pIB/V5-His TOPO®
TA Expression® Kit

Version F
13 April, 2004
25-0331

pIB/V5-His TOPO® TA Expression Kit

For Five-Minute Cloning of *Taq* Polymerase-Amplified PCR Products for Stable Expression in Lepidopteran Insect Cell Lines

Catalog no. K890-20

Invitrogen™
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Table of Contents

Kit Contents

Shipping and Storage

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

Store Box 1 at -20°C. Store Box 2 at -80°C.

TOPO TA Cloning® Reagents

pIB/V5-His TOPO TA Cloning® reagents (Box 1) are listed below. Note that the user must supply *Taq* polymerase. **Store Box 1 at -20°C.**

Item	Concentration	Amount
pIB/V5-His-TOPO® vector, linearized	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 phenol red	25 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
Sterile Water	--	1 ml
OpIE2 Forward Sequencing Primer	Lyophilized in TE Buffer, pH 8	2 µg
OpIE2 Reverse Sequencing Primer	Lyophilized in TE Buffer, pH 8	2 µg
Expression Control Plasmid (pIB/V5-His/CAT)	0.5 µg/µl in TE buffer, pH 8	10 µl
Control PCR Primers	0.1 µg/µl each in TE Buffer, pH 8	10 µl
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8	10 µl

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Kit Contents and Storage, continued

Primer Sequences

The sequence of each primer is provided below:

Primer	Sequence	pMoles Supplied
OpIE2 Forward	5´-CGCAACGATCTGGTAAACAC-3´	329
OpIE2 Reverse	5´-GACAATACAAACTAAGATTTAGTCAG-3´	250

One Shot® TOP10 Reagents

The table below describes the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit. Transformation efficiency is at least 1 x 10^9 cfu/µg DNA. Note that TOP10 One Shot® cells may be ordered separately (Catalog no. C4040-03).

Store Box 2 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 x 50 µl
pUC19 Control DNA	10 ng/µl	10 µl

Genotype

TOP10: Use this strain for general cloning of PCR products in pIB/V5-His-TOPO®.

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

One Shot® Electrocomp™

TOP10 cells are also available as electrocompetent cells in a One Shot® format (Catalog no. C4040-52). Transformation efficiency is 1 x 10^9 cfu/µg supercoiled DNA.

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Reagents Supplied by the User

Be sure to have the following reagents and equipment on hand before starting experiments:

- Express Five® Serum-Free Medium (recommended)
- Grace's Medium (Optional)
- Fetal Bovine Serum (FBS) (Optional)
- 5, 10, and 25 ml sterile pipettes
- Cryovials
- Hemacytometer and Trypan Blue (see page 25)
- Table-top centrifuge
- 60 mm tissue culture plates (other flasks and plates may be used)
- Sterile microcentrifuge tubes (1.5 ml)
- Cell Lysis Buffer (see page 26)
- PBS (see page 26)
- Cloning cylinders (optional)
- 96-well plates (optional)

Accessory Products

Products Available Separately

The following products are available separately from Invitrogen.

Product	Amount	Catalog no.
Sf9 Cells, frozen	1 ml vial, 1 x 10^7 cells/ml	B825-01
Sf21 Cells, frozen	1 ml vial, 1 x 10^7 cells/ml	B821-01
High Five™ Cells, frozen	1 ml vial, 3 x 10^6 cells/ml	B855-02
Grace's Insect Cell Culture Medium, Unsupplemented	500 ml	11595-030
Sf-900 II SFM	1 liter	10902-088
Express Five® SFM	1 liter	10486-025
Cellfectin® Reagent	1 ml	10362-010
Blasticidin S	50 mg	R210-01

Other InsectSelect™ Kits

Several other kits that allow you to clone and stably express your gene of interest using the InsectSelect™ technology are available from Invitrogen. These kits include InsectSelect™ vectors with different antibiotic resistance genes. In addition, the pIZT/V5-His Vector Kit enables expression of a gene of interest and a cycle 3-GFP/Zeocin™ fusion gene. This allows both visual monitoring of transfection efficiency and generation of a stable cell line. For more information about the various InsectSelect™ vector kits available from Invitrogen, visit our World Wide Web site (www.invitrogen.com) or call Technical Service (see page 39). See the table below for ordering information.

Product	Catalog no.
pIB/V5-His Vector Kit	V8020-01
pMIB/V5-His Vector Kit	V8030-01
pIZ/V5-His Vector Kit	V8000-01
pIZT/V5-His Vector Kit	V8010-01

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Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The table below describes the antibodies available for detection of C-terminal fusion proteins expressed using pIB/V5-His-TOPO®. Horseradish peroxidase (HRP)-conjugated antibodies allow one-step detection using colorimetric or chemiluminescent detection methods.

Fifty microliters of each antibody is supplied which is sufficient for 25 Westerns.

Product	Epitope	Catalog no.
Anti-V5 Antibody	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 Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997) HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His (C-term)-AP Antibody		R932-25

Purification of 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 the InsectSelect™ System, the ProBond™ Purification System or the ProBond™ resin in bulk are available separately. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Metal-Binding Resin	50 ml	R801-01
(precharged resin provided as a 50% slurry in 20% ethanol)	150 ml	R801-15
ProBond™ Purification System	6 purifications	K850-01
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The pIB/V5-His TOPO® TA Expression Kit combines the InsectSelect™ System with TOPO® Cloning technology to provide a highly efficient, rapid cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for stable expression in insect cell lines. No ligase, post-PCR procedures, or PCR primers containing special, additional sequences are required.

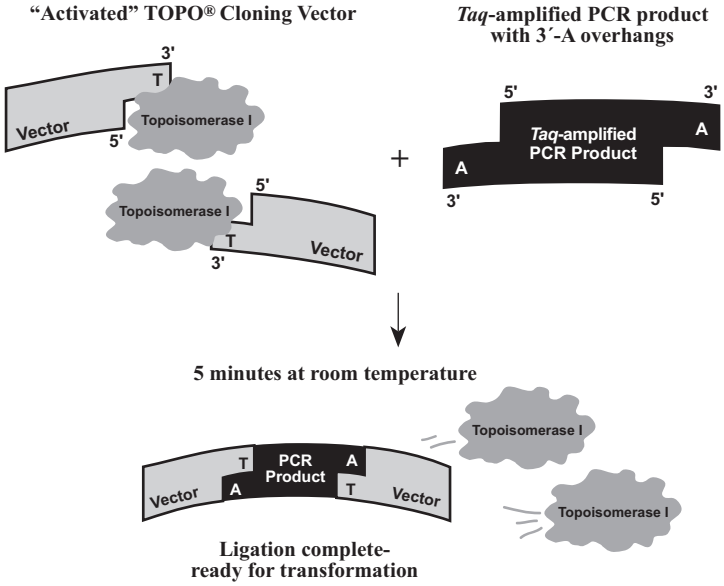
TOPO® Cloning

pIB/V5-His-TOPO® is supplied linearized with:

- Single 3´ thymidine (T) overhangs for TA Cloning®
- Topoisomerase I 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.

TOPO® Cloning exploits the ligation activity of topoisomerase by providing an "activated", linearized TA vector using proprietary technology (Shuman, 1994). Ligation of the vector with a PCR product containing 3´ A overhangs is very efficient and occurs spontaneously within 5 minutes at room temperature. The TOPO® Cloning reaction can be transformed into chemically competent cells (provided) or electroporated directly into electrocompetent cells (see page v).



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Overview, continued

InsectSelect™ System

The InsectSelect™ System allows you to express your protein of interest in insect cell lines transiently or stably. The system utilizes the expression vector, pIB/V5-His-TOPO® to express your gene of interest. In addition to the TOPO® Cloning feature, this 3.5 kb vector has the following additional features:

- *OpIE2* promoter for high-level, constitutive expression of the gene of interest (Theilmann and Stewart, 1992)
- *OpIE1* promoter for expression of the blasticidin resistance gene (see next bullet) (Theilmann and Stewart, 1991)
- Blasticidin resistance gene for selection of stable cell lines (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965)
- EM7 promoter for expression of ampicillin (or blasticidin) resistance in *E. coli*
- Ampicillin resistance gene for selection of transformants in *E. coli*
- Optional C-terminal peptide containing the V5 epitope and 6xHis tag for detection and purification of your protein of interest

For more information and a map of the vector, see pages 33-34.

Description of System

The PCR product of interest is TOPO® Cloned into pIB/V5-His-TOPO® and transfected into Sf9, Sf21, or High Five™ cells using lipid-mediated transfection. After transfection, cells can be assayed for expression of the PCR product. Once you have confirmed that your PCR product expresses, you can select for a stable polyclonal population or stable clonal cell lines using blasticidin as a selection agent. Stable cell lines can be used to express the protein of interest in either adherent culture or suspension culture.

Description of Promoters

Baculovirus immediate-early promoters utilize the host cell transcription machinery and do not require viral factors for activation. Both the *OpIE2* and *OpIE1* promoters are from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV). The virus' natural host is the Douglas fir tussock moth; however, the promoters allow protein expression in *Lymantria dispar* (LD652Y), *Spodoptera frugiperda* cells (Sf9) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), Sf21 (Invitrogen), *Trichoplusia ni* (High Five™) (Invitrogen), *Drosophila* (Kc1, S2) (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997), and mosquito cell lines (unpublished data). The *OpIE2* promoter has been shown to be about 5- to 10-fold stronger than the *OpIE1* promoter (Pfeifer *et al.*, 1997). Both promoters have been sequenced and analyzed. For more detailed information on the *OpIE2* and *OpIE1* promoters, see page 36 and page 37, respectively.

Expression Levels

The *OpIE2* promoter provides relatively high levels of constitutive expression, although not all proteins will express as high as might be expected from baculovirus late promoters such as polyhedrin or very late promoters such as p10 (Jarvis *et al.*, 1996). However, some researchers have found that the InsectSelect™ System expresses some proteins better than baculovirus systems. To date, reported expression levels range from 1-2 μg/ml (human IL-6; Invitrogen) to 8-10 μg/ml (human melanotransferrin) (Hegedus *et al.*, 1999).

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Overview, continued

Blasticidin Resistance

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 (Kimura and Yamaguchi, 1996; Yamaguchi *et al.*, 1975).

Experimental Outline

The table below describes the general steps needed to clone and express your gene of interest. For more details, refer to the pages indicated. Information on how to culture insect cell lines may be found in our Insect Cell Lines manual. This manual may be downloaded from our Web site (www.invitrogen.com).

Step	Action	Page
1	Establish culture of Sf9, Sf21, or High Five™ cells.	4
2	Design primers to produce your PCR product.	5-6
3	Amplify your PCR product.	7
4	TOPO® Clone your PCR product and transform into TOP10 One Shot® competent cells. Select on LB plates containing 50-100 μg/ml ampicillin.	8-10
5	Isolate plasmid DNA and analyze for the presence and orientation of the insert.	11
6	Sequence your recombinant expression vector to confirm that your gene is in frame with the DNA encoding the C-terminal peptide (if desired).	11
7	Transfect Sf9, Sf21, or High Five™ cells.	14-16
8	Assay for transient expression of your protein.	17-18
9	Create stable cell lines expressing the protein of interest by selecting with blasticidin.	19-22
10	Scale-up expression for purification.	23
11	Purify your recombinant protein by chromatography on metal-chelating resin (*e.g.* ProBond™).	23-24

Methods

Culturing Insect Cells

Introduction

Before you start your cloning experiments, be sure to have cell cultures of Sf9, Sf21, or High Five™ cells growing and have frozen master stocks available.

Cells for Transfection

You will need log-phase cells with >95% viability to perform a successful transfection. Review pages 14-16 to determine how many cells you will need for transfection.

Insect Cell Lines Manual

For additional information on insect cell culture, refer to the Insect Cell Lines manual. This manual contains information on:

- Thawing frozen cells
- Maintaining and passaging cells
- Freezing cells
- Using serum-free medium
- Growing cells in suspension
- Scaling up cell culture

This manual is available from our Web site (www.invitrogen.com) or you may request the manual from Technical Service (see page 39).

Designing PCR Primers

Introduction

It is important to properly design your PCR primers to ensure that you obtain the recombinant protein you need for your studies. Use the information below and the diagram on page 6 to design your PCR primers. **Remember that your PCR product will have single 3´ adenine overhangs (see page 6).**

General Molecular Biology Techniques

For help with *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into either pIB/V5-His-TOPO®.

Translation Initiation

Your PCR product should contain a Kozak translation initiation sequence and an ATG start codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). You may need to design a Kozak sequence into your forward PCR primer. An example of a Kozak consensus sequence is provided below. Note that other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG start codon is shown underlined.

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

Fusion to the C-terminal Peptide

If you wish to include the C-terminal peptide for detection with either the V5 or His(C-term) antibodies or purification using the 6xHis tag, you must design your reverse PCR primer to remove the native stop codon and maintain the frame through the DNA encoding the C-terminal peptide.

If you do not wish to include the C-terminal peptide, include the native stop codon in the reverse PCR primer or design the primer to anneal downstream of the native stop codon.

Secretion of Recombinant Protein

If your protein of interest is normally secreted, you may design your forward PCR primer to include the native secretion signal. To date, all mammalian secretion signals tested have functioned properly in insect cells. We have successfully expressed human interleukin-6 (IL6) using the native secretion signal to levels of 1-2 μg/ml.

In addition, we recommend that you design a forward primer to create a PCR product without the secretion signal to express your protein intracellularly in the event that your protein is not secreted utilizing the native secretion signal.

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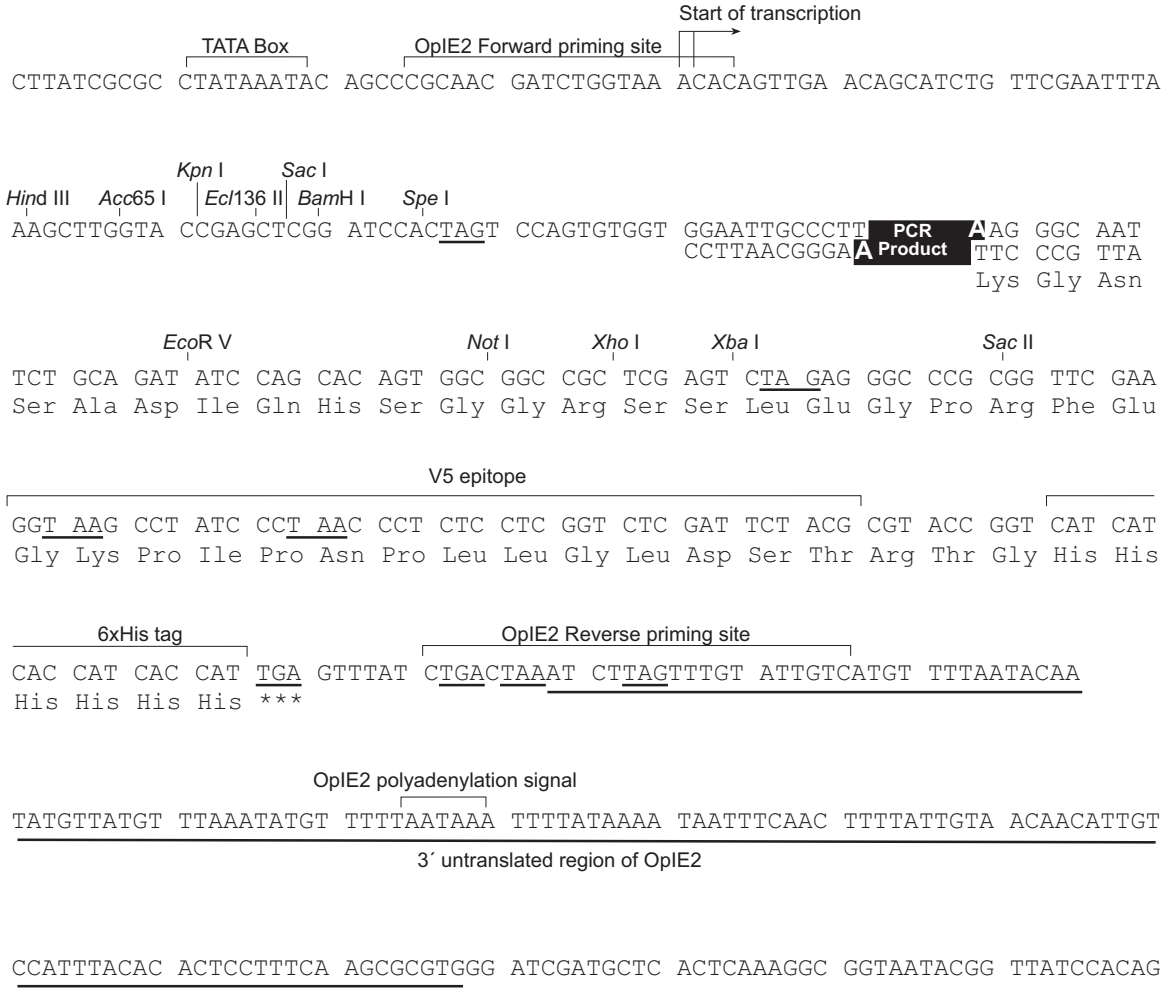
Designing PCR Primers, continued

TOPO® Cloning Site for pIB/V5-His-TOPO®

The diagram below is supplied to help you design appropriate PCR primers to correctly clone and express your PCR product using pIB/V5-His-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. Stop codons are underlined. For a map of pIB/V5-His-TOPO®, see page 33. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (page 39).**



Producing PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers you are ready to produce your PCR product.

Materials Supplied by the User

You will need the following reagents and equipment.

- *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, *Taq* must be used 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 a proof-reading polymerase only, you can add 3′ A-overhangs using the method on page 29.

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)	1 µM 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 pIB/V5-His TOPO® TA Expression Kit (see page 27). 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 (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR. Call Technical Service for more information (page 39).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into pIB/V5-His-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. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 30-32 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 also 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.

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. **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 below).** For this reason two different TOPO® Cloning reactions are provided to help you obtain the best possible results. Read the following information carefully.

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. 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 next page).

Materials Supplied by the User

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

- 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

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TOPO® Cloning Reaction and Transformation, continued

Note

There is no blue-white screening for the presence of inserts. Individual recombinant plasmids need to be analyzed by restriction analysis or sequencing for the presence and orientation of insert. Sequencing primers included in each kit can be used to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparation 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*.
- 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.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® cells for each transformation.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent TOP10 One Shot® *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 13.

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	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 or +4°C.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°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) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.
2. Place the reaction on ice and proceed to the **One Shot® Chemical Transformation** (next page) or **Transformation by Electroporation** (next page). **Note**: You may store the TOPO® Cloning reaction at -20°C overnight.

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TOPO® Cloning Reaction and Transformation, continued

TOP10 One Shot® Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from Step 2 previous page into a vial of One Shot® 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 do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion (see page 13).
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 25-200 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. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, 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 formation of bubbles.**
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see next page.
3. Immediately add 250 µl of room temperature SOC medium.
4. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance gene.
5. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

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TOPO® Cloning Reaction and Transformation, continued

Note

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

Analysis of Positive Clones

1. Culture 10 transformants overnight in 2-5 ml LB or SOB medium containing 50-100 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 the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids for the presence and orientation of insert by restriction analysis. We recommend sequencing your constructs to confirm that your gene of interest is cloned in frame with the C-terminal peptide. Sequencing primers are included to help you sequence your insert (see page v). To use the sequencing primers, resuspend in 20 µl sterile water to prepare a 0.1 µg/µl stock solution. Refer to the diagram on page 6 for the sequence surrounding the TOPO® Cloning site.

   If you need help with setting up restriction enzyme digests or DNA sequencing, 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. You may use either the forward **or** reverse sequencing primers included in the kit **and** a primer that hybridizes 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. Both false positive and false negative results can 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 *Taq* polymerase. Use a 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. (Don't forget to make a patch plate to preserve the colonies for further analysis.)
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using the appropriate conditions (see text above).
5. For the final extension, incubate at 72°C for 10 minutes. Hold at +4°C.
6. Visualize by agarose gel electrophoresis.

continued on next page

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 30-32. 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 LB plates containing 50-100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 µg/ml ampicillin. Grow until 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, 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. 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

Transient Expression in Insect Cells

Introduction

Once you have TOPO® Cloned your gene of interest into pIB/V5-His-TOPO®, you are ready to transfect your construct into Sf9 or High Five™ cells using lipid-mediated transfection and test for expression of your protein.

Plasmid Preparation

Plasmid DNA for transfection into insect cells must be very 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.™ MiniPrep Kit (Catalog no. K1900-01) or other resin-based DNA purification systems. The S.N.A.P.™ MiniPrep Kit is a small-scale plasmid isolation kit that isolates 10-15 µg of plasmid DNA from 10-15 ml of bacterial culture. Plasmid can be used directly for transfection of insect cells.

Method of Transfection

We recommend lipid-mediated transfection with Cellfectin® Reagent. Note that other lipids may be substituted, although transfection conditions may need to be optimized.

Expected Transfection Efficiency using Cellfectin® Reagent:

- **40-60% for Sf9 cells**
- **40-60% for High Five™ cells**

Note: Other transfection methods (*e.g.* calcium phosphate and electroporation (Mann and King, 1989)) have been tested with High Five™ cells, but no significant increase in transfection efficiency was demonstrated.

Control of Plasmid Quality

To test the quality of a plasmid DNA preparation, include a 'DNA only' control in all transfection experiments. At about 24 to 48 hours posttransfection, compare the 'DNA only' control with cells transfected with plasmid. If the plasmid preparation contains contaminants, then the cells will appear unhealthy and start to lyse.

Before Starting

You will need the following for each transfection experiment:

- ~1 µg of highly purified plasmid DNA (~1 µg/µl in TE buffer)
- Either log-phase Sf9 cells (1.6-2.5 x 10^6 cells/ml, >95% viability) or log-phase High Five™ cells (1.8-2.3 x 10^6 cells/ml, >95% viability)
- Serum-free medium (see next page)
- 60 mm tissue-culture dishes
- 1.5 ml sterile microcentrifuge tubes
- Rocking platform only (NOT orbital)
- 27°C incubator
- Inverted Microscope
- Paper towels and air-tight bags or containers
- 5 mM EDTA, pH 8

continued on next page

Transient Expression in Insect Cells, continued

Serum-Free Media

Several serum-free media are available from Invitrogen for use in transfection experiments with the pIB/V5-His TOPO® vector. Express Five® SFM (Catalog no. 10486-025) is recommended for use with High Five™ cells while Sf-900 II SFM (1X) (Catalog no. 10902-088) is optimized for use with Sf9 and Sf21 cells. Other serum-free media may be used, although you may have to optimize conditions for transfection and selection. Note that if you wish to transfect Sf9 or Sf21 cells in serum-free medium, you will need to adapt the cells to serum-free medium before transfection (see the Insect Cell Lines manual for a protocol).

Prepare Cells

For each transfection, use log-phase cells with greater than 95% viability. We recommend that you set up enough plates to perform a time course for expression of your gene of interest. Test for expression 2, 3, and 4 days posttransfection. You will need at least one 60 mm plate for each time point.

1. For Sf9 cells or High Five™ cells, seed 2 x 10^6 cells in appropriate serum-free medium in a 60 mm dish.

   Rock gently from side to side for 2 to 3 minutes to evenly distribute the cells. Cells should be 50 to 60% confluent.
2. Incubate the cells for at least 15 minutes without rocking to allow the cells to fully attach to the bottom of the dish to form a monolayer of cells.
3. Verify that the cells have attached by inspecting them under an inverted microscope.

Positive and Negative Controls

We recommend that you include the following controls:

- pIB/V5-His/CAT vector as a positive control for transfection and expression
- Lipid only as a negative control
- DNA only to check for DNA contamination

Note

- If you use another lipid besides Cellfectin® Reagent, review the protocol on the next page and consult the manufacturer's instructions to adapt the protocol for your use. You may have to empirically determine the optimal conditions for transfection.
- **Do not linearize** the plasmid prior to transfection. Linearizing the plasmid appears to decrease protein expression. The reason for this is not known.

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Transfection Procedure

Plasmid DNA and Cellfectin® Reagent are mixed together in the appropriate medium (see below) and incubated with freshly seeded insect cells. The amount of cells, liposomes, and plasmid DNA has been optimized for 60 mm culture plates. It is important that you optimize transfection conditions if you use plates or flasks other than 60 mm plates.

Note: If you are using serum-free medium, we recommend using Sf-900 II SFM to transfect Sf9 cells and Express Five® SFM to transfect High Five™ cells. If you are using Grace's Medium, be sure to use Grace's Medium without supplements. The proteins in the FBS and supplements will interfere with the liposomes, causing the transfection efficiency to decrease.

1. To prepare each transfection mixture, use a 1.5 ml microcentrifuge tube. Add the following reagents:

Grace's Insect Media (Sf9) **OR** Appropriate serum-free medium	1 ml
pIB/V5-His-TOPO® construct (~1 μg/μl in TE, pH 8)	1 μl (1 μg)
Cellfectin® Reagent (**mix well before use and always add last**)	20 μl

2. Gently mix the transfection mixture for 10 seconds.
3. Incubate the transfection mixture at room temperature for 15 minutes.
4. Carefully remove all of the medium from the cell monolayer and add the entire transfection mix dropwise into the 60 mm dish. Repeat for all transfections.

   (Distribute the drops evenly over the monolayer. This method reduces the chances of disturbing the monolayer.)
5. Incubate the dishes at room temperature for 4 hours on a side-to-side, rocking platform. Adjust speed to ~2 side to side motions per minute. **Note**: If you do not have a rocker, manually rock the dishes periodically.
6. Following the 4-hour incubation period, add 1-2 ml of complete TNM-FH medium or the appropriate serum-free medium to each 60 mm dish. Place the dishes in a sealed plastic bag with moist paper towels to prevent evaporation and incubate at 27°C. **Note**: It is not necessary to remove the transfection solution as Cellfectin® Reagent is not toxic to the cells. If you are using a different lipid and observe loss of viability, then remove the transfection solution after 4 hours, rinse two times with medium, and replace with 1-2 ml of fresh medium.
7. Harvest the cells 2, 3, and 4 days posttransfection and assay for expression of your gene (see next page). There's no need to add fresh medium if the cells are sealed in an airtight plastic bag with moist paper towels.

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Testing for Expression

Use the cells from one 60 mm plate for each expression experiment. Before starting prepare Cell Lysis Buffer and SDS-PAGE sample buffer. Recipes are provided on page 26 for your convenience, but other recipes are suitable.

1. Prepare an SDS-PAGE gel that will resolve your expected recombinant protein.
2. Remove the medium from the cells. **If your protein is predicted to be secreted, be sure to save and assay both the medium and the cell pellet.**
3. Add 100 µl Cell Lysis Buffer to the plate and slough (or scrape) the cells into a microcentrifuge tube. Vortex the cells to ensure they are completely lysed.
4. Centrifuge at maximum speed for 1-2 minutes to pellet nuclei and cell membranes. Transfer the supernatant to a new tube. **Note**: If you are expressing a membrane protein, it may be located in the pellet. Be sure to assay the pellet (see below).
5. Assay the lysate for protein concentration. You may use the Bradford, Lowry, or BCA assays (Pierce).
6. To assay your samples, mix them with SDS-PAGE sample buffer as follows:
   - Lysate: 30 µl lysate with 10 µl **4X SDS-PAGE** sample buffer.
   - Pellet: Resuspend pellet in 100 µl **1X SDS-PAGE** sample buffer.
   - Medium: 30 µl medium with 10 µl **4X SDS-PAGE** sample buffer. **Note**: Because of the volume of medium, it is difficult to normalize the amount loaded on an SDS-PAGE gel. If you are concerned about normalization, concentrate the medium.
7. Heat the samples for 5 minutes at 70°C. Centrifuge briefly.
8. Load approximately 3 to 30 µg protein per lane. For the cell pellet sample, load the same volume as the lysate. Amount to load depends on the amount of your protein produced.
9. Electrophorese your samples, blot, and probe with antibody to your protein, antibody to the V5 epitope, or antibody to the C-terminal histidine tag (see page viii).
10. Visualize proteins using your desired method.

Note

The C-terminal tag containing the V5 epitope and 6xHis tag will increase the size of your protein by ~3 kDa. Note that any additional amino acids between your protein and the tags are not included in this molecular weight calculation.

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Assay for CAT

If you use pIB/V5-His/CAT as a positive control vector, you may assay for CAT expression using your method of choice. Commercial kits to assay for CAT protein are available.There is also a novel, rapid radioactive assay (Neumann *et al.*, 1987).

CAT can be detected by Western blot using antibodies against the C-terminal fusion tag or an antibody against CAT (Catalog no. R902-25). The CAT/V5-His protein fusion migrates around 34 kDa on an SDS-PAGE gel.

Troubleshooting

Cells Growing Too Slowly (Or Not At All).

For troubleshooting guidelines regarding cell culture, refer to the Insect Cell Lines manual. This manual can be downloaded from our Web site (www.invitrogen.com).

Low Transfection Efficiency.

If the transfection efficiencies are too low, check the following:

- **Impure DNA.** Transfected cells will appear unhealthy when compared to the negative control (DNA only). Use clean, pure DNA isolated by resin based DNA isolation kits (*e.g.* S.N.A.P. MidiPrep Kit).
- **Poor Cell Viability.** Be sure to test cells for viability and make sure you use log-phase cells. Refer to the Insect Cell Lines manual to troubleshoot cell culture.
- **Method of Transfection**. Optimize transfection.

Low or No Protein Expression

- **Gene not cloned in frame with the C-terminal sequence.** If it is not in frame with the C-terminal peptide sequence, expression will not be detected using the antibody to the V5 epitope or the C-terminal histidine tag.
- **No Kozak sequence for proper initiation of translation.** Translation will be inefficient and the protein will not be expressed at its optimal level.
- **Optimize expression.** If you've tried a time course to optimize expression, try switching cell lines. Proteins may express better in a different cell line.
- **Proteins are degraded.** Include protease inhibitors in the Cell Lysis buffer to prevent degradation of recombinant protein.
- **Poor secretion.** Check the cell pellet as well as the medium when analyzing secreted expression. Protein may be trapped in the cell and not secreted. To improve secretion, try a different cell line (*e.g.* High Five™).

Selecting Stable Cell Lines

Introduction

Once you have demonstrated that your protein is expressed in Sf9, Sf21, or High Five™ cells, you may wish to create stable expression cell lines for long-term storage and large-scale production of the desired protein.

Nature of Stable Cell Lines

Note that stable cell lines are created by multiple copy integration of the vector. Amplification as in the case with calcium phosphate transfection and hygromycin resistance in *Drosophila* is generally not observed.

Before Starting

Review the information on blasticidin S on page 38. Prepare a stock solution of blasticidin S as described.

Effect of Blasticidin on Sensitive and Resistant Cells

Cytopathic effects should be visible within 3-5 days depending on the concentration of blasticidin in the medium. Sensitive cells will enlarge and become filled with vesicles. The outer membrane will show signs of blebbing, and cells will eventually detach from the plate.

Blasticidin-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes between blasticidin-resistant cells compared to cells not under selection with blasticidin.

Suggested Blasticidin Concentrations

In general, concentrations around 10 μg/ml will kill Sf9 cells (in complete TNM-FH medium) and concentrations around 20 μg/ml will kill High Five™ cells (in Express Five® SFM) within one week, although a few cells will remain that exclude trypan blue. To obtain faster and more thorough killing, we recommend using 50-80 μg/ml blasticidin. After selection of foci, the concentration of blasticidin can be lowered and maintained at 10 μg/ml. If you use other media or have trouble selecting cells using the concentrations above, we recommend that you perform a kill curve (see below).

Blasticidin Selection Guidelines

If you wish to test your cell line for sensitivity to blasticidin, perform a kill curve as described below. Assays can be done in 24-well tissue culture plates.

- Prepare TNM-FH medium or the serum-free medium of choice supplemented with concentrations ranging from 0 to 100 μg/ml blasticidin. Generally, concentrations that effectively kill lepidopteran insect cells within a week are in the 50 to 80 μg/ml range. **Note**: While concentrations under 10 μg/ml will kill cells within a week, higher concentrations will result in faster and more thorough killing. In addition, using higher concentrations of blasticidin may result in enrichment of clones containing multiple integrations of your gene of interest.
- Test varying concentrations of blasticidin on the cell line to determine the concentration that kills your cells within a week (kill curve).
- Use the concentration of drug that kills your cells within a week.

Note

Do not linearize the plasmid prior to transfection. Linearizing the plasmid appears to decrease protein expression. The reason for this is not known.

continued on next page

Selecting Stable Transformants, continued

Stable Transfection

For stable transfections, follow the steps below. Include a mock transfection and a positive control (pIB/V5-His/CAT).

1. Follow the transfection procedure on page 16, Steps 1 to 6.
2. Forty-eight hours posttransfection, remove the transfection solution and add fresh medium (**no blasticidin**).
3. Split cells 1:5 (20% confluent) and let cells attach for at least 15 minutes before adding selective medium.
4. Remove medium and replace with medium containing blasticidin at the appropriate concentration. Incubate cells at 27°C.
5. Replace selective medium every 3 to 4 days until you observe foci forming. At this point you may use cloning cylinders or dilution to isolate clonal cell lines (next page) or you can let resistant cells grow out to confluence for a polyclonal cell line (2 to 3 weeks).
6. To isolate a polyclonal cell line, let the resistant cells grow to confluence and split the cells 1:5 and test for expression. **Important**: Always use medium **without** blasticidin when splitting cells. Let the cells attach before adding selective medium.
7. Expand resistant cells into flasks to prepare frozen stocks. **Always use medium containing blasticidin when maintaining stable lepidopteran cell lines. You may drop the concentration of blasticidin to 10 μg/ml for maintenance.**

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Isolation of Clonal Cell Lines Using Cloning Cylinders

If you elect to select clonal cell lines, try to isolate as many foci (colonies) as possible for expression testing. As in mammalian cell culture, the location of integration may affect expression of your gene.

Tip: Perform selections in small plates or wells. When you remove the medium, you must work quickly to prevent the cells from drying out. Using smaller plates or wells limits the number of colonies you can choose at a time. To select more colonies, increase the number of plates or wells, not the size.

To select colonies:

1. Examine the closed plate under a microscope and mark the location of each colony on the top of the plate. Transfer the markings to the bottom of the plate. Be sure to include orientation marks. **Note**: Each colony will contain 50 to 200 cells. Sf9 cells tend to spread more than High Five™ cells.
2. Move the culture dish to the sterile cabinet and remove the lid.
3. Apply a thin layer of sterile silicon grease to the bottom of the cloning cylinder (Scienceware, Catalog no. 378747-00 or Belco, Catalog no. 2090-00608), using a sterile cotton-tipped wooden applicator. The layer should be thick enough to retard the flow of liquid from the cylinder, without obscuring the opening on the inside. **Tip**: Cloning cylinders and silicon grease can be sterilized together by placing a small amount of grease in a glass petri dish and placing the cloning cylinders upright in the grease. After autoclaving, the grease will have spread out in a thin layer to coat the bottom of the cylinders.
4. Aspirate the culture medium and place the cylinder firmly and directly over the marked area. Use a microscope if it is available to help you direct placement of the cylinder.
5. Use 20 to 100 µl of medium (no blasticidin) to slough the cells. Try to hold the pipette tip away from the sides of the cloning cylinder to avoid the grease (this will take a little practice).
6. Remove the cells and medium and transfer to a microtiter plate and let the cells attach. Remove medium and replace with selective medium for culturing. Expand the cell line and test for expression of your gene of interest. **Important**: Always use medium **without** blasticidin when splitting cells. Let the cells attach before adding selective medium.

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Isolation of Clonal Cell Lines Using a Dilution Method

You may also select clonal cell lines using a quick dilution method. The objective of this method is to dilute the cells so that under selective pressure only one stable viable cell per well is achieved. **Note that the higher your transfection efficiency, the more you should dilute out your cells. The protocol below works well with cells transfected at 5-10% efficiency.**

1. Forty-eight hours after transfection, dilute the cells to 1 x 10^4 cells/ml in medium **without** blasticidin. **Note**: Other dilutions of the culture should also be used as transfection efficiency will determine how many transformed cells there will be per well.
2. Add 100 µl of the cell solution from Step 1 to 32 wells of a 96-well microtiter plate (8 rows by 4 columns).
3. Dilute the remaining cells 1:1 with medium **without** blasticidin and add 100 µl of this solution to the next group of 32 wells (8 x 4).
4. Once again, dilute the remaining cells 1:1 with medium **without** blasticidin and add 100 µl of this solution to the last group of 32 wells. **Note**: Although the cells can be diluted to low numbers, cell density is critical for viability. If the density drops below a certain level, the cells will not grow.
5. Let the cells attach for at least 15 minutes, then remove the medium and replace with medium containing blasticidin. **Note**: Removing and replacing medium may be tedious. If you slough the cells gently, it is possible to dilute the cells directly into selective medium.
6. Wrap the plate and incubate at 27°C for 1 week. It's not necessary to change the medium or place in a humid environment.
7. Check the plate after a week and mark the wells that have only one colony.
8. Continue to incubate the plate until the colony fills most of the well.
9. Harvest the cells and transfer to a 24-well plate with 0.5 ml of fresh medium containing blasticidin.
10. Continue to expand the clone to 12- and 6-well plates, and finally to a T-25 flask.

Assay for Expression

Assay each of your cell lines for yield of the desired protein and select the one with the highest yield for scale-up and purification of recombinant protein. **If your protein is secreted, remember to assay the cell pellet as well as the medium.** You may wish to compare the yield of protein in the cells and medium.

Yield of Expressed Protein

In general, the level of secreted protein is comparable to that obtained with viral expression systems in insect cells. We have obtained stable cell lines that express and secrete human interleukin-6 to levels of 1-2 µg/ml. Human melanotransferrin has been expressed to levels of 8-10 µg/ml (Hegedus *et al.*, 1999).

Important

Remember to prepare master stocks and working stocks of your stable cell lines prior to scale-up and purification. Refer to the Insect Cell Lines manual for information on freezing your cells and scaling up for purification.

Scale-Up and Purification

Introduction

Once you have obtained stable cell lines expressing the protein of interest and prepared frozen stocks of your cell lines, you are ready to purify your protein. General information for protein purification is provided below. Eventually, you may expand your stable cell line into larger flasks, spinners, shake flasks, or bioreactors to obtain the desired yield of protein. If your protein is secreted, you may culture cells in serum-free medium to simplify purification.

Important

As you expand your stable cell line, you can maintain the concentration of blasticidin at 10 μg/ml.

Serum-Free Medium

If your protein is secreted, use Express Five® Serum-Free Medium to facilitate expression and purification (Catalog no. 10486-017).

Adapting Cells to Different Medium

Cells can be switched from complete TNM-FH to serum-free medium during passage. Refer to the Insect Cell Lines manual for more information on how to adapt cells to different medium.

CAUTION

If you plan to use a metal-chelating resin such as ProBond™ to purify your secreted protein from serum-free medium, **note that adding serum-free medium directly to the column will strip the nickel ions from the resin.** See the information below in **Purification of 6xHis-tagged Proteins from Medium** for a general recommendation to address this issue.

Purifying Proteins from Medium

Many protocols are suitable for purifying proteins from the medium. The choice of protocol depends on the nature of the protein being purified. Note that the culture volume needed to purify sufficient quantities of protein is dependent on the expression level of your protein and the method of detection. To purify 6xHis-tagged proteins from the medium, see below.

Purification of 6xHis-tagged Proteins from Medium

To purify 6xHis-tagged recombinant proteins from the culture medium, we recommend that you perform dialysis or ion exchange chromatography prior to affinity chromatography on metal-chelating resins.

Dialysis allows:

- Removal of media components that strip Ni^{+2} from metal-chelating resins

Ion exchange chromatography allows:

- Removal of media components that strip Ni^{+2} from metal-chelating resins
- Concentration of sample for easier manipulation in subsequent purification steps

Conditions for successful ion exchange chromatography will vary depending on the protein. For more information, refer to *Current Protocols in Protein Science* (Coligan *et al.*, 1998), *Current Protocols in Molecular Biology*, Unit 10 (Ausubel *et al.*, 1994) or the *Guide to Protein Purification* (Deutscher, 1990).

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Metal-chelating Resin

You may use the ProBond™ Purification System (Catalog no. K850-01) or a similar product to purify your 6xHis-tagged protein. The ProBond™ Purification System contains ProBond™, a metal-chelating resin specifically designed to purify 6xHis-tagged proteins. Before starting, be sure to consult the ProBond™ Purification System manual to familiarize yourself with the buffers and the binding and elution conditions. If you are using another resin, consult the manufacturer's instructions.

Note

Many insect cell proteins are naturally rich in histidines, with some containing stretches of six histidines. When using the ProBond™ Purification System or other similar products to purify 6xHis-tagged proteins, these histidine-rich proteins may co-purify with your protein of interest. The contamination can be significant if your protein is expressed at low levels. We recommend that you add 5 mM imidazole to the binding buffer prior to addition of the protein mixture to the column. Addition of imidazole may help to reduce background contamination by preventing proteins with low specificity from binding to the metal-chelating resin.

Purification of Intracellularly Expressed Proteins

If you are expressing your 6xHis-tagged protein intracellularly, you may lyse the cells and add the lysate directly to the ProBond™ column. You will need 5 x 10^6 to 1 x 10^7 cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual).

1. Seed 2 x 10^6 cells in two or three 25 cm^2 flasks.
2. Grow the cells in selective medium until they reach confluence (4 x 10^6 cells).
3. Wash cells once with PBS.
4. Harvest the cells by sloughing.
5. Transfer the cells to a sterile centrifuge tube.
6. Centrifuge the cells at 1000 x g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -80°C until needed.

Scale-Up

To scale up insect cell culture, refer to the Insect Cell Lines manual.

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

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

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

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.
5. To add X-gal to the plate, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml X-gal stock solution (see below), spread evenly, and let dry 15 minutes. Protect plates from light.

X-Gal Stock Solution

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

Trypan Blue Exclusion Assay

1. Prepare a 0.4% stock solution of trypan blue in phosphate buffered saline, pH 7.4
2. Mix 0.1 ml of trypan blue solution with 1 ml of cells and examine under a microscope at low magnification.
3. Dead cells will take up trypan blue while live cells will exclude it. Count live cells versus dead cells. Cell viability should be at least 95-99% for healthy log-phase cultures.

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Cell Lysis Buffer

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

1. This solution can be prepared 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 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.

To prevent proteolysis, you may add 1 mM PMSF, 1 μM leupeptin, and 0.1 μM aprotinin before use.

1X PBS

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

1. Dissolve: 8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   in 800 ml deionized water.
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter. You may wish to filter-sterilize or autoclave the solution to increase shelf life.

4X SDS-PAGE Sample Buffer

Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

Yield is ~10 ml.

Aliquot and freeze at -20°C until needed.

Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 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. Three simple protocols are provided below.

Note

Note that cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 7).

Using the S.N.A.P.™ MiniPrep Kit

The S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) allows you to rapidly purify PCR products from regular agarose gels. You will need to prepare 6 M sodium iodide in sterile water before starting. Add sodium sulfite to a final concentration of 10 mM to the NaI solution to prevent oxidation.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel. **Note**: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of 6 M NaI.
3. Add 1.5 volumes Binding Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
7. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
8. Centrifuge again at maximum speed for 1 minute to fully dry the resin.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 9.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 9). Be sure to make the gel slice as small as possible for best results.

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Low-Melt Agarose Method

Note that gel purification will result in a dilution of your PCR product. Use only chemically competent cells for transformation.

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 9.
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 TOP10 One Shot® cells using the method on page 10.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

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

Before Starting

You will need the following items:

- *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 a proofreading polymerase (*e.g. Vent*®, Platinum® *Pfx*, *Pfu*), 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 ligation into pIB/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 previous page). 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.

pIB/V5-His TOPO® Control Reactions

Introduction

If you have trouble obtaining transformants or vector containing insert, perform the following control reactions to help troubleshoot your experiment. Performing the control reactions involves producing a control PCR product containing the *lac* promoter and the LacZα fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product in either direction will yield blue colonies on LB agar plates containing antibiotic and X-gal.

Before Starting

Be sure to prepare LB plates containing 50-100 µg/ml ampicillin **and X-gal** (see page 25 for recipe) before performing the control reaction:

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

2. Overlay with 70 µl (1 drop) of mineral oil.
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

pIB/V5-His TOPO® Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the 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
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 TOP10 One Shot® cells (page 10).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin and X-Gal (see page 25). 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.

Analysis of Results

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

The "vector only" plate should yield very few colonies (<15% of the vector + PCR insert plate) and these should be all white.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform with 10 pg per 50 µl of cells using the protocol on page 10. Plate 10 µl of the transformation mixture plus 20 µl of SOC to help ensure even spreading on LB plates containing 50 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA for TOP10 cells.

continued on next page

Factors Affecting Cloning Efficiency

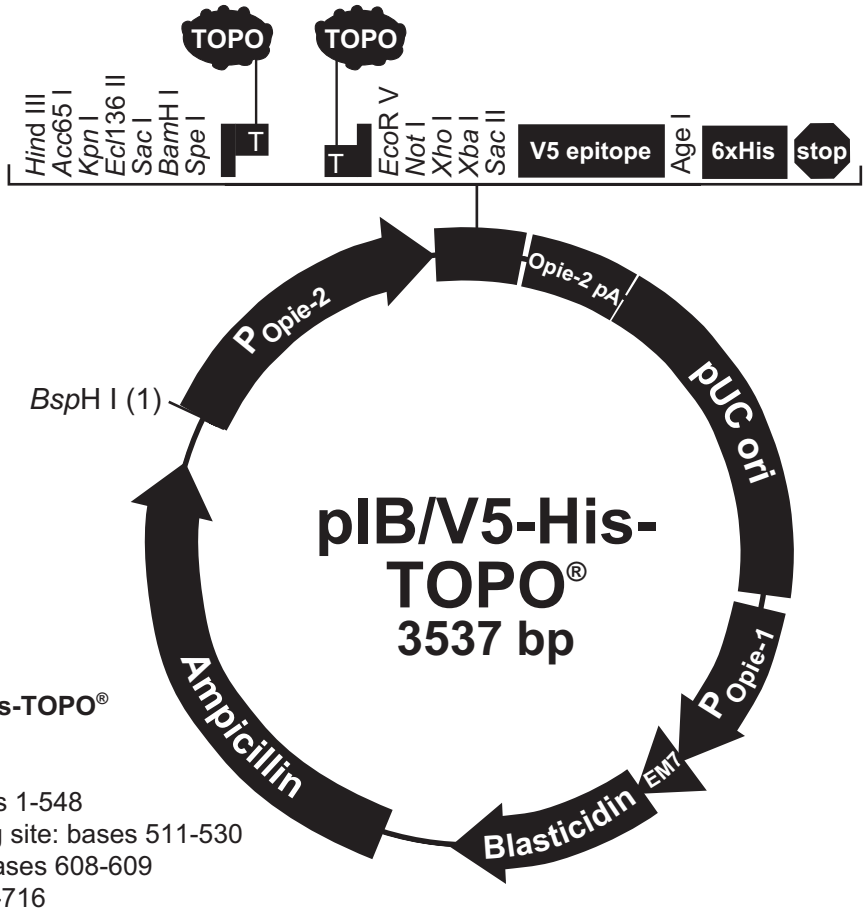
Note that lower cloning efficiencies will result from the following variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 85% (or more) cloning efficiency.

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 page 27.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product. Note that you may add up to 4 µl of your PCR to the TOPO® Cloning reaction (page 9).
Cloning blunt-ended fragments	Add 3′ A-overhangs by incubating with *Taq* polymerase (page 29).
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 (page 27) or optimize your PCR. If your template DNA carries an ampicillin marker, carryover into the TOPO® Cloning reaction from the PCR may lead to false positives. Linearize the template DNA prior to PCR to eliminate carryover.
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).

pIB/V5-His-TOPO® Map and Features

Map

The figure below summarizes the features of the pIB/V5-His-TOPO® vector (3537 bp). Vector is supplied linearized between nucleotides 608 and 609 (TOPO® Cloning site). For a more detailed explanation of each feature, see the next page. **The complete sequence of pIB/V5-His-TOPO® is available from our Web site (www.invitrogen.com) or from Technical Service (see page 39).**



Features of pIB/V5-His-TOPO®
3537 nucleotides

Opie-2 promoter: bases 1-548
OpIE2 Forward priming site: bases 511-530
TOPO® Cloning site: bases 608-609
V5 epitope: bases 675-716
6xHis tag: bases 726-743
OpIE2 Reverse priming site: bases 753-778
Opie-2 polyadenylation sequence: bases 761-890
pUC origin: bases 959-1632 (c)
Opie-1 promoter: bases 1681-1972
EM7 promoter: bases 1987-2045
Blasticidin resistance gene (*bsd*): bases 2064-2463
Ampicillin resistance gene (*bla*): bases 2582-3442

continued on next page

pIB/V5-His-TOPO® Map and Features, continued

Features

The features of pIB/V5-His-TOPO® (3537 bp) are described below. All features have been functionally tested. The multiple cloning site has been tested by restriction analysis.

Features	Function
OpIE2 promoter	Provides constitutive expression of the gene of interest in lepidopteran insect cells (Theilmann and Stewart, 1992).
OpIE2 Forward priming site	Allows sequencing of the insert from the 5′ end.
TOPO® Cloning site	Allows cloning of PCR product.
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of your recombinant protein with the Anti-V5 Antibody (Catalog no. R960-25) or Anti V5-HRP Antibody (Catalog no. R961-25) (Southern *et al.*, 1991).
6xHis tag	Allows purification of your recombinant protein on metal-chelating resin such as ProBond™. In addition, the C-terminal 6xHis tag is the epitope for the Anti-His(C-term) Antibody (Catalog no. R930-25) and the Anti-His(C-term)-HRP Antibody (Catalog no. R931-25).
OpIE2 Reverse priming site	Allows sequencing of the insert from the 3′ end.
OpIE2 polyadenylation sequence	Efficient transcription termination and polyadenylation of mRNA (Theilmann and Stewart, 1992).
pUC (pMB1-derived) origin	Replication, maintenance, and high copy number in *E. coli*.
OpIE1 promoter	Provides constitutive expression of the blasticidin resistance gene in lepidopteran insect cells (Theilmann and Stewart, 1991).
EM7 promoter	Allows efficient expression of the ampicillin and blasticidin resistance genes in *E. coli*.
Blasticidin resistance gene (*bsd*)	Allows generation of stable insect cell lines.
Ampicillin resistance gene (*bla*)	Selection of transformants in *E. coli*. **Note**: The native promoter has been removed. Transcription is assumed to start from the EM7 promoter.

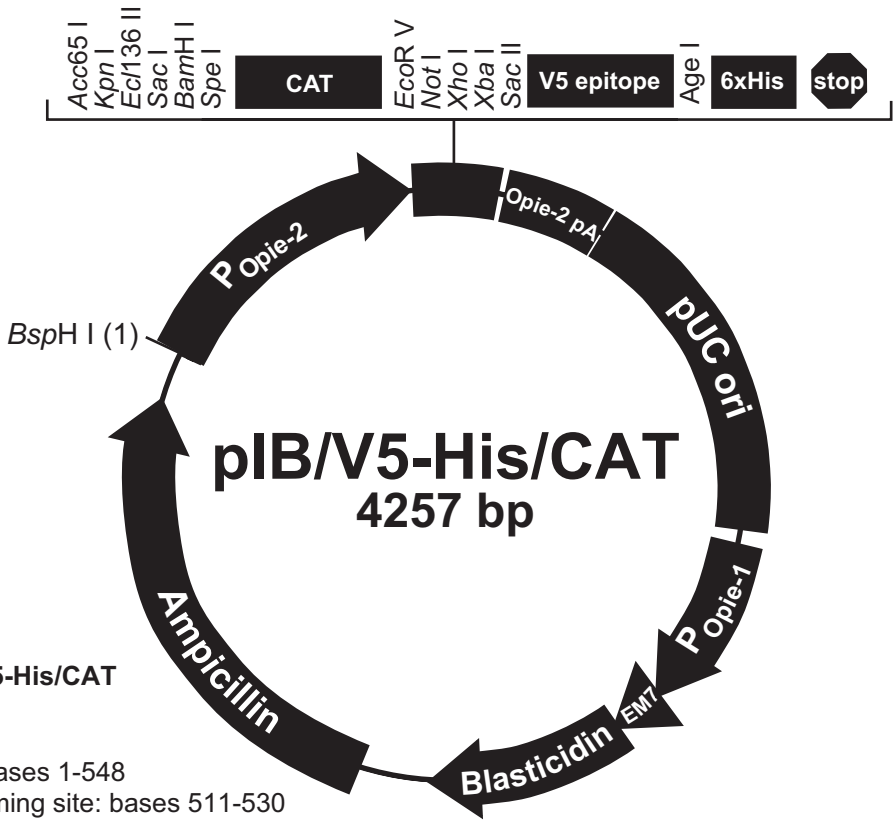
pIB/V5-His/CAT Map

Description

pIB/V5-His/CAT is a 4257 bp control vector expressing chloramphenicol acetyltransferase (CAT). The CAT gene was amplified using PCR and TOPO® Cloned into pIB/V5-His-TOPO®. CAT is expressed as a fusion to the V5 epitope and 6xHis tag. The molecular weight of the protein is 34 kDa.

Map

The figure below summarizes the features of the pIB/V5-His/CAT vector. **The complete nucleotide sequence for pIB/V5-His/CAT is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 39).**



Features of pIB/V5-His/CAT
4257 nucleotides

Opie-2 promoter: bases 1-548
OpIE2 Forward priming site: bases 511-530
CAT ORF: bases 666-1322
V5 epitope: bases 1395-1436
6xHis tag: bases 1446-1463
OpIE2 Reverse priming site: 1473-1498
Opie-2 polyadenylation sequence: bases 1481-1610
pUC origin: bases 1679-2352 (c)
Opie-1 promoter: bases 2401-2692
EM7 promoter: bases 2707-2765
Blasticidin resistance gene (*bsd*): bases 2784-3183
Ampicillin resistance gene (*bla*): bases 3302-4162

OpIE2 Promoter

Description

The *OpIE2* promoter has been analyzed by deletion analysis using a CAT reporter in both *Lymantria dispar* (LD652Y) and *Spodoptera frugiperda* (Sf9) cells. Expression in Sf9 cells was much higher than in LD652Y cells. Deletion analysis revealed that sequence up to -275 base pairs from the start of transcription is necessary for maximal expression (Theilmann and Stewart, 1992). Additional sequence beyond -275 may broaden the host range expression of this plasmid to other insect cell lines (Tom Pfeifer, personal communication).

In addition, an 18 bp element appears to be required for expression. This 18 bp element is repeated almost completely in three different locations and partially at six other locations. These are marked in the figure below. Elimination of the three major 18 bp elements reduces expression to basal levels (Theilmann and Stewart, 1992). The function of these elements is not known.

Primer extension experiments revealed that transcription initiates equally from either the C or the A indicated. These two transcriptional start sites are adjacent to a CAGT sequence motif that has been shown to be conserved in a number of early genes (Blissard and Rohrmann, 1989).

```
1    GGATCATGAT GATAAACAAT GTATGGTGCT AATGTTGCTT CAACAACAAT TCTGTTGAAC

61   TGTGTTTTCA TGTTTGCCAA CAAGCACCTT TATACTCGGT GGCCTCCCCA CCACCAACTT

121  TTTTGCACTG CAAAAAAACA CGCTTTTGCA CGCGGGCCCA TACATAGTAC AAACTCTACG

181  TTTCGTAGAC TATTTTACAT AAATAGTCTA CACCGTTGTA TACGCTCCAA ATACACTACC

241  ACACATTGAA CCTTTTTGCA GTGCAAAAAA GTACGTGTCG GCAGTCACGT AGGCCGGCCT

301  TATCGGGTCG CGTCCTGTCA CGTACGAATC ACATTATCGG ACCGGACGAG TGTTGTCTTA

361  TCGTGACAGG ACGCCAGCTT CCTGTGTTGC TAACCGCAGC CGGACGCAAC TCCTTATCGG

421  AACAGGACGC GCCTCCATAT CAGCCGCGCG TTATCTCATG CGCGTGACCG GACACGAGGC

                              TATA                    Start of Transcription
481  GCCCGTCCCG CTTATCGCGC CTATAAATAC AGCCCGCAAC GATCTGGTAA ACACAGTTGA

541  ACAGCATCTG TTCGAATTTA
```

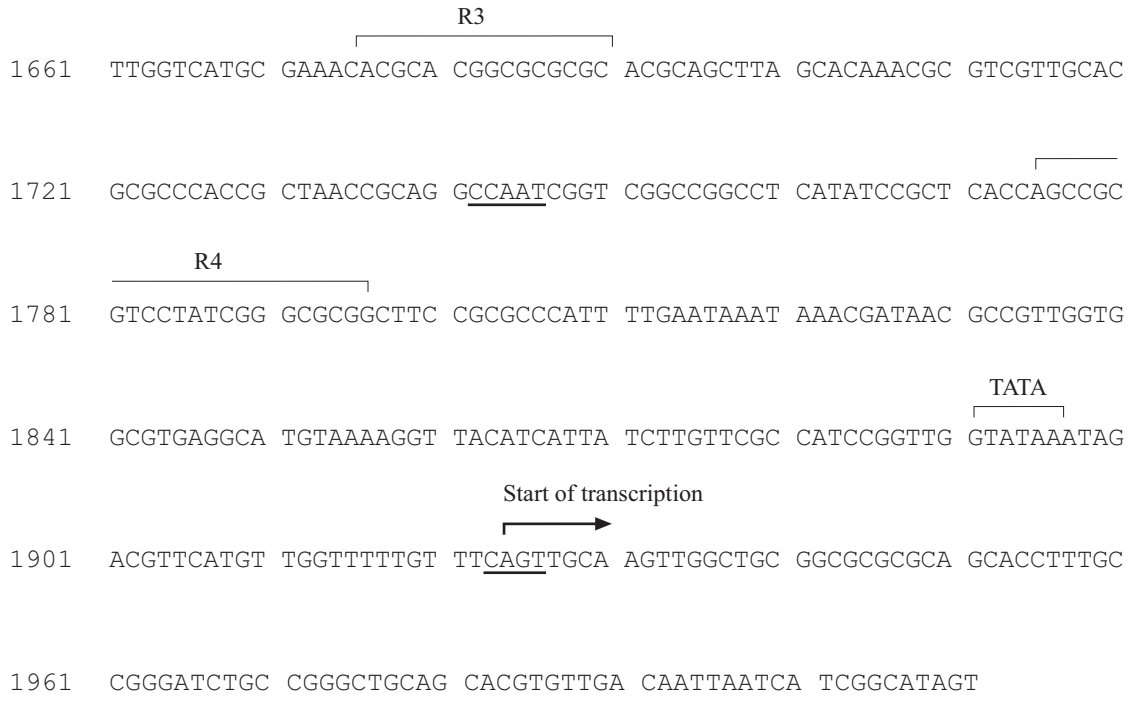
OpIE1 Promoter

Description

The *OpIE1* promoter has been analyzed by deletion analysis using a CAT reporter in both *Lymantria dispar* (LD652Y) and *Spodoptera frugiperda* (Sf9) cells. Deletion analysis revealed that sequence between -186 and -106 is important for maximum transcription in Sf9 cells (Theilmann and Stewart, 1991).

This region contains a canonical CCAAT site (underlined) (Johnson and McKnight, 1989)and an element (R4) that is homologous to the proposed binding site of the *Drosophila* transcription factor Adf-1 (England *et al.*, 1990). Three other Adf-1-like elements are found at three other distal locations. These elements are referred to as R1, R2, R3, and R4. R3 and R4 are marked in the figure below. R1 and R2 are not present in pIB/V5-His-TOPO® but do not appear to be important for expression in Sf9 cells. The function of these elements has not been determined.

Primer extension experiments revealed that transcription initiates from the A in the CAGT sequence. This CAGT sequence motif has been shown to be conserved in a number of early genes (Blissard and Rohrmann, 1989).



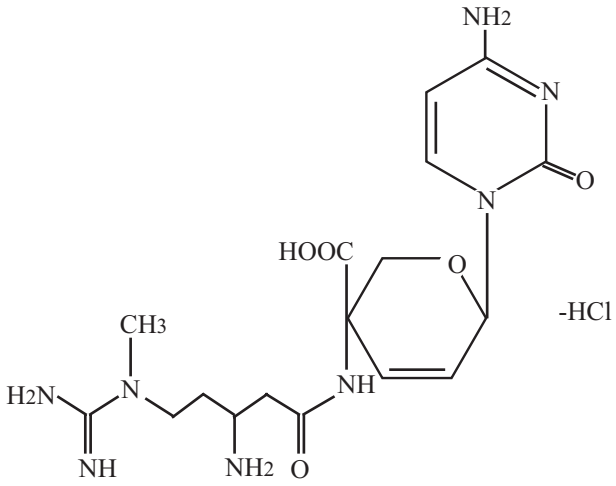
Blasticidin S

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 protective clothing (*e.g.* a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

To inactivate blasticidin for disposal, add sodium bicarbonate.

Preparing and Storing Stock Solutions

- Blasticidin S is soluble in water and acetic acid. Water is generally used to prepare stock solutions of 5 to 10 mg/ml.
- Dissolve blasticidin S in sterile water and filter-sterilize the solution.
- Blasticidin S is unstable in solutions with a pH greater than 8. Be sure the pH of the solution is below 7.
- Aliquot in small volumes (see 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.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store at +4°C. Discard after 1-2 weeks.

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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continued on next page

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Limited Use Label License No: 51 Blasticidin and the Blasticidin Selection Marker

Blasticidin and the blasticidin resistance gene (bsd) are the subject of U.S. Patent No. 5,527,701sold under patent license for research purposes only. Inquiries for commercial use should be directed to: Kaken Pharmaceutical Company, Ltd., Bunkyo Green Court, Center Office Building, 19-20 Fl, 28-8 Honkomagome 2-chome, Bunkyo-ku, Tokyo 113-8650, Japan, Tel: 81 3-5977-5008; Fax: 81 3-5977-5008

continued on next page

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Product Qualification

Introduction

Invitrogen qualifies the pIB/V5-His TOPO® TA Expression Kit as described below.

Restriction Digest

The parental supercoiled pIB/V5-His and pIB/V5-His/CAT are qualified by restriction digest prior to adaptation with topoisomerase. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pIB/V5-His	pIB/V5-His/CAT
*Acc*65 I (linearizes)	3520 bp	not tested
Xba I (linearizes)	3520 bp	not tested
*Hin*d III (linearizes)	not tested	4256 bp
Xho I (linearizes)	not tested	4256 bp
Drd I	339, 1086, 2095 bp	339, 1086, 2831 bp
Pst I	1368, 2152 bp	1368, 3158 bp

TOPO® Cloning Efficiency

Once the pIB/V5-His vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in the kit. Under conditions described on pages 30-32, a 500 bp control PCR product was TOPO® Cloned into pIB/V5-His-TOPO® and subsequently transformed into the One Shot® competent E. coli included with the kit.

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

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the 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 ~1 x 10^9 cfu/µg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

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