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pEF6/V5-His TOPO® TA Expression Kit

Five-minute cloning of *Taq* polymerase-amplified PCR products for high-level expression in mammalian cells

Catalog no. K9610-20

Rev. Date: 28 June 2010
Manual part no. 25-0279

MAN0000111

User Manual

Table of Contents

Fast Start

Introduction

The procedure below is designed to get experienced users quickly started with the pEF6/V5-His TOPO® TA Expression Kit. Information is provided elsewhere in the manual if you need help with any of the steps.

Before Starting

- Determine a strategy for PCR (pages 3–4) and generate the PCR product containing your gene of interest (page 5).
- Prepare LB plates containing 50–100 µg/mL ampicillin (see page 28). Store the plates at 4°C. Prior to transformation, warm the plates at 37°C for 30 minutes (2 plates for each transformation).
- Prepare or purchase chemically competent or electrocompetent TOP10 cells. For convenient high-efficiency transformation, we recommend One Shot® TOP10 Chemically Competent *E. coli* (supplied with the kit) or One Shot® TOP10 Electrocompetent Cells, which are available separately from Invitrogen (see page 30 for ordering information). Prior to transformation, thaw the competent cells on ice (1 vial of cells for each transformation).
- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator (if you are using 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).
- Warm the vial of SOC medium from Box 2 to room temperature.

TOPO® Cloning Reaction

1. Set up your TOPO® Cloning reaction (6 µL) according to the table below. Mix the reaction gently and incubate it for 5 minutes at room temperature.
   Note: See page 11 for additional information on optimizing the TOPO® Cloning reaction for your needs.

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

*Store all reagents at –20°C when finished. Store the salt solutions and water at room temperature or 4°C.

2. Place the reaction on ice and proceed to **Transformation** (next page).
   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Continued on next page

Transformation

1. Add 2 µL of each TOPO® Cloning reaction to a separate tube of competent cells (40–50 µL), and transform using your method of choice (see page 8).
2. Spread 10–50 µL from each transformation on a pre-warmed selective plate and incubate the plates overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate has well-spaced colonies.
3. Proceed to **Analyzing Positive Clones**, below.

Analyzing Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 50 µg/mL ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification or PureLink™ HiPure Plasmid Miniprep kits (see page 30 for ordering information).
3. Analyze the plasmids by restriction analysis or by sequencing. The T7 Promoter and BGH Reverse sequencing primers are included to help you sequence your insert. Refer to the diagram on page 4 for the sequence surrounding the TOPO® Cloning site. For the complete sequence of the vector, visit www.invitrogen.com or contact Technical Support (see page 32).

Long-Term Storage

After you have identified the correct clone, purify the colony and make a glycerol stock for long term storage. Keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB agar plate containing 50–100 µg/mL ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50–100 µg/mL ampicillin.
3. Grow the cells until the culture reaches stationary phase (OD_{600} = 1–2).
4. Mix 0.85 mL of the culture with 0.15 mL of sterile glycerol and transfer the mix to a cryovial.
5. Store the glycerol stocks at –80°C.

Kit Contents

Shipping/Storage

The pEF6/V5-His TOPO® TA Expression Kit is shipped on dry ice. Each kit contains a box with pEF6/V5-His TOPO TA Cloning® reagents (Box 1) and a box with One Shot® TOP10 chemically competent cells (Box 2).

Store Box 1 at –20°C and Box 2 at –80°C.

pEF6/V5-His TOPO TA Cloning® Reagents

pEF6/V5-His TOPO TA Cloning® reagents (Box 1) are listed below. **Note that the kit does not contain *Taq* polymerase.**

Store at –20°C.

Item	Composition	Amount
pEF6/V5-His-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 phenol red	20 µL
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 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
T7 Promoter Primer	0.1 µg/µL in TE Buffer, pH 8.0*	20 µL
BGH Reverse Primer	0.1 µg/µL in TE Buffer, pH 8.0	20 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
Expression Control Plasmid (pEF6/V5-His-TOPO®/*lacZ*)	0.5 µg/µL in TE Buffer, pH 8.0	10 µL

*__TE buffer, pH 8.0__: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

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Kit Contents, continued

One Shot® Reagents

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

Store at –80°C.

Item	Composition	Amount
SOC Medium (may be stored at room temperature or 4°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

Sequencing Primers

The table below provides the sequence and pmoles of the T7 Promoter primer and the BGH Reverse primer. Two micrograms of each primer are supplied.

Primer	Sequence	pMoles Supplied
T7 Promoter	5´-TAATACGACTCACTATAGGG-3´	328
BGH Reverse	5´-TAGAAGGCACAGTCGAGG-3´	358

Genotype of TOP10 Cells

Use this strain for general cloning of PCR products in pEF6/V5-His-TOPO®. Note that this strain cannot be used for single-strand rescue of DNA.

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

Methods

Description of the System

System Overview

The pEF6/V5-His TOPO® TA Expression Kit provides a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for high-level expression in mammalian cells. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Once cloned, analyzed, and transfected into a mammalian host cell line, the PCR product can be constitutively expressed.

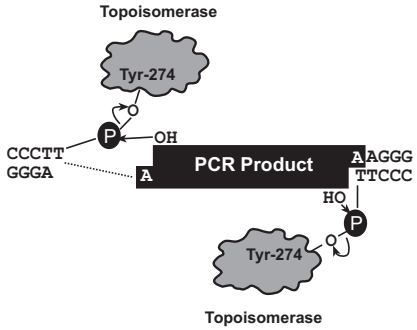
How It Works

The plasmid vector (pEF6/V5-His-TOPO®) is supplied linearized with:

- Single 3´ thymidine (T) overhangs for TA Cloning®
- Topoisomerase covalently bound to the vector (this is referred to as "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the 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).



Once the PCR product is cloned into pEF6/V5-His-TOPO® and transformants analyzed for the correct orientation, the plasmid may be transfected into the mammalian cell line of choice. The strong human EF-1α promoter in pEF6/V5-His-TOPO® allows high-level expression of your PCR product across a broad range of cell types (Goldman *et al.*, 1996; Mizushima and Nagata, 1990). The PCR product may be expressed as a fusion to the C-terminal V5 epitope and polyhistidine (6×His) tag for detection and purification; or, by designing the 3´ PCR primer with a stop codon, the PCR product may be expressed as a native protein.

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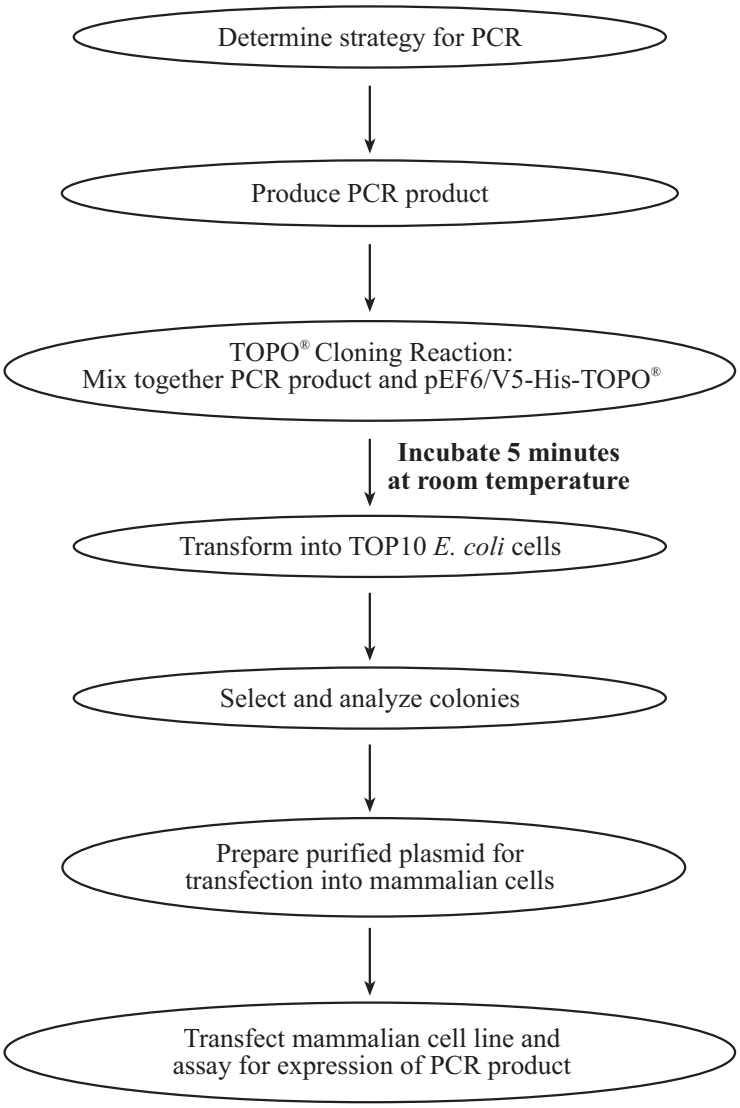
Experimental Outline

The flow chart below outlines the experimental steps necessary to clone and express your PCR product



Designing PCR Primers

Designing Your PCR Primers

The cloning of a PCR product into a pEF6/V5-His-TOPO® vector is a rapid and efficient process. However, to ensure proper expression of your recombinant protein, it is important to pay attention to the general considerations outlined below:

- Design of PCR primers to clone your PCR product of interest is critical for expression. The pEF6/V5-His-TOPO® vector is a C-terminal fusion vector that does not contain an ATG initiation codon. If there is no ATG start codon or optimal sequences for translation initiation (Kozak sequences) in the DNA to be amplified, then these features need to be incorporated into your forward primer (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

  (G/A)NN<u>ATG</u>**G**

- Clone in frame with the V5 epitope and polyhistidine tag (C-terminal peptide) in order to detect and/or purify your fusion PCR product.

  OR

- Include the native stop codon to express the native protein.
- Do **not** add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into the pEF6/V5-His-TOPO® vector.
- Cloning efficiencies may vary depending on the primer nucleotide sequence (see **Factors Affecting Cloning Efficiency**, page 19).
- Use the diagram on the next page to design your PCR primers. After you have designed your PCR primers, proceed to **Producing PCR Products**, page 5.

Note

The MembranePro™ Functional Protein Expression System is optimized for use with the pEF6 vector. However, cloning your gene into the pEF6/V5-His-TOPO® vector without a stop codon and in frame with the polylinker will result in a fusion protein with V5 and polyhistidine (6×His) tags on the C-terminus of your protein. As the C-terminus of your transmembrane protein will likely be inside the VLP, these tags will be inaccessible to purification resins and antibodies. In theory, these tags could be used to identify and isolate a fusion membrane protein after denaturing the VLP; however, the MembranePro™ Functional Protein Expression System does not support using the tags for extraction and purification.

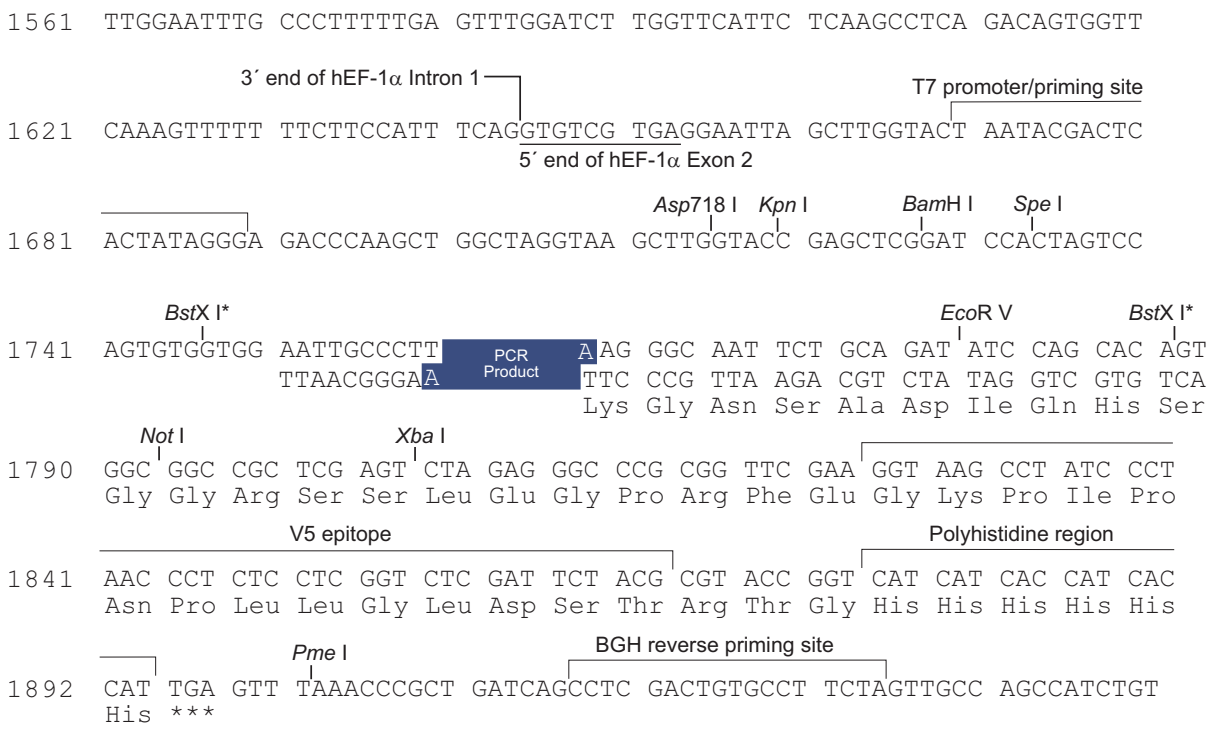
Continued on next page

Designing PCR Primers, continued

TOPO® Cloning Site of pEF6/ V5-His-TOPO®

The diagram below is supplied to help you design the appropriate PCR primers to correctly clone and express your PCR product. Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 1,760 and 1,761. This is the TOPO® Cloning site.

For a map and a description of the features of pEF6/V5-His-TOPO®, refer to the **Appendix**, pages 25–26. **The complete sequence of pEF6/V5-His-TOPO® is available for downloading at www.invitrogen.com or by contacting Technical Support (see page 32).**



*Note that there are two *BstX* I sites flanking the TOPO® Cloning site.

Producing PCR Products

Materials Needed

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, you must use *Taq* in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

If you use polymerase mixtures that do not have enough *Taq* polymerase or only have a proofreading polymerase, you can add 3′ A-overhangs to your PCR product post-amplification using the method on page 22.

Producing PCR Products

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 and 3´ adenylated.

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
Taq Polymerase (1 unit/µL)	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, refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the pEF6/V5-His TOPO® TA Expression Kit (see page 20). 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 from Invitrogen can help you optimize your PCR (see page 30 for ordering information). For more information, visit www.invitrogen.com or contact Technical Support (page 32).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR product, ligate it into pEF6/V5-His-TOPO®, and transform the recombinant vector into *E. coli* all in one day. It is important to have everything you need set up and ready to use to ensure you obtain the best possible results. If this is the first time you have TOPO® Cloned, you may wish to perform the control reactions on pages 17–18 in parallel with your samples.

Note

Recent experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. We have also observed that in the presence of salt, incubation times of greater than 5 minutes can increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Inclusion of salt allows for longer incubation times because it 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.

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. **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.** For this reason, two different TOPO® Cloning reactions are provided to help you obtain the best possible results.

Materials Needed

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50–100 µg/mL ampicillin (two for each transformation)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubator
- General microbiological supplies (i.e., plates, spreaders)

Preparation

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

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

Continued on next page

Important

- 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 $MgCl_2$.
- For TOPO® Cloning and transformation of electrocompetent *E. coli*, you **must reduce the amount of salt** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing. Dilute the Salt Solution 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µL) for eventual transformation into chemically competent TOP10 One Shot® *E. coli* (provided) or electrocompetent *E. coli*. See page 11 for additional information on optimizing the TOPO® Cloning reaction for your needs.

Note: The red or yellow color of the TOPO® vector solution is normal and is used to visualize the solution.

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

*Store all reagents at –20°C when finished. Store the salt solutions and water at room temperature or 4°C.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature.

   Note: For most applications, 5 minutes yields plenty of colonies for analysis. Depending on your needs, you can vary the length of the TOPO® Cloning reaction from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time yields more colonies.

2. Place the reaction on ice and proceed to **One Shot® Chemical Transformation** or **Transformation by Electroporation** (next page).

   Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Continued on next page

One Shot® TOP10 Chemical Transformation

1. Add 2 µL of the TOPO® Cloning reaction from Step 2, above, into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate the transformation mix on ice for 5 to 30 minutes.
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 to the transformation mix.
6. Cap the tube tightly and shake it horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10–50 µL from each transformation on a pre-warmed selective plate and incubate the plates overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate has well-spaced colonies.
8. An efficient TOPO® Cloning reaction produces hundreds of colonies. Pick ~10 colonies for analysis (see **Analyzing Transformants**, next page).

Transformation by Electroporation

1. Add 2 µL of the TOPO® Cloning reaction into a 0.1 cm cuvette containing 50 µL of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid forming bubbles.**
2. Electroporate your samples using your own protocol and your electroporator. If you have problems with arcing, see **Note** below.
3. Immediately add 250 µL of room temperature SOC medium to the transformations.
4. Transfer the solution to a 15 mL snap-cap tube (i.e., Falcon) and shake for at least 1 hour at 37°C to allow the expression of the antibiotic resistance gene.
5. Spread 10–50 µL from each transformation on a pre-warmed selective plate and incubate the plates overnight at 37°C. You may add a small amount of SOC to the transformation mix before plating to ensure even spreading of small volumes. We recommend that you plate two different volumes to ensure that at least one plate has well-spaced colonies.
6. An efficient TOPO® Cloning reaction produces hundreds of colonies. Pick ~10 colonies for analysis (see **Analyzing Transformants**, next page).

Note

Adding 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 µL and 80 µL (0.1 cm cuvettes) or 100 µL to 200 µL (0.2 cm cuvettes). If you experience arcing during transformation, try one of the following:

- 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 re-suspend it in water prior to electroporation

Analyzing Transformants

Note

There is no blue-white screening for the presence of inserts. Analyze individual recombinant colonies by restriction analysis or sequencing for the presence and orientation of the insert in pEF6/V5-His-TOPO®. You may use the T7 Promoter and BGH Reverse sequencing primers supplied in the kit to sequence across an insert in the TOPO® Cloning site to confirm that your insert is fused in frame with the C-terminal peptide. Refer to page 5 for the location and sequence of the priming sites.

Analyzing Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 50 µg/mL ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification or PureLink™ HiPure Plasmid Miniprep kits (see page 30 for ordering information). Refer to www.invitrogen.com or contact Technical Support for more information on a large selection of plasmid purification columns.
3. Analyze the plasmids by restriction analysis or by sequencing. The T7 Promoter and BGH Reverse sequencing primers are included to help you sequence your insert. Refer to the diagram on page 4 for the sequence surrounding the TOPO® Cloning site. For the complete sequence of the vector, visit www.invitrogen.com or contact Technical Support (see page 32).

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions to troubleshoot your experiment (see pages 17–18).

Continued on next page

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use a combination of the T7 Promoter or the BGH Reverse sequencing primer with a primer that binds within your insert**. You will have to determine the amplification conditions.** 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 also suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µL reaction volume. Multiply by the number of colonies to be analyzed.
2. Pick 10 colonies and resuspend them individually in 20 µL of the PCR cocktail. **Prepare 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 to inactivate the nucleases.
4. Amplify your samples for 20 to 30 cycles using the amplification conditions you have determined.
5. For the final extension, incubate the reaction at 72°C for 10 minutes. Store at the reactions at 4°C.
6. Visualize the results by agarose gel electrophoresis.

Long-Term Storage

After you have identified the correct clone, purify the colony and make a glycerol stock for long term storage. Keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB agar plate containing 50–100 µg/mL ampicillin. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50–100 µg/mL ampicillin.
3. Grow the cells until the culture reaches stationary phase (OD_{600} = 1–2).
4. Mix 0.85 mL of the culture with 0.15 mL of sterile glycerol and transfer the mix to a cryovial.
5. Store the glycerol stocks at –80°C.

Optimizing the TOPO® Cloning Reaction

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 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 the reaction on ice for only 5 minutes.

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

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, 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. Adding 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

Introduction

After you have confirmed that your construct is in the correct orientation and that it is fused to the C-terminal peptide (if desired), you are ready to transfect your cell line of choice. We recommend that you include the positive control vector (see next page) and a mock transfection to evaluate your results.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants may kill the cells, and salt interferes with lipid complexing, decreasing the transfection efficiency.

When isolating plasmid DNA from *E. coli* strains (such as TOP10) that are wild type for endonuclease 1 (*end*A1+) with commercially available kits, ensure that the Lysis or Resuspension Buffer contains 10 mM EDTA. EDTA inactivates the endonuclease and avoids DNA nicking and vector degradation.

We recommend using the PureLink™ HQ Mini Plasmid Purification or the PureLink™ HiPure Plasmid Miniprep kits for isolating pure plasmid DNA (see page 30 for ordering information). Refer to www.invitrogen.com or contact Technical Support (page 32) for more information on a large selection of plasmid purification columns.

Methods of Transfection

For established cell lines (e.g., HeLa, COS-1), consult the original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow the protocol for your cell line exactly. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. For more information, see *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). Invitrogen offers a large selection of reagents for transfection; for more information on the reagents available, visit www.invitrogen.com or call Technical Support (see page 32).

Lipofectamine™ 2000

The Lipofectamine™ 2000 reagent is a proprietary, cationic lipid-based formulation suitable for the transfection of nucleic acids into eukaryotic cells. It is supplied with the MembranePro™ kits and is also available separately from Invitrogen (see page 30). Using Lipofectamine™ 2000 to transfect eukaryotic cells offers the following advantages:

- You can add the DNA-Lipofectamine™ 2000 complexes directly to cells in culture medium in the presence of serum.
- You do not have to remove the complexes or change or add medium following transfection; however, you may remove the complexes 4–6 hours after transfection without loss of activity.
- Provides the highest transfection efficiency in 293FT cells.

Continued on next page

Positive Control

pEF6/V5-His-TOPO®/*lacZ* is provided as a positive control vector for mammalian transfection and expression and it may be used to optimize transfection and expression conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the control of the human EF-1α promoter. A successful transfection results in β-galactosidase expression that you can be easily assay.

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the β-Gal Assay Kit and the β-Gal Staining Kit for fast and easy detection of β-galactosidase expression (see page 30 for ordering information).

Analyzing Recombinant Protein

Detecting Fusion Proteins

To detect the expression of your fusion protein from pEF6/V5-His-TOPO®, you may use the Anti-V5 Antibodies or the Anti-His(C-term) Antibodies available from Invitrogen (see page 31 for ordering information) or an antibody to your protein of interest.

To detect the fusion protein by Western blot, prepare a cell lysate from the transfected cells. A sample protocol is provided below. Other protocols may also be suitable. Refer to *Antibodies: A Laboratory Manual* (Harlow and Lane, 1988) for additional information. We recommend that you perform a time course to optimize the expression of your fusion protein (e.g., 24, 48, 72 hours, etc. after transfection). To lyse cells:

1. Wash the cell monolayers (~10^6 cells) once with phosphate-buffered saline (PBS, see page 30 for ordering information).
2. Scrape the cells into 1 mL of PBS and centrifuge them at 1,500 × g for 5 minutes to pellet. Discard the supernatant.
3. Resuspend the cell pellet in 50 µL of Cell Lysis Buffer (see **Appendix**, page 29, for a recipe). Other cell lysis buffers are also suitable.
4. Incubate the cell suspension at 37°C for 10 minutes to lyse the cells.
   Note: If degradation of your protein is a potential problem, you may prefer to lyse the cells at room temperature or on ice.
5. Centrifuge the cell lysate at 10,000 × g for 10 minutes to pellet 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 the lysate to a final concentration of 1X and heat the sample at 70°C for 5 minutes.
7. Load 20 µg of the lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine (6×His) tag adds approximately 5 kDa to the size of your protein.

Purification

You need 5 × 10^6 to 1 × 10^7 transfected cells for purifying your protein on a 2 mL ProBond™ column (or other metal-chelating column). Refer to the manufacturer's instructions before attempting to purify your fusion protein. To prepare the cells for lysis, refer to the protocol on page 16.

Creating Stable Cell Lines

Introduction

After you have established that your construct can be expressed in the mammalian cell line of choice, you may wish to generate a stable cell line that overexpresses your protein of interest. The pEF6/V5-His-TOPO® vector contains the blasticidin resistance gene (*bsd*) to allow the selection of stable cell lines using blasticidin (Kimura *et al.*, 1994). For more information about blasticidin, refer to the **Appendix**, page 23.

Determining Antibiotic Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of blasticidin required to kill your untransfected host cell line. Typically, concentrations between 2 µg/mL and 10 µg/mL of blasticidin are sufficient to kill the untransfected host cell line. Test a range of concentrations (see below) to ensure that you determine the minimum concentration of blasticidin necessary to prevent the growth of your untransfected cell line. Refer to the **Appendix**, page 23, for instructions on how to prepare and store blasticidin.

1. Plate or split a confluent plate so that the cells are approximately 25% confluent. Prepare a set of 6 plates.
2. The next day, substitute the culture medium with medium containing varying concentrations of blasticidin (e.g., 0, 1, 3, 5, 7.5, and 10 µg/mL blasticidin).
3. Replenish the selective medium every 3–4 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 1–2 weeks after addition of the antibiotic.

Possible Sites for Linearization

To obtain stable transfectants, you may choose to linearize your pEF6/V5-His-TOPO® construct before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the gene of interest or other elements required for expression in mammalian cells. The table below lists unique sites that may be used to linearize your construct prior to transfection. Other restriction sites are also possible. **Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.**

Enzyme	Restriction Site (bp)	Location
Ssp I	3	Upstream of EF-1α promoter
Aat II	121	Upstream of EF-1α promoter
*Bst*1107 I	3,767	End of SV40 polyA
Sap I	4,030	Backbone
*Eam*1105 I	5,039	Ampicillin gene
Fsp I	5,261	Ampicillin gene
Sca I	5,519	Ampicillin gene

Continued on next page

Selecting Stable Integrants

After you have determined the appropriate concentration of blasticidin to use for selection, generate a stable cell line expressing your pEF6/V5-His-TOPO® construct.

1. Transfect mammalian cells with your pEF6/V5-His-TOPO® construct using the desired protocol. Remember to include a plate of untransfected cells as a negative control.
2. 24 hours after transfection, wash the cells and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing blasticidin at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Feed the cells with selective medium every 3–4 days until you can identify foci.
5. Pick and expand at least 20 foci to test for expression of the protein of interest.

Preparing the Cells for Lysis

Use the procedure below to prepare stably transfected cells for lysis prior to purifying your protein on ProBond™. You need 5×10^6 to 1×10^7 cells for purifying of your protein on a 2 mL ProBond™ column. For more information, refer to the ProBond™ Purification System manual.

1. Seed cells (from a stable cell line) in five T-75 flasks or two to three T-175 flasks.
2. Grow the cells in selective medium until they are 80–90% confluent.
3. Harvest the cells by treating them with trypsin-EDTA or TrypLE™ Express dissociation reagent for 2 to 5 minutes, or by scraping the cells in PBS.
4. Inactivate the dissociation reagent by diluting with fresh medium (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1,500 × g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at –70°C until needed.

Lysing the Cells

If you are using ProBond™ resin, refer to the ProBond™ Purification System manual for details about sample preparation for chromatography. If you are using other metal-chelating resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

pEF6/V5-His TOPO TA Cloning® Control Reactions

Introduction

If you have trouble obtaining transformants or vector containing insert, we recommend performing the following control TOPO® Cloning reactions to help you evaluate your results. Performing the control reactions involves producing a control PCR product containing the *lac* promoter and the α fragment of β-galactosidase using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product yields blue colonies on LB agar plates containing ampicillin and X-gal.

Before Starting

Prepare the following reagents before performing the control reaction:

- 40 mg/mL X-gal in dimethylformamide (see page 28 for a recipe)
- LB plates containing 50–100 µg/mL ampicillin and X-gal (2 plates per transformation)

To add X-gal to previously made agar plates, warm the plate to 37°C. Pipette 40 µL of 40 mg/mL X-Gal stock solution onto the plate. Spread evenly and let dry 15 minutes. Protect the plates from light.

Producing 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)	1 µL
Sterile Water	41.5 µL
Taq Polymerase (1 unit/µL)	1 µL
Total Volume	50 µL

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 it 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

pEF6/V5-His TOPO TA Cloning® Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pEF6/V5-His-TOPO® vector, set up two 6 µL TOPO® Cloning reactions as described below.

1. Set up the 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
pEF6/V5-His-TOPO® vector	1 µL	1 µL

2. Incubate the reactions at room temperature for **5 minutes** and place them on ice.
3. Transform 2 µL of each reaction into One Shot® TOP10 chemically competent or electrocompetent *E. coli* (see page 8).
4. Spread 10–50 µL of each transformation mix onto LB plates containing 50–100 µg/mL ampicillin and X-Gal (see page 28). 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 assure even spreading.
   Note: No IPTG is required.
5. Incubate the plates overnight at 37°C.

Expected Results

The "Vector + PCR Insert" reaction should produce hundreds of colonies. Greater than 90% of these will be blue and contain the 500 bp insert.

The "Vector Only" reaction should yield very few colonies (<10% of the number of colonies found on the "Vector + PCR Insert" plate).

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 DNA using the protocol on page 8. Plate 10 µL of the transformation mixture plus 20 µL SOC on LB plates containing 50–100 µg/mL ampicillin. Transformation efficiency should be ~1 × 10^9 cfu/µg DNA.

Continued on next page

Factors Affecting Cloning Efficiency

Lower transformation and/or cloning efficiencies result from the following variables. Most of these are easily corrected, but if you are cloning large inserts, you may not obtain the expected 90% (or more) cloning efficiency.

Variable	Solution
pH>9 in PCR amplification reaction	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>3 kb)	Increase amount of insert. Or gel-purify as described on pages 20–21.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. **Note:** You may use up to 4 µL of your PCR reaction in a TOPO® Cloning reaction.
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 22).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (pages 20–21) or optimize your PCR.
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	*Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).
	Do not use a 2-step cycling program (denaturation and annealing only) to produce PCR products. Use only a 3-step cycling program (denaturation, annealing, and extension). *Taq* polymerase is more likely to add nontemplate 3´ A residues in a 3-step cycling program than in a 2-step cycling program.

Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Two simple protocols are provided below.

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit allows you to rapidly purify PCR products from regular agarose gels (see page 30 for ordering information).

1. Equilibrate a water bath or heat block to 50°C.
2. Cut the area of the gel containing the desired DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment. Weigh the gel slice.
3. Add Gel Solubilization Buffer (GS1) supplied in the kit as follows:
   - For ≤ 2% agarose gels, place up to 400 mg gel into a sterile, 1.5-mL polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µL of Gel Solubilization Buffer (GS1) for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5-mL polypropylene tubes, and add 60 µL of Gel Solubilization Buffer (GS1) for every 10 mg of gel.
4. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After gel slice appears dissolved, incubate the tube for an **additional** 5 minutes.
5. Preheat an aliquot of TE Buffer to 65–70°C
6. Place a Quick Gel Extraction Column into a Wash Tube. Pipette the mixture from Step 4 onto the column. Use one column per 400 mg agarose.
7. Centrifuge the column at >12,000 × g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
8. **Optional:** Add 500 µL of Gel Solubilization Buffer (GS1) to the column and incubate it at room temperature for 1 minute. Centrifuge the column at >12,000 × g for 1 minute and discard the flow-through. Place the column back into the Wash Tube.

Procedure continued on next page

Continued on next page

Using the PureLink™ Quick Gel Extraction Kit, continued

Procedure continued from previous page

9. Add 700 µL of Wash Buffer (W9) with ethanol (add 96–100% ethanol to the Wash Buffer according to instructions on the label of the bottle) to the column and incubate it at room temperature for 5 minutes. Centrifuge the column at >12,000 × g for 1 minute. Discard the flow-through.
10. Centrifuge the column at >12,000 × g for 1 minute to remove any residual buffer. Place the column into a 1.5 mL Recovery Tube.
11. Add 50 µL **warm** (65–70°C) TE Buffer to the center of the cartridge. Incubate the column at room temperature for 1 minute.
12. Centrifuge the column at >12,000 × g for 2 minutes. **The Recovery Tube contains the purified DNA.** Store the DNA at –20°C. Discard the column.
13. Use 4 µL of the purified DNA for the TOPO® Cloning reaction.

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that the gel purification results in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µL of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 7.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µL directly into chemically competent One Shot® TOP10 cells using the method on page 8.

Note

Note that cloning efficiency may decrease with purification of the PCR product. Optimize your PCR to produce a single band (see **Producing PCR Products**, page 5).

Adding 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO TA Cloning® vectors is often difficult because of very low cloning efficiencies. These low efficiencies are caused by the 3´ to 5´ exonuclease activity of proofreading polymerases which removes the 3´ A-overhangs necessary for TOPO TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Materials Needed

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7–1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8–10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for TOPO® Cloning into pEF6/V5-His-TOPO®.

   Note: If you plan to store your sample(s) overnight before proceeding with TOPO® Cloning, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

Note

You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see pages 20–21). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10–15 minutes at 72°C. Use 4 µL in the TOPO® Cloning reaction.

Vent® is a registered trademark of New England Biolabs.

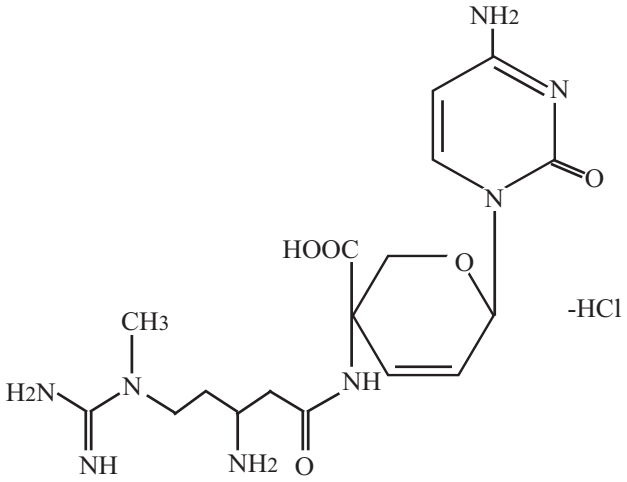
Blasticidin

Blasticidin

Blasticidin S HCl, isolated from *Streptomyces griseochromogenes,* is a nucleoside antibiotic which inhibits the protein synthesis in prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by the 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

The formula for blasticidin is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Preparing and Storing Stock Solutions

Blasticidin is available from Invitrogen in 50 mg aliquots (see page 30). Use sterile water to prepare stock solutions of 5 to 10 mg/mL.

- Always wear gloves, mask, goggles, and protective clothing (e.g., a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.
- Dissolve blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at –20°C for long-term storage or store at 4°C for short-term storage.
- Aqueous **stock** solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at –20°C.
- pH of the aqueous solution should not exceed 7 to prevent the 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.
- You may store medium containing blasticidin at 4°C for up to 2 weeks.

Human EF-1α Promoter

Description

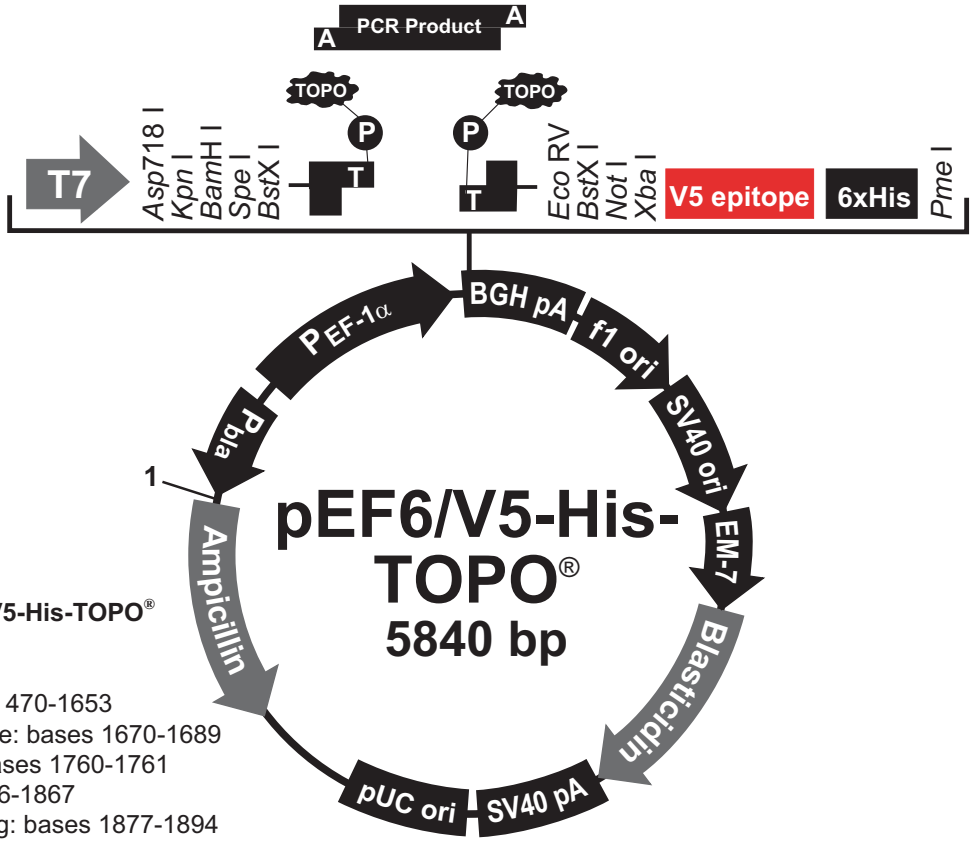
The diagram below shows the features of the human EF-1α promoter (Mizushima and Nagata, 1990) used in the pEF6/V5-His-TOPO® vector. Features are marked as described in Uetsuki, *et al.*, 1989.

pEF6/V5-His-TOPO® Vector

Map of pEF6/V5-His-TOPO®

The figure below summarizes the features of the pEF6/V5-His-TOPO® vector. The vector is supplied linearized between base pairs 1,760 and 1,761. This is the TOPO® Cloning site. Unique restriction sites flanking the TOPO® Cloning site are shown.

The complete sequence for pEF6/V5-His-TOPO® is available for downloading at www.invitrogen.com or by contacting Technical Support (see page 32).



Comments for pEF6/V5-His-TOPO®
5840 nucleotides

EF-1α promoter: bases 470-1653
T7 promoter/priming site: bases 1670-1689
TOPO® Cloning site: bases 1760-1761
V5 epitope: bases: 1826-1867
Polyhistidine (6xHis) tag: bases 1877-1894
BGH reverse priming site: bases 1917-1934
BGH polyadenylation signal: bases 1923-2147
f1 origin of replication: bases 2193-2621
SV40 promoter and origin: bases 2626-2970
EM-7 promoter: bases 3012-3078
Blasticidin resistance gene: bases 3079-3477
SV40 early polyadenylation signal: bases 3635-3765
pUC origin: bases 4148-4821 (complementary strand)
bla promoter: bases 21-105 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4966-5826 (complementary strand)

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pEF6/V5-His-TOPO® Vector, continued

Features of pEF6/V5-His-TOPO®

pEF6/V5-His-TOPO® (5,840 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human elongation factor 1α (hEF-1α) promoter	Permits overexpression of your recombinant protein in a broad range of mammalian cell types (Goldman *et al.*, 1996; Mizushima and Nagata, 1990)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
TOPO® Cloning site	Allows insertion of your PCR product in frame with the C-terminal V5 epitope and polyhistidine (6×His) tag
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of the fusion protein with the Anti-V5 Antibody or the Anti-V5-HRP Antibody (Southern *et al.*, 1991) (see page 31 for ordering information)
C-terminal polyhistidine (6×His) tag	Permits purification of your fusion protein on metal-chelating resins (i.e., ProBond™) In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody and the Anti-His(C-term)-HRP Antibody (Lindner *et al.*, 1997) (see page 31 for ordering information)
BGH reverse priming site	Permits sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM-7 promoter	For expression of the blasticidin resistance gene in *E. coli*
Blasticidin resistance gene (*bsd*)	Selection of stable transfectants in mammalian cells (Kimura *et al.*, 1994)
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene
Ampicillin resistance gene (β-lactamase)	Selection of transformants in *E. coli*

pEF6/V5-His-TOPO®/*lacZ* Vector

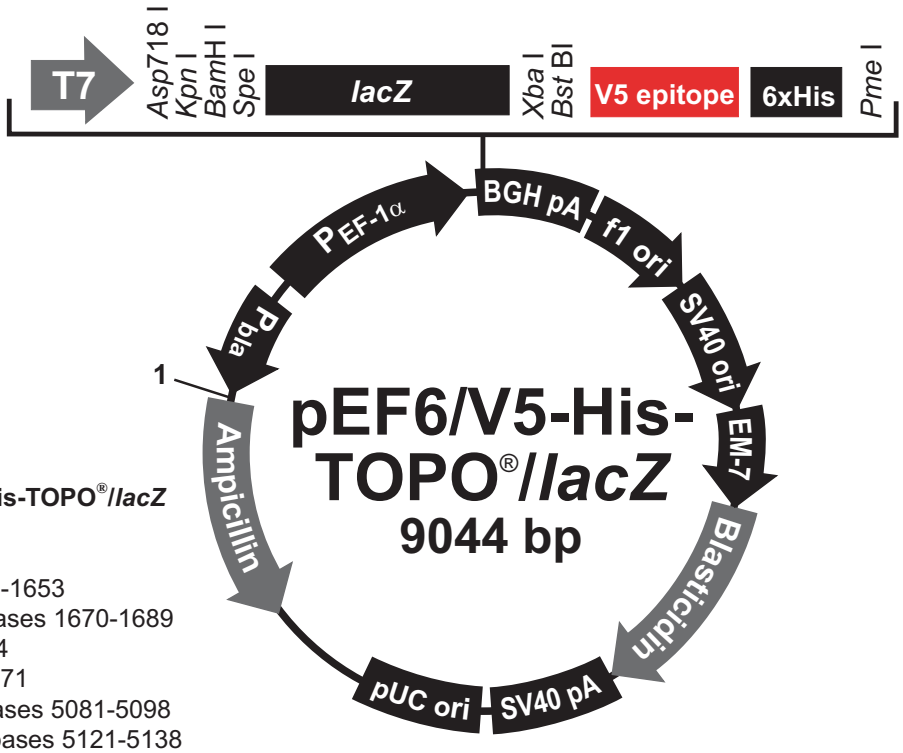
Description

pEF6/V5-His-TOPO®/*lacZ* is a 9,044 bp control vector containing the gene for β-galactosidase. The *lacZ* gene was amplified and TOPO® Cloned into pEF6/V5-His-TOPO® such that it is in frame with the C-terminal peptide containing the V5 epitope and the polyhistidine (6×His) tag.

Map of pEF6/V5-His-TOPO®/lacZ

The figure below summarizes the features of the pEF6/V5-His-TOPO®/*lacZ* vector. Unique restriction sites flanking the *lacZ* gene are shown.

The complete sequence for pEF6/V5-His-TOPO®/*lacZ* is available for downloading at www.invitrogen.com or by contacting Technical Support (see page 32).



Comments for pEF6/V5-His-TOPO®/*lacZ*
9044 nucleotides

EF-1α promoter: bases 470-1653
T7 promoter/priming site: bases 1670-1689
lacZ ORF: bases 1761-4964
V5 epitope: bases: 5030-5071
Polyhistidine (6xHis) tag: bases 5081-5098
BGH reverse priming site: bases 5121-5138
BGH polyadenylation signal: bases 5127-5351
f1 origin of replication: bases 5397-5825
SV40 promoter and origin: bases 5835-6179
EM-7 promoter: bases 6216-6282
Blasticidin resistance gene: bases 6283-6681
SV40 early polyadenylation signal: bases 6839-6969
pUC origin: bases 7352-8025 (complementary strand)
bla promoter: bases 21-105 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 8170-9030 (complementary strand)

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 the solution on liquid cycle for 20 minutes at 15 psi. Allow the solution to cool to 55°C and add antibiotic if needed (50–100 µg/mL ampicillin).
4. Store the medium 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 the medium plus agar on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool the medium to ~55°C, add antibiotic (50–100 µg/mL ampicillin), and pour into 10 cm plates.
4. Let the agar harden, then invert the plates and store them at 4°C, in the dark.

X-Gal Stock Solution

1. To make a 40 mg/mL stock solution, dissolve 400 mg X-Gal in 10 mL of dimethylformamide. Protect the X-Gal solution from light by storing it in a brown bottle at –20°C.
2. To add X-Gal to previously made agar plates, warm the plate to 37°C. Pipette 40 µL of the 40 mg/mL X-Gal stock solution onto the plate, spread it evenly, and let it dry for 15 minutes. Protect the plates from light.

Continued on next page

Cell Lysis Buffer

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

1. You can prepare this solution from the following common 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 of the solution up to 90 mL with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume of the solution up to 100 mL. Store the solution at room temperature.

Note: You may add protease inhibitors to the Cell Lysis Buffer at the following concentrations:

1 mM PMSF
1 µg/mL pepstatin
1 µg/mL leupeptin

Accessory Products

Additional Products

A number of products included with the pEF6/V5-His TOPO® TA Expression Kit as well as other reagents that may be used with the kit are available separately from Invitrogen. See the table below for ordering information.

Item	Amount	Cat. no.
T7 Promoter Primer	2 mg	N560-02
BGH Reverse Primer	2 mg	N575-02
PCR Optimizer™ Kit	100 reactions	K1220-01
One Shot® TOP10 Chemically Competent *E. coli*	20 reactions	C4040-03
One Shot® TOP10 Electrocomp™ *E. coli*	20 reactions	C4040-52
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink™ HQ Mini Plasmid Purification	100 preps	K2100-01
PureLink™ Quick Gel Extraction Kit	50 preps	K2100-12
Lipofectamine™ 2000 Transfection Reagent	0.75 mL 1.5 mL 15 mL	11668-027 1668-019 11668-500
Calcium Phosphate Transfection Kit	75 reactions	K2780-01
S.O.C. Medium	10 × 10 mL	15544-034
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
Blasticidin	50 mg	R210-01
Phosphate-Buffered Saline (PBS), pH 7.4	50 mL	10010-023
Trypsin-EDTA (0.05% Trypsin, EDTA•4Na)	100 mL	25300-054
TrypLE™ Express Dissociation Enzyme	100 mL	12604-013
β-Gal Staining Kit	1 kit	K1465-01
β-Gal Assay Kit	80 mL	K1455-01

MembranePro™ Functional Protein Expression System

The MembranePro™ Functional Protein Expression System allows the expression and display of mammalian cell surface membrane proteins, including G-protein coupled receptors (GPCRs), in an aqueous-soluble format. The MembranePro™ Functional Protein Expression System is optimized for use with the pEF6 vector, and the pEF6/V5-His TOPO® TA Vector Kit allows you to directly insert a *Taq* polymerase-amplified PCR product into the pEF6/V5-His TOPO® vector in a TOPO® Cloning reaction to generate your expression vector. For more information, visit www.invitrogen.com or contact Technical Support (page 32).

Product	Amount	Cat. no.
MembranePro™ Functional Protein Expression Kit	10 reactions	A11667
MembranePro™ Functional Protein Support Kit	10 reactions 60 reactions 600 reactions	A11668 A11669 A11670

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Products for Detecting Recombinant Proteins

Once cloned into pEF6/V5-His-TOPO®, you can detect the expression of your PCR product using an antibody to the protein itself or to the appropriate epitope. The table below describes the antibodies available for use with pEF6/V5-His-TOPO®. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods. The amount of antibody supplied is sufficient for 25 western blots.

Antibody	Epitope	Cat. no.
Anti-V5	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991): GKPIPNPLLGLDST	R960-25
Anti-V5-HRP		R961-25
Anti-His(C-term)	Detects the C-terminal polyhistidine tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP		R931-25

Products for Purifying Recombinant Protein

The metal binding domain encoded by the polyhistidine tag allows simple, easy purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using Invitrogen's ProBond™ Resin (see below). To purify proteins expressed using pEF6/V5-His-TOPO®, the ProBond™ Purification System is available separately. Additional ProBond™ resin is available in bulk. See the table below for ordering information.

Product	Amount	Cat. no.
ProBond™ Purification System (includes six 2 mL precharged, prepacked Pro resin columns and buffers for native and denaturing purification)	6 purifications	K850-01
ProBond™ Purification System with Anti-V5-HRP Antibody	1 Kit	K854-01
ProBond™ Metal-Binding Resin (precharged resin provided as a 50% slurry in 20% ethanol)	50 mL 150 mL	R801-01 R801-15
Purification Columns (10 mL polypropylene columns)	50 columns	R640-50

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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SDS

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

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product and is searchable by product lot number, which is printed on each box. CofAs are available on our website at www.invitrogen.com/support.

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Notes

Notes

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