pUB/Bsd TOPO®
Cloning Kit
Version C
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pUB/Bsd TOPO® Cloning Kit

Five-minute cloning of blunt-end PCR products into a vector containing the blasticidin selection marker for generating stable mammalian cell lines

Catalog no. K512-20

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Table of Contents

Kit Contents and Storage

Shipping and Storage

The pUB/Bsd TOPO® Cloning Kit is shipped on dry ice. Each kit contains a box with pUB/Bsd TOPO® Cloning reagents (Box 1) and a box with One Shot® TOP10 Chemically Competent *E. coli* (Box 2). Store **Box 1 at -20°C** and **Box 2 at -80°C.**

TOPO® Cloning Reagents

pUB/Bsd TOPO® Cloning reagents (Box 1) are listed below. **Please note that the user must supply a thermostable proofreading polymerase.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pUB/Bsd-TOPO®	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP; 12.5 mM dCTP; 12.5 mM dGTP; 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl; 0.06 M $MgCl_2$	50 µl
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8.0	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8.0	10 µl
Sterile Water	--	1 ml

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Kit Contents and Storage, Continued

One Shot® Reagents

The table below describes the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2).

Store at -80°C.

Item	Composition	Amount
SOC Medium (may be stored at +4°C or room temperature)	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 *E. coli*	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

Genotype of TOP10 Cells

TOP10: Use this strain for general cloning. Please note that this strain cannot be used for rescue of single-strand DNA.

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

Accessory Products

Additional Products

The table below lists additional products available from Invitrogen which you may use in conjunction with the pUB/Bsd TOPO® Cloning Kit.

Item	Amount	Catalog no.
One Shot® TOP10 Electrocomp™ *E. coli*	10 reactions	C4040-50
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
Lipofectamine™ 2000 Reagent	1.5 ml	11668-019
	0.75 ml	11668-027
Calcium Phosphate Transfection Kit	75 reactions	K2780-01
Blasticidin S HCl	50 mg	R210-01
S.N.A.P.™ MiniPrep Kit	25 reactions	K1900-25
	100 reactions	K1900-01
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
ThermalAce™ DNA Polymerase	200 units	E0200
	1000 units	E1000
PCR Optimizer™ Kit	100 reactions	K1220-01

Introduction

Overview

Introduction

The pUB/Bsd TOPO® Cloning Kit provides a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of blunt-end PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Once cloned, analyzed, and transfected, the PCR product can be stably maintained in mammalian cell lines.

pUB/Bsd-TOPO®

pUB/Bsd-TOPO® is a 4.3 kb vector derived from pUB/Bsd. The vector contains the following elements:

- TOPO®-adapted for rapid cloning of blunt-end PCR products
- Human ubiquitin C promoter (hUbC) for expression of the blasticidin resistance gene across a broad range of species and cell types (Schorpp *et al.*, 1996; Wulff *et al.*, 1990) (see page 22 for more information).
- EM7 synthetic promoter for expression of the blasticidin resistance gene in *E. coli*
- Blasticidin resistance gene for selection of stable cell lines
- The ampicillin (*bla*) resistance gene for selection in *E. coli*
- pUC origin for high copy replication and maintenance of the plasmid in *E. coli*

For a map and more details on pUB/Bsd-TOPO®, please see pages 20-21.

Important

The pUB/Bsd-TOPO® vector does not contain any elements to allow expression of your PCR product in mammalian cells. If you need to express your PCR product in a mammalian cell line of choice, the PCR product must contain a suitable eukaryotic promoter and a polyadenylation sequence.

Applications of pUB/Bsd TOPO®

The pUB/Bsd-TOPO® vector allows you to:

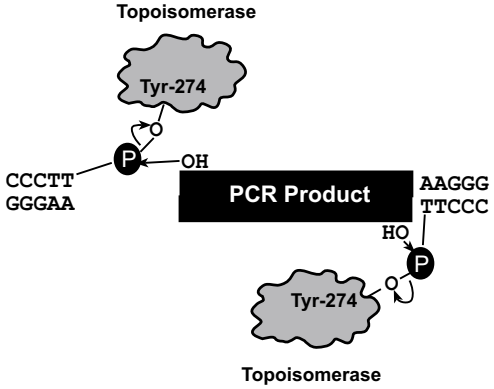
- Maintain an archival copy of your PCR product
- Create stable cell lines containing your PCR product
- Clone your TOPO® Tools linear DNA construct
- Sequence your PCR product

Continued on next page

How TOPO® Cloning Works

The plasmid vector (pUB/Bsd-TOPO®) is supplied linearized with *Vaccinia* virus DNA topoisomerase I covalently bound to the 3′ end of each DNA strand (referred to as "TOPO®-activated" vector).

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products (see below). The TOPO® Cloning Reaction can be transformed into chemically competent cells or electroporated directly into electrocompetent cells.



Once the PCR product is cloned into the pUB/Bsd-TOPO® vector and transformants analyzed for the presence of the insert, the plasmid can be transfected into mammalian cells and used for any downstream applications.

Continued on next page

Experimental Outline

The table below outlines the experimental steps necessary to TOPO® Clone your PCR product into the pUB/Bsd-TOPO® vector. Please refer to the indicated pages for more details on each step.

Step	Action	Page
1	Amplify your gene of interest with a thermostable proofreading polymerase. If you are using the linear DNA construct from TOPO® Tools technology, you can directly clone your PCR product obtained after secondary amplification into the pUB/Bsd-TOPO® vector.	4
2	Verify the integrity and concentration of your PCR product.	5
3	TOPO® Clone your PCR product into the pUB/Bsd-TOPO® vector.	6
4	Transform the TOPO® Cloning reaction into One Shot® TOP10 Chemically Competent *E. coli.*	8
5	Analyze transformants by restriction digestion or PCR.	10
6	Select the correct clone and isolate plasmid DNA for transfection.	13
7	Transfect your construct into a mammalian cell line of choice.	13
8	Generate a stable cell line, if desired.	16

Methods

Producing PCR Products

Introduction

To produce your PCR product, you will need to decide on a PCR strategy and synthesize appropriate primers. The sequence surrounding the TOPO® Cloning site is provided below to help you design the PCR primers. Please note that if you need to express your PCR product in mammalian cells, the PCR product must contain a suitable eukaryotic promoter and a polyadenylation sequence.

If you are TOPO® Cloning your linear DNA construct generated using the TOPO® Tools technology, you do not need to perform this step. Use the linear DNA construct obtained after performing the TOPO® Tools procedure and proceed directly to **TOPO® Cloning your PCR Product**, page 6.

Materials Supplied by the User

You will need the following reagents and equipment:

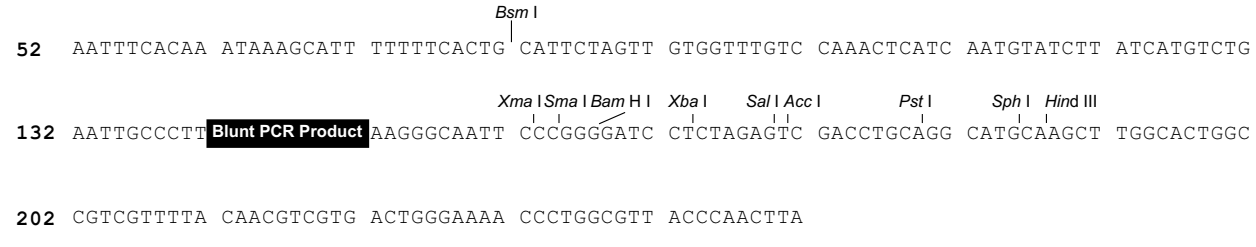
- Thermostable proofreading DNA polymerase (see below)
- Thermocycler
- DNA template and primers for the PCR product

Thermostable DNA Polymerase

Please note that pUB/Bsd-TOPO® is a blunt vector. You need to use a proofreading polymerase for amplification to generate blunt-end PCR product. We recommend using ThermalAce™ DNA Polymerase (see page vi for ordering information). **Note:** You will obtain a lower TOPO® Cloning efficiency, if you are using a mixture of *Taq* and a proofreading DNA polymerase to produce your
PCR product.

TOPO® Cloning Site

The figure below illustrates the TOPO® Cloning site of pUB/Bsd-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 141-142. This is the TOPO® Cloning site. **Please note that the full sequence of pUB/Bsd-TOPO® may be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (see page 28).** A map of pUB/Bsd-TOPO® is provided on pages 20-21.



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PCR Reaction

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Primers	100-200 ng each
Sterile water	add to a final volume of 49 µl
Thermostable Proofreading DNA Polymerase	1 µl
Total Volume	50 µl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band, please see the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR reaction, you may gel-purify your fragment before using the pUB/Bsd-TOPO® vector. 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 vi) from Invitrogen can help you optimize your PCR. See Technical Service for more information (page 28).

TOPO® Cloning your PCR Product

Introduction

TOPO® Cloning technology allows you to ligate your PCR product into pUB/Bsd-TOPO® and transform the recombinant vector into TOP10 *E. coli* in one day. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. At this point you should have already performed the following steps:

- Amplified your gene of interest using a proofreading DNA polymerase
- Verified the integrity and concentration of your PCR product

If this is the first time you are using the TOPO® Cloning technology, perform the control reactions on page 18 in parallel with your sample.

Before Starting

- You should have a single discrete band corresponding to the correct size of your PCR product after amplification. If you do not see a single band, please refer to the **Note** on the previous page.
- pUB/Bsd-TOPO® vector contains blasticidin and ampicillin resistance markers. If you use a plasmid template to generate your PCR product that carries either the ampicillin or blasticidin resistance marker, we recommend purifying your PCR product or using a selection agent not contained in the plasmid template to select for transformants. For example, if the plasmid template contains the blasticidin resistance marker, then use ampicillin to select for transformants.

Salt Solution

Recent experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction results in the following:

- A 2- to 3-fold increase in the number of transformants.
- Allows for longer incubation times (up to 30 minutes). Longer incubation times can result in an increase in the number of transformants obtained.

Including salt in the TOPO® Cloning reaction prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

If you do **not** include salt in the TOPO® Cloning reaction, the number of transformants obtained generally decreases as the incubation time increases beyond 5 minutes.

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. Please note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see next page). For this reason two different TOPO® Cloning reactions are provided to help you obtain the best possible results.

Continued on next page

TOPO® Cloning your PCR Product, Continued

Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt must be reduced to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during elextroporation. The Salt Solution is diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see below).

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for transformation into either One Shot® TOP10 Chemically Competent *E. coli* provided in the kit or electrocompetent *E. coli*. For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (*e.g.* add 5 µl of the Salt Solution to 15 µl sterile water). Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 12. **Note**: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl

*Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature (22 - 25°C).
   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb), increasing the reaction time will yield more colonies.
2. Place the reaction on ice and proceed to **One Shot® TOP10 Chemical Transformation** (see page 9).
   Note: You may store the TOPO® Cloning reaction at -20°C overnight.

Transforming the TOPO® Cloning Reaction

Introduction

Once you have TOPO® cloned your PCR product into the pUB/Bsd-TOPO® vector, you are ready to transform the TOPO® Cloning reaction into One Shot® TOP10 Chemically Competent *E. coli* provided in the kit. If you wish to use electroporation, you will need electrocompetent cells. Please note that the TOPO® Cloning reaction contains salt solution which may cause arcing if you are using electroporation. Please refer to the **Note** on the next page for some tips to prevent arcing.

Materials Supplied by the User

In addition to general microbiological supplies (i.e. plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath
- Low Salt LB plates containing 100 µg/ml blasticidin or LB plates containing 100 µg/ml ampicillin, two for each transformation (see page 24 for a recipe)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Individual recombinant plasmids need to be analyzed by restriction analysis, PCR, or sequencing for the presence of insert.

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® cells for each transformation.

Important

For selection of blasticidin-resistant *E. coli*, use Low Salt LB medium containing 100 µg/ml blasticidin (see page 24 for a recipe).

Please note that the salt concentration of the medium must remain low (<90 mM) and the pH should not exceed 7.0. Failure to lower the salt content of your LB medium will result in non-selection due to inhibition of the drug unless a higher concentration of blasticidin is used.

Continued on next page

Transforming the TOPO® Cloning Reaction, Continued

One Shot® TOP10 Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from Step 2, page 7 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion (see page 12).
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Proceed to **Analyzing Positive Clones**, next page.

Note

When transforming electrocompetent *E. coli*, addition of the Dilute Salt Solution in the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol-precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Continued on next page

Transforming the TOPO® Cloning Reaction, Continued

Analyzing Positive Clones

1. Pick 10 colonies and culture them overnight in Low Salt LB medium containing 100 µg/ml blasticidin or LB medium containing 50 µg/ml ampicillin (3-5 ml).
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using the S.N.A.P.™ MiniPrep Kit (see page vi for ordering information).
3. Analyze the plasmids for the presence of the insert using restriction analysis, PCR, or sequencing. Please refer to the diagram on page 4 for restriction sites and sequence surrounding the TOPO Cloning® site. For the complete sequence of the vector, please see our Web site (www.invitrogen.com) or contact Technical Service (page 28).

   If you need help with setting up restriction enzyme digests or DNA sequencing, please refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. Choose the appropriate PCR primers for your PCR product. You will have to determine the amplification conditions for your PCR product.

If this is the first time you have used this technique, we recommend that you perform restriction analysis in parallel to confirm that PCR gives you the correct result. Artifacts may be obtained because of mispriming or contaminating template.

The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and a thermostable polymerase. Use 20 µl reaction volume. Multiply by the number of colonies to be analyzed (*e.g.* 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. (Do not 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 to 30 cycles using parameters previously determined.
5. For the final extension, incubate at 72°C for 10 minutes.
6. Visualize by agarose gel electrophoresis.

Continued on next page

Important

If you have problems obtaining transformants, perform the control reactions described on page 18. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to isolate a single colony and prepare 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 Low Salt LB plates containing 100 µg/ml blasticidin or LB plates containing 50 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of low salt LB containing 100 µg/ml blasticidin or LB containing 50 µg/ml ampicillin. Grow until the culture reaches stationary phase.
3. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
4. Store at -80°C.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high cloning efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products or toxic genes, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.
- Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Transfection Guidelines

Introduction

Once you have the desired construct, you are ready to transfect the plasmid into the mammalian cells of choice. Please note the following guidelines for transfection.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipids, decreasing transfection efficiency. We recommend isolating plasmid DNA using the S.N.A.P.™ MidiPrep Kit (see page vi) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (e.g. HeLa), please consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). If you wish to use a lipid-based reagent for transfection of adherent or suspension cells, we recommend using Lipofectamine™ 2000 Reagent available from Invitrogen (see page vi for ordering information). For more information on other transfection reagents available from Invitrogen, please visit our Web site (www.invitrogen.com) or call Technical Service (see page 28).

Expressing your PCR Product

Introduction

If your PCR product contains an appropriate eukaryotic promoter and a polyadenylation sequence, you can express your PCR product in any mammalian cell line of choice. Expression of your PCR product can be performed in either transiently transfected cells or stable cell lines (see **Creating Stable Cell Lines**, page 16). You may use a functional assay to detect the protein encoded by your PCR product or a Western blot analysis if you have an antibody to the protein.

We recommend testing 3-4 clones for expression and selecting the clone that expresses your protein of interest at high levels for purification or creating stable cell lines.

Detecting Proteins

To detect the protein by Western blot, you will need to prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of the protein (e.g. 24, 48, 72 hours, etc. after transfection).

A large selection of unconjugated and conjugated secondary antibodies is available from Invitrogen for detecting your recombinant protein containing a suitable epitope tag. Please visit our Web site (www.invitrogen.com) or call Technical Service (see page 28) for more details.

Preparing Cell Lysates

To detect your protein by Western blot, you need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols are suitable. To lyse cells:

1. Wash cell monolayers (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (PBS, see page 24 for a recipe).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see page 24 for a recipe).
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.
   Note: You may lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes at +4°C to pellet the nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration.
   Note: Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer to a final concentration of 1X and boil the samples for 5 minutes.
7. Load 20 µg of the lysate onto an SDS/PAGE gel and electrophorese according to the manufacturer's recommendation. Use the appropriate percentage of acrylamide to resolve your protein of interest.

Continued on next page

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® Novex and Novex® Tris-Glycine polyacrylamide gels are available from Invitrogen. The NuPAGE® gel electrophoresis system avoids the protein modifications associated with Laemmli-type SDS-PAGE, ensuring optimal separation for protein analysis. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use for visualizing your protein, please refer to our Web site at (www.invitrogen.com) or call Technical Service (see page 28).

Creating Stable Cell Lines

Introduction

The pUB/Bsd-TOPO® vector contains the blasticidin resistance gene (*bsd*) to allow for selection of stable cell lines using blasticidin. If you wish to create stable cell lines, transfect your pUB/Bsd-TOPO® vector into a mammalian cell line of choice and select for stable transfectants using blasticidin. General information and guidelines are provided below for your convenience. For more information on blasticidin and its mechanism of action, see page 23.

Blasticidin Selection Guidelines

Blasticidin is available from Invitrogen (see page vi for ordering information). Use the antibiotic as follows:

- Prepare a stock solution of 5-10 mg/ml of blasticidin in sterile water and filter-sterilize the solution.
- Test varying concentrations of blasticidin on your cell line to determine the concentration that kills your cells (see below).
- Use 2-10 µg/ml of blasticidin in complete medium depending on your cell line.

Effect of blasticidin should be visible within 3-7 days and complete selection can take up to 7 days depending on the concentration of blasticidin in the medium.

Determination of Blasticidin Sensitivity

To successfully generate a stable cell line, you need to determine the minimum concentration of blasticidin required to kill your untransfected host cell line. We recommend that you test a range of concentrations to ensure that you determine the minimum concentration necessary for your host cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Prepare a set of 6 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of blasticidin (0, 2, 4, 6, 8, and 10 µg/ml blasticidin).
3. Replenish the selective media every 2-3 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of blasticidin that prevents growth within 7 days after addition of blasticidin.

Continued on next page

Creating Stable Cell Lines, Continued

Possible Sites for Linearization

To obtain stable transfectants, we recommend linearizing your pUB/Bsd-TOPO® construct before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the chances that the vector will integrate in a way that does not disrupt the gene of interest or other elements required for selection or expression in mammalian cells. The table below lists unique sites that may be used to linearize your construct before transfection. Other restriction sites are possible. **Be sure that your insert does not contain the restriction site you wish to use to linearize your vector.**

Enzyme	Location	Supplier
Sca I	Ampicillin gene	Invitrogen (Catalog no. 15436-017)
*Alw*N I	pUC origin	Invitrogen (Catalog no. 45200-029)
*Eco*R V	Backbone	Invitrogen (Catalog no. 15425-010)
Apa I	Backbone	Invitrogen (Catalog no. 15440-019)
Not I	Backbone	Invitrogen (Catalog no. 15441-025)
Sac I	Backbone	Invitrogen (Catalog no. 15222-011)

Selecting Stable Integrants

Once you have determined the appropriate concentration of blasticidin to use for selection, you can generate a stable cell line carrying your pUB/Bsd-TOPO® construct.

1. Transfect mammalian cells with your pUB/Bsd-TOPO® construct using the desired protocol. Remember to include a plate of untransfected cells as a negative control.
2. After 24 hours of transfection, wash the cells and add fresh medium to the cells.
3. Split the cells into fresh medium after 48 hours of transfection. Split the cells such that they are no more than 25% confluent. If the cells are too dense, the blasticidin will not kill the cells. Blasticidin works best on actively dividing cells.
4. Incubate the cells at 37°C for at least 2-3 hours until they have attached to the culture dish.
5. Remove the medium and add fresh medium containing blasticidin at the pre-determined concentration required for your cell line.
6. Feed the cells with selective medium every 3-4 days until foci can be identified.
7. Pick and expand at least 20 foci to test for expression of the protein of interest.

Appendix

pUB/Bsd-TOPO® Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions using the reagents included in the kit involves producing a control PCR product containing the *lac* promoter and the LacZα protein. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing ampicillin and X-gal.

Before Starting

Be sure to prepare the following reagents before performing the control reaction:

- 40 mg/ml X-gal in dimethylformamide (see page 24 for recipe)
- LB plates containing 100 µg/ml ampicillin and X-gal (two per transformation)
- To add X-gal to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

Producing the Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter and LacZα, set up the following 50 µl PCR:

Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg/µl each)	2 µl
Sterile Water	40.5 µl
Thermostable Proofreading DNA Polymerase	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if necessary.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	60°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

Continued on next page

pUB/Bsd-TOPO® Control Reactions, Continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pUB/Bsd-TOPO© vector set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution or Dilute Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pUB/Bsd-TOPO© vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10 *E. coli* (page 8).
4. Spread 25-100 µl of each transformation mix onto LB plates containing 100 µg/ml ampicillin and X-gal. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
5. Incubate overnight at 37°C.

Analyzing Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 85% of these will be blue and contain the 500 bp insert.

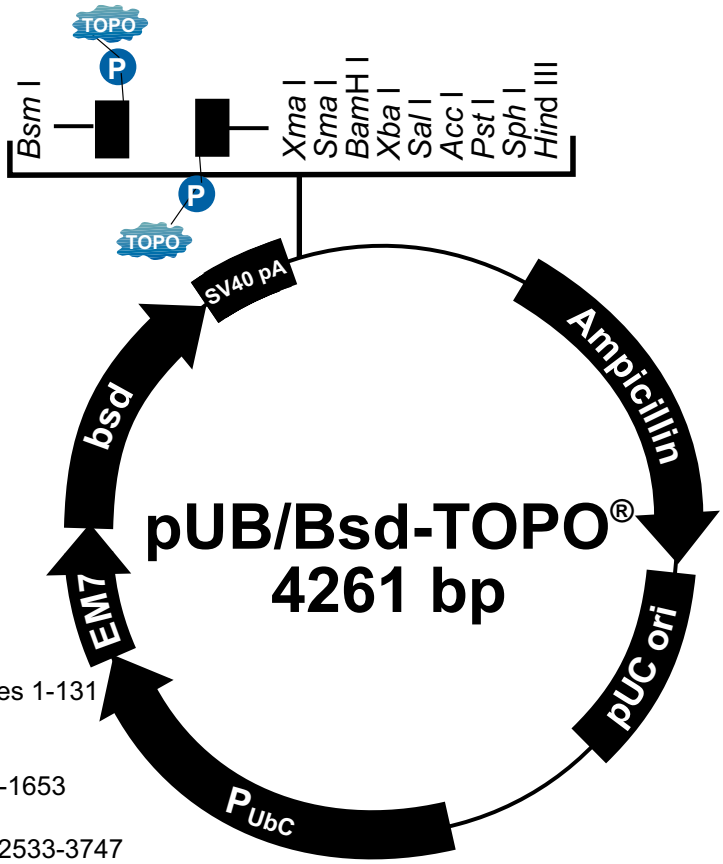
Transformation Control

The pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 Chemically Competent *E. coli*. Transform one vial of One Shot® TOP10 *E. coli* with 10 pg of pUC19 using the protocol on page 8. Plate 10 µl of the transformation mixture plus 20 µl SOC on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

pUB/Bsd-TOPO©

Map

The figure below summarizes the features of the pUB/Bsd-TOPO© vector. The vector is supplied linearized between base pairs 141-142. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from our Web site at (www.invitrogen.com) or from Technical Service (page 28).**



Comments for pUB/Bsd-TOPO®
4261 nucleotides

SV40 early polyadenylation sequence: bases 1-131
TOPO® cloning site: bases 141-142
bla promoter: bases 694-798
Ampicillin (*bla*) resistance gene: bases 793-1653
pUC origin: bases 1798-2471
Human ubiquitin C (*UbC*) promoter: bases 2533-3747
EM7 promoter: bases 3753-3803
Blasticidin (*bsd*) resistance gene: bases 3822-4220

Continued on next page

Features of pUB/Bsd-TOPO©

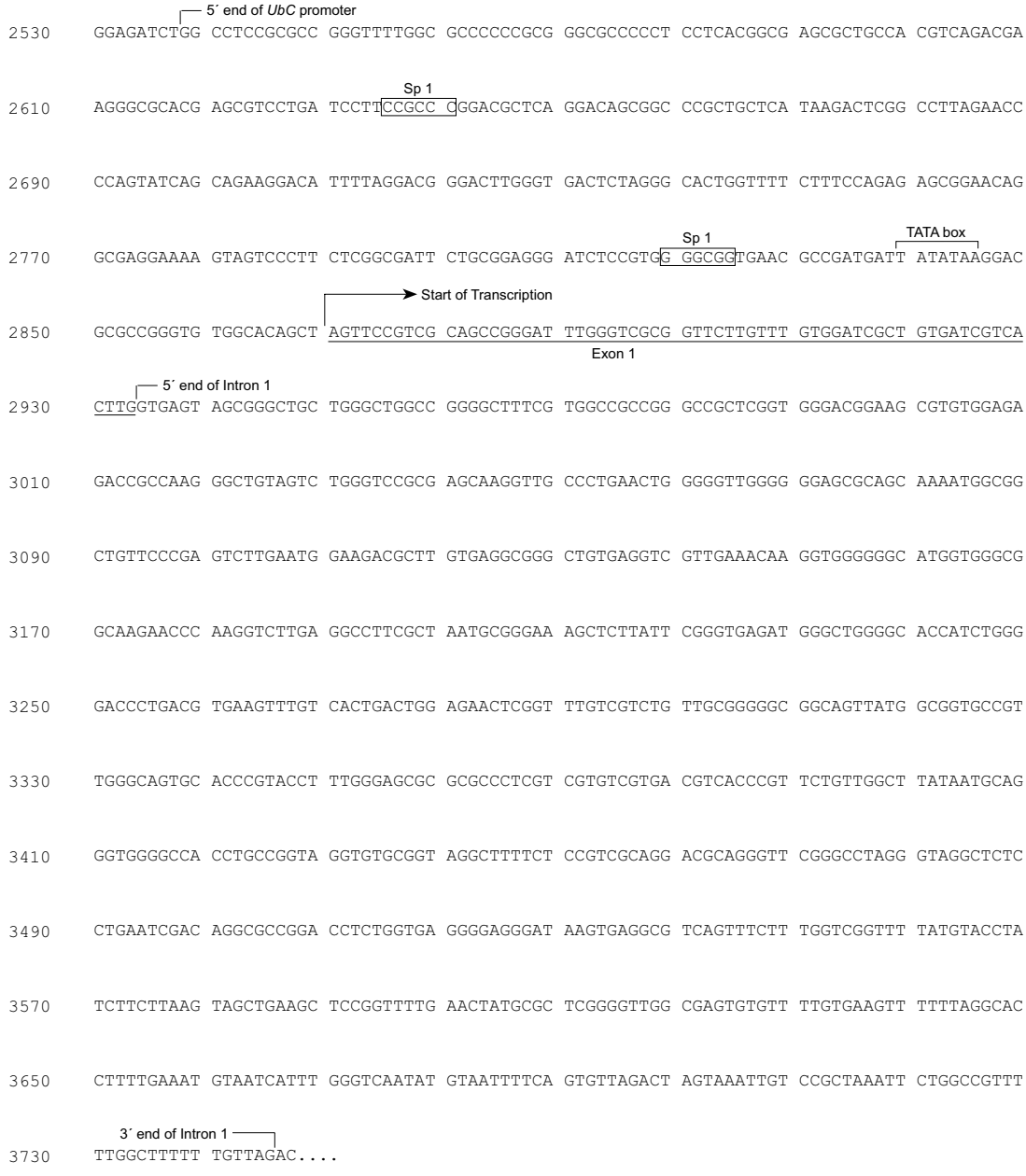
pUB/Bsd-TOPO© contains the following elements. All features have been functionally tested.

Feature	Benefit
TOPO® Cloning site	Allows insertion of your PCR product.
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene.
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli.*
pUC origin	High-copy number replication and growth in *E. coli.*
Human ubiquitin C (UbC) promoter	Allows expression of the blasticidin resistance gene in mammalian cells. This promoter drives expression of the human ubiquitin C gene (Nenoi *et al.*, 1996; Schorpp *et al.*, 1996).
EM7 promoter	Synthetic promoter based on the bacteriophage T7 promoter for expression of the blasticidin resistance gene in *E. coli.*
Blasticidin (*bsd*) resistance gene	Allows selection by blasticidin in *E. coli*, yeast, plants, and mammalian hosts (Kimura *et al.*, 1994; Kimura and Yamaguchi, 1996).
SV40 early polyadenylation sequence	Allows efficient transcription termination and polyadenylation of mRNA in mammalian cells.

Human Ubiquitin Promoter

Description

The human UbC promoter allows high-level expression of recombinant protein in most mammalian cell lines (Wulff *et al.*, 1990) and in virtually all tissues tested in transgenic mice (Schorpp *et al.*, 1996). The diagram below shows the features of the UbC promoter used in pUB/Bsd TOPO® vector. Features are marked as per Nenoi, *et al.*, 1996 .



Blasticidin

Description

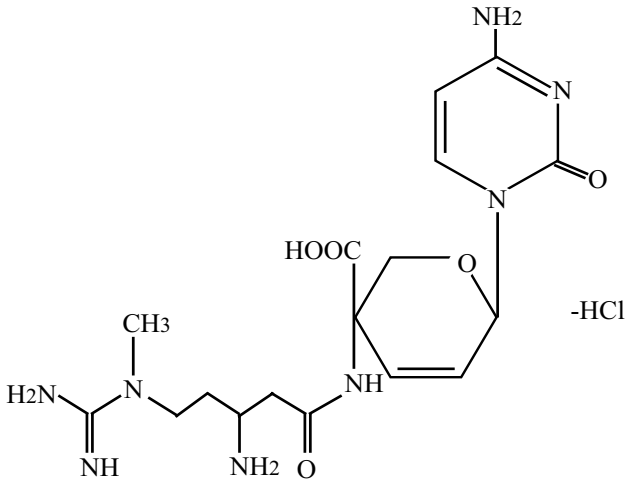
Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *BSD* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

Merck Index: 12: 1350

MW: 458.9

Formula: $C_{17}H_{26}N_8O_5$-HCl



Handling Blasticidin

Always wear gloves, mask, goggles, and a laboratory coat when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

- Blasticidin is soluble in water and acetic acid.
- Prepare a stock solution of 5 to 10 mg/ml blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use and freeze at -20°C for long-term storage or store at +4°C for short term storage.
- Aqueous stock solutions are stable for 1 week at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should not exceed 7 to prevent inactivation of blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and discard the unused portion.
- Medium containing blasticidin may be stored at +4°C for up to 2 weeks.

Recipes

Low Salt LB Medium and Plates with Blasticidin

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

1. Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust pH to 7.0 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding blasticidin to 100 µg/ml final concentration.
4. Store plates at +4°C in the dark. Plates with blasticidin are stable up to 2 weeks.

LB medium and Plates with Ampicillin

10 g Tryptone
10 g NaCl
5 g Yeast Extract
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. For plates, add 15 g/L agar before autoclaving.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add the appropriate concentration of ampicillin.
4. Store plates at +4°C in the dark.

Cell Lysis Buffer

50 mM Tris
150 mM NaCl
1% Nonidet P-40
pH 7.8

1. This solution can be prepared from the following stock solutions. For 100 ml, combine:

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

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

1 mM PMSF
1 µg/ml pepstatin
1 µg/ml leupeptin

Continued on next page

X-gal Stock Solution

1. To make a 40 mg/ml stock solution, dissolve 400 mg X-gal in 10 ml dimethylformamide.
2. Protect from light by storing in a brown bottle at -20°C.

To add to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

Phosphate-Buffered Saline (PBS)

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
pH 7.4

1. Dissolve the following in 800 ml of deionized water:
   - 8 g NaCl
   - 0.2 g KCl
   - 1.44 g Na_2HPO_4
   - 0.24 g KH_2PO_4
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter and autoclave for 20 minutes on liquid cycle.
4. Store at +4°C or room temperature.

Product Qualification

Introduction

Invitrogen qualifies the pUB/Bsd-TOPO® Cloning Kit as described below.

Vectors

The pUB/Bsd (parental vector of pUB/Bsd-TOPO©) is qualified by restriction enzyme digestion. The pUB/Bsd plasmid is qualified prior to adaptation with topoisomerase. The table below lists the restriction enzymes and the expected fragments. Please note that restriction sites used to qualify the parental vector may no longer be present in the topoisomerase-adapted vector.

Restriction Enzyme	pUB/Bsd
*Eco*R V	4245 bp
*Hin*d III	4245 bp
Pvu II	3747 bp, 498 bp
Xho I	4245 bp

TOPO® Cloning Efficiency

Once the pUB/Bsd vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in the kit. Under conditions described on page 18, a 500 bp control PCR product is TOPO® Cloned into pUB/Bsd-TOPO® and subsequently transformed into the One Shot® TOP10 Chemically Competent *E. coli* included with the kit.

Each lot of vector must yield greater than 85% cloning efficiency.

One Shot® TOP10 Competent *E. coli*

All competent cells are tested for transformation efficiency using the control plasmid included in the One Shot® kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1×10^9 cfu/µg plasmid DNA.

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and lack of phage contamination.

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Blasticidin and the Blasticidin Selection Marker

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Technical Service

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Technical Service, Continued

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