized (Calmels *et al.*, 1991; Drocourt *et al.*, 1990). This 13,665-Da protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), binds stoichiometrically to Zeocin™ selective antibiotic and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™ selective antibiotic.

Molecular weight, formula, and structure

The formula for Zeocin™ selective antibiotic is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1527.5. The structure of Zeocin™ selective antibiotic is shown below.



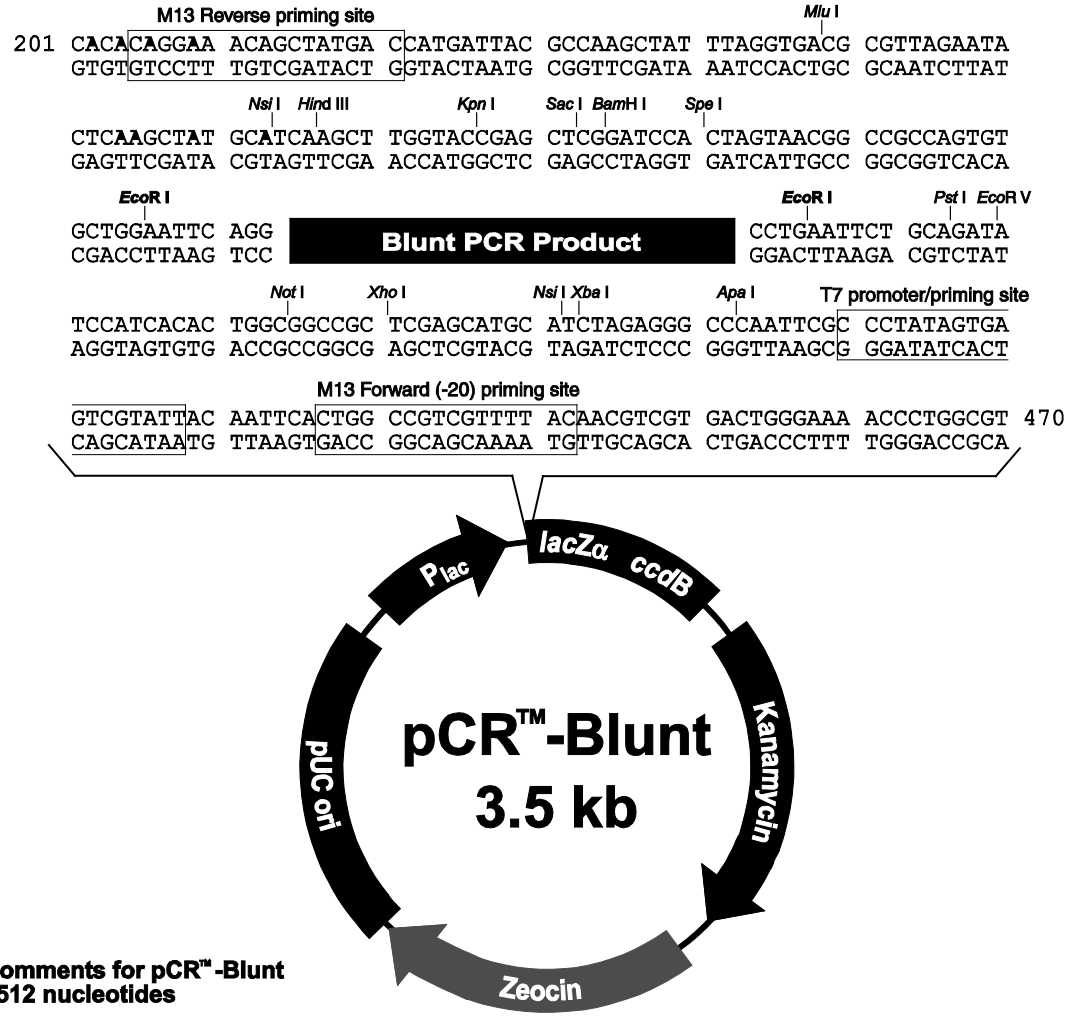
Handling Zeocin™ selective antibiotic

- High ionic strength and acidity or basicity inhibit the activity of Zeocin™ selective antibiotic. Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see page 21 for a recipe).
- Store Zeocin™ selective antibiotic at –20°C and thaw on ice before use.
- Zeocin™ selective antibiotic is light sensitive. Store the drug and plates or medium containing the drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses when handling solutions that contain Zeocin™.
- Do not ingest or inhale solutions containing Zeocin™.

Map and Features of pCR®-Blunt

pCR™-Blunt map

The following map summarizes the features of the pCR®-Blunt vector. Restriction sites that are only found in the polylinker are shown. **The complete sequence of the vector** is available from **www.lifetechnologies.com/support** or by contacting Technical Support (page 27).



Comments for pCR™-Blunt
3512 nucleotides

Lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
*Lac*Z-alpha ORF: bases 217-570
T7 promoter priming site: bases 400-419
M13 Forward (-20) priming site: bases 427-442
Fusion joint: bases 571-579
*ccd*B lethal gene ORF: bases 580-882
Kanamycin resistance ORF: bases 1231-2025
Zeocin resistance ORF: bases 2231-2605
pUC origin: bases 2673-3386

Continued on next page

Map and Features of pCR®-Blunt, Continued

Features of pCR®-Blunt

The table below describes the features of pCR®-Blunt. All features have been functionally tested.

Features	Benefit
lac promoter	Allows expression of *lac*Zα-*ccd*B gene fusion.
*lac*Zα-*ccd*B fusion gene	Provides positive selection against non-recombinant vector. ccdB function is disrupted (inactivated) by an insert. Includes the M13 forward and reverse priming sites for sequencing of the insert.
Multiple Cloning Site	Allows insertion of PCR fragment to disrupt expression of the *ccd*B gene and allows convenient screening, restriction mapping, and excision of cloned insert.
T7 promoter	Allows *in vivo* or *in vitro* transcription and translation.
T7, M13 Forward (–20), M13 Reverse priming sites	Allows sequencing of the insert.
Kanamycin resistance gene	Allow selection and maintenance in *E. coli*.
Sh ble (Zeocin™ resistance gene)	Allow selection and maintenance in *E coli*. Expression is driven using the *bla* (β-lactamase) promoter.
pUC origin	Allows high copy replication and maintenance of the plasmid in *E. coli.*

Accessory Products

Additional products

The following table lists additional products that may be used with TOPO® TA Cloning Kits. For more information, visit **www.lifetechnologies.com/support** or contact Technical Support (page 27).

Item	Quantity	Cat. no.
TA Cloning Dual Promoter Kit	40 reactions	K460040
ExpressLink™ T4 DNA Ligase	30 reactions	A13726
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
	250 units	11708-021
	500 units	11708-039
AccuPrime™ *Pfx* DNA Polymerase	200 reactions	12344-024
	1000 reactions	12344-032
AccuPrime™ *Pfx* SuperMix	200 reactions	12344-040
PCR SuperMix High Fidelity	100 reactions	10790-020
The PCR Optimizer™ Kit	100 reactions	K1220-01
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions	C4040-50
	20 reactions	C4040-52
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C8620-03
TOPO® TA Cloning® Kit	20 reactions	K4500-01
Kanamycin	5 g	11815-024
	25 g	11815-032
	100 ml (10 mg/ml)	15160-054
Zeocin™ Selection Reagent	8 x 1.25 ml	R250-01
	50 ml	R250-05

Technical Support

Obtaining support

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

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 Data Sheets (SDS)

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

Certificate of analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

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ccdB selection technology is described in Bernard et al., "Positive Selection Vectors Using the F Plasmid ccdB Killer Gene" Gene 148 (1994) 71-74. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). For licensing information for use in other than research, please contact: Out Licensing, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008; Phone (760) 603-7200 or e-mail **outlicensing@lifetech.com**.

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Notes

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