invitrogen™

pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits

Five-minute, TOPO® Cloning of *Taq* polymerase-amplified PCR products into vectors for high-level expression in the Expressway™ Cell-Free *E. coli* Expression System or in *E. coli*

Catalog nos. V960-05, V960-06, K9900-96, and K9900-98

Version B
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User Manual

Table of Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Produce PCR product</td><td>Produce PCR products using Taq polymerase and your own protocol. End the PCR reaction with a final 7 to 30 minute extension step.</td></tr>
<tr><td>Perform the TOPO® Cloning Reaction</td><td>1. Set up one of the following TOPO® Cloning reactions using the reagents in the order shown. For electroporation, dilute Salt Solution 4-fold to prepare Dilute Salt Solution.

<table>
<tr><th>Reagent</th><th>Chemical Transformation</th><th>Electroporation</th></tr>
<tr><td>Fresh PCR product</td><td>0.5 to 4 µl</td><td>0.5 to 4 µl</td></tr>
<tr><td>Salt Solution</td><td>1 µl</td><td>--</td></tr>
<tr><td>Dilute Salt Solution</td><td>--</td><td>1 µl</td></tr>
<tr><td>Water</td><td>to a final volume of 5 µl</td><td>to a final volume of 5 µl</td></tr>
<tr><td>TOPO® Vector</td><td>1 µl</td><td>1 µl</td></tr>
<tr><td>Total volume</td><td>6 µl</td><td>6 µl</td></tr>
</table>
2. Mix gently and incubate for 5 minutes at room temperature.
3. Place on ice and proceed to transform One Shot® chemically competent E. coli, below.</td></tr>
<tr><td>Transform One Shot® Chemically Competent E. coli</td><td>1. For each transformation, thaw one vial of One Shot® E. coli cells on ice.
2. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® chemically competent E. coli and mix gently.
3. Incubate on ice for 5 to 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Incubate at 37°C for 1 hour with shaking.
7. Spread 10-50 µl of bacterial culture on a prewarmed LB agar plate containing 100 µg/ml ampicillin, and incubate overnight at 37°C.</td></tr>
</table>

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 25-26 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products. Catalog numbers K9900-96 and K9900-98 are also supplied with the Expressway™ Maxi Cell-Free *E. coli* Expression System and the Expressway™ NMR Cell-Free *E. coli* Expression System, respectively. For a detailed description of the components included with the Expressway™ Maxi or Expressway™ NMR Cell-Free *E. coli* Expression Systems and their use, refer to the manual included with each kit. For a description of the components included with the pEXP5-TOPO® TA Expression Kits, see the rest of this section.

Product	Amount	Catalog no.
pEXP5-NT/TOPO® TA Expression Kit	10 reactions	V960-05
pEXP5-CT/TOPO® TA Expression Kit	10 reactions	V960-06
Expressway™ Maxi Cell-Free *E. coli* Expression System *with pEXP5-NT/TOPO® and pEXP5-CT/TOPO®*	1 kit	K9900-96
Expressway™ NMR Cell-Free *E. coli* Expression System *with pEXP5-NT/TOPO® and pEXP5-CT/TOPO®*	1 kit	K9900-98

System Kit Components

The Expressway™ Cell-Free *E. coli* Expression Systems include the following components. For a detailed description of the contents of the pEXP5-TOPO® TA Expression Kits, see pages viii-ix.

	Catalog no.			
Components	**V960-05**	**V960-06**	**K9900-96**	**K9900-98**
pEXP5-NT/TOPO® TA Expression Kit	✓		✓	✓
pEXP5-CT/TOPO® TA Expression Kit		✓	✓	✓
Expressway™ Maxi Cell-Free *E. coli* Expression System			✓	
Expressway™ NMR Cell-Free *E. coli* Expression System				✓

Shipping/Storage

Each pEXP5-TOPO® TA Expression Kit is shipped on dry ice. Upon receipt, store the components as detailed below.

Item	Storage
pEXP5-NT/TOPO® and/or pEXP5-CT/TOPO® Reagents	-20°C
One Shot® TOP10 Chemically Competent *E. coli*	-80°C

continued on next page

Kit Contents and Storage, continued

pEXP5-TOPO® Reagents

The following reagents are supplied with the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector. **Note that the user must supply *Taq* polymerase. Store at -20°C.**

Item	Concentration	Amount
pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector, TOPO®-adapted	5-10 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM phenol red	10 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
Water	---	1 ml
T7 Forward Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
T7 Reverse Primer **(pEXP5-NT/TOPO® kit only)**	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
T7 Term Reverse Primer **(pEXP5-CT/TOPO® kit only)**	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8.0	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8.0	10 µl

continued on next page

Kit Contents and Storage, continued

Primer Sequences

The table below provides the sequences of the primers included with the kits.

Primer	Sequence	pmoles Supplied
T7 Forward	5´-TAATACGACTCACTATAGGG-3´	327
T7 Reverse	5´-TAGTTATTGCTCAGCGGTGG-3´	325
T7 Term Reverse	5′-ATCCGGATATAGTTCCTCCTTTC-3′	434

One Shot® Reagents

The following reagents are included with the One Shot® TOP10 Chemically Competent *E. coli* kit. Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store at -80°C.**

Reagent	Composition	Amount
S.O.C. 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	--	11 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

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

Accessory Products

Introduction

The products listed in this section may be used with the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA Expression Kit. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 36).

Additional Products

Some of the reagents supplied in the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA Expression Kit and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Note: Other reagent quantities may be available.

Item	Quantity	Catalog no.
Platinum® *Taq* DNA Polymerase	100 reactions 250 reactions 500 reactions	10966-018 10966-026 10966-034
Taq DNA Polymerase, Recombinant	100 units 250 units 500 units	10342-053 10342-012 10342-020
Platinum® *Taq* DNA Polymerase High Fidelity	100 units 500 units	11304-011 11304-029
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions	C4040-50
BL21 Star™(DE3) One Shot® Chemically Competent *E. coli*	20 reactions	C6010-03
BL21 Star™(DE3)pLysS One Shot® Chemically Competent *E. coli*	20 reactions	C6020-03
BL21-AI™ One Shot® Chemically Competent *E. coli*	20 reactions	C6070-03
LB Broth	500 ml	10855-021
LB Agar	500 g	22700-025
Ampicillin	200 mg	11593-019
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
AcTEV™ Protease	1,000 units 10,000 units	12575-015 12575-023
Expressway™ Maxi Cell-Free *E. coli* Expression System	100 reactions	K9900-97
Expressway™ NMR Cell-Free *E. coli* Expression System	5 reactions	K9900-99

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Accessory Products, continued

Products to Detect Recombinant Fusion Proteins

If you have cloned your gene of interest in frame with the N- or C-terminal 6xHis tag in pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, respectively, you may use an antibody to the appropriate epitope to detect your recombinant fusion protein. The table below describes the antibodies available from Invitrogen for detection. The amount of antibody supplied is sufficient for 25 western blots.

Product	Epitope	Catalog no.
Anti-HisG Antibody	Detects the N-terminal polyhistidine (6xHis) tag followed by glycine: HHHHHHG	R940-25
Anti-HisG-HRP Antibody		R941-25
Anti-HisG-AP Antibody		R942-25
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

Products to Purify Recombinant Fusion Proteins

If you clone your gene of interest in frame with the N-terminal or C-terminal 6xHis tag in pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, respectively, you may purify your recombinant fusion protein using one of Invitrogen's metal-chelating resins. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Nickel-Chelating Resin	50 ml 150 ml	R801-01 R801-15
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K853-01
Ni-NTA Agarose	10 ml 25 ml 100 ml	R901-01 R901-15 R901-10
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Purification System with Anti-His(C-term)-HRP Antibody	1 kit	K953-01
Polypropylene Columns (empty)	50	R640-50

Introduction

Overview

Introduction

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits provide a highly efficient, five-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for T7-based, high-level expression of recombinant fusion proteins in the Expressway™ Cell-Free *E. coli* Expression System or for inducible expression in *E. coli*. No ligase, post-PCR procedures, or PCR primers containing special, additional sequences are required to generate the expression construct.

A choice of kits allows you to fuse your gene of interest to an N-terminal or C-terminal tag for easy detection and purification of recombinant fusion proteins (see table below).

Vector	Fusion Peptide	Fusion Tag	Benefit
pEXP5-NT/TOPO®	N-terminal	6xHis, TEV recognition site	Cleavable detection and purification tag
pEXP5-CT/TOPO®	C-terminal	6xHis	Detection and purification tag

For more information about TOPO® Cloning and how it works, see the next page. For more information about the Expressway™ Cell-Free *E. coli* Expression System or T7-based expression in *E. coli*, see pages 2 and 3, respectively.

Features of the Vectors

Features of the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors include:

- Bacteriophage T7 promoter for high-level, inducible expression of the recombinant protein of interest in the Expressway™ Cell-Free *E. coli* Expression System or in *E. coli*
- Ribosome binding site (RBS) optimally spaced from the initiation ATG in the N-terminal tag for efficient translation of the recombinant fusion protein (pEXP5-NT/TOPO® only)
- N-terminal or C-terminal fusion tags for detection and purification of recombinant fusion proteins (choice of tag varies depending on the particular vector; see above)
- Tobacco Etch Virus (TEV) recognition site for cleavage of the N-terminal peptide from your recombinant fusion protein (pEXP5-NT/TOPO® only)
- TOPO® Cloning site for rapid and efficient cloning of *Taq*-amplified PCR products (see the next page for more information)
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

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Overview, continued

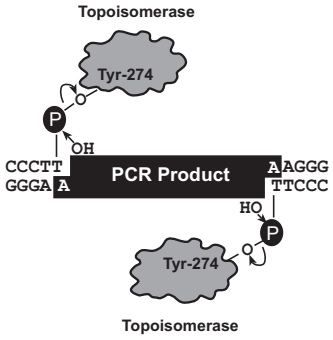
How TOPO® Cloning Works

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors are supplied linearized with:

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

Taq polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in each 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 (CCCTT) and cleaves the phosphodiester backbone 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.



The Expressway™ Cell-Free *E. coli* Expression Systems

The Expressway™ Maxi and NMR Cell-Free *E. coli* Expression Systems facilitate T7-based, *in vitro* transcription and translation of target DNA to protein in a single tube. The System uses an optimized *E. coli* extract, a reaction buffer containing an ATP regenerating system, amino acids, and an optimized Feed Buffer to facilitate production of recombinant protein in 3-6 hours without the need for specialized equipment. The recombinant protein produced is suitable for use in downstream structural proteomics applications including x-ray crystallography, mass spectrometry, and NMR spectroscopy.

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits are supplied with the Expressway™ Maxi (Catalog no. K9900-96) or Expressway™ NMR (Catalog no. K9900-98) Cell-Free *E. coli* Expression Systems, but each System is also available separately from Invitrogen (see page x for ordering information).

For more information about the Expressway™ Maxi or NMR Cell-Free *E. coli* Expression Systems, refer to the manual for each product. Manuals are included with Catalog nos. K9900-96 and K9900-98, but are also available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).

T7-Regulated Expression

The Basis of T7-Regulated Expression

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors allow expression of your gene of interest in *E. coli* under the control of the strong bacteriophage T7 promoter. In bacteriophage T7, the T7 promoter drives expression of gene 10 (ϕ10). T7 RNA polymerase specifically recognizes this promoter. To express the gene of interest in *E. coli*, you may use a bacterial host that expresses T7 RNA polymerase or infect the cell with phage expressing T7 RNA polymerase.

We generally use a BL21-derived *E. coli* strain as the host for the expression construct. These strains express T7 RNA polymerase in a regulated manner, thus facilitating regulated expression of the gene of interest. Many BL21-derived strains are available from Invitrogen. For more information about some of the options available, see the next page. For more information about T7-based expression systems, see published references (Studier *et al.*, 1990).

Use of TOP10 Cells

One Shot® TOP10 Chemically Competent *E. coli*, which do not contain T7 RNA polymerase, are included with each pEXP5-TOPO® TA Expression Kit to serve as a host for stable propagation and maintenance of recombinant plasmids. **Do not** use BL21-derived *E. coli* strains to maintain your expression construct as the presence of T7 RNA polymerase, even at basal levels, can lead to expression of the gene of interest even in the absence of inducer. In general, this is not a problem. However, if the gene of interest is toxic to the *E. coli* host, plasmid instability and/or cell death results.

After you have TOPO® Cloned your PCR product into the pEXP5-TOPO® vector, we recommend that you transform, characterize, and maintain your expression construct in TOP10 cells. When you are ready to perform an expression experiment, transform your expression construct into a BL21 *E. coli* strain. See the next page for more information.

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T7-Regulated Expression, continued

BL21 *E. coli* Strains

You may use the BL21 (Grodberg and Dunn, 1988; Studier and Moffatt, 1986) *E. coli* strain or any suitable BL21 derivative as a host to express your recombinant protein. Consider the following factors when choosing a suitable BL21 strain to use for expression:

- **Ability to induce T7 RNA polymerase expression:** Use a BL21-derived strain that contains the DE3 bacteriophage lambda lysogen. The λDE3 lysogen contains the T7 RNA polymerase under the control of the *lacUV5* promoter, allowing expression of T7 RNA polymerase to be induced by isopropyl β-D-thiogalactoside (IPTG).
- **Expressing a slightly toxic or toxic gene:** Use a BL21-derived strain that expresses T7 RNA polymerase in a tightly regulated manner (*e.g.* BL21-AI™). Alternatively, use a BL21-derived strain containing the pLysS or pLysE plasmid. The pLysS and pLysE plasmids express varying levels of T7 lysozyme, which binds to T7 RNA polymerase and inhibits transcription. BL21 strains that contain the pLysS or pLysE plasmids exhibit reduced basal levels of T7 RNA polymerase, enabling expression of slightly toxic or toxic genes, respectively.
- **Protein yield desired:** To express the highest levels of recombinant protein, use a BL21-derived strain that helps prevent mRNA degradation (*e.g.* BL21 Star™) or one that does not contain the pLysS or pLysE plasmid.

For recommended BL21-derived strains available from Invitrogen, see the next page.

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T7-Regulated Expression, continued

Recommended BL21 Strains

Many BL21-derived strains are available from Invitrogen for expression of recombinant proteins from pEXP5-NT/TOPO® or pEXP5-CT/TOPO®. Choose one of the following recommended BL21-derived strains depending on your needs. If you are expressing your recombinant protein for the first time and are uncertain about its effect on the cells (*i.e.* toxicity), we recommend using the BL21-AI™ *E. coli* strain. This strain allows tightly regulated expression of recombinant protein, thus making it ideal for use to express toxic proteins.

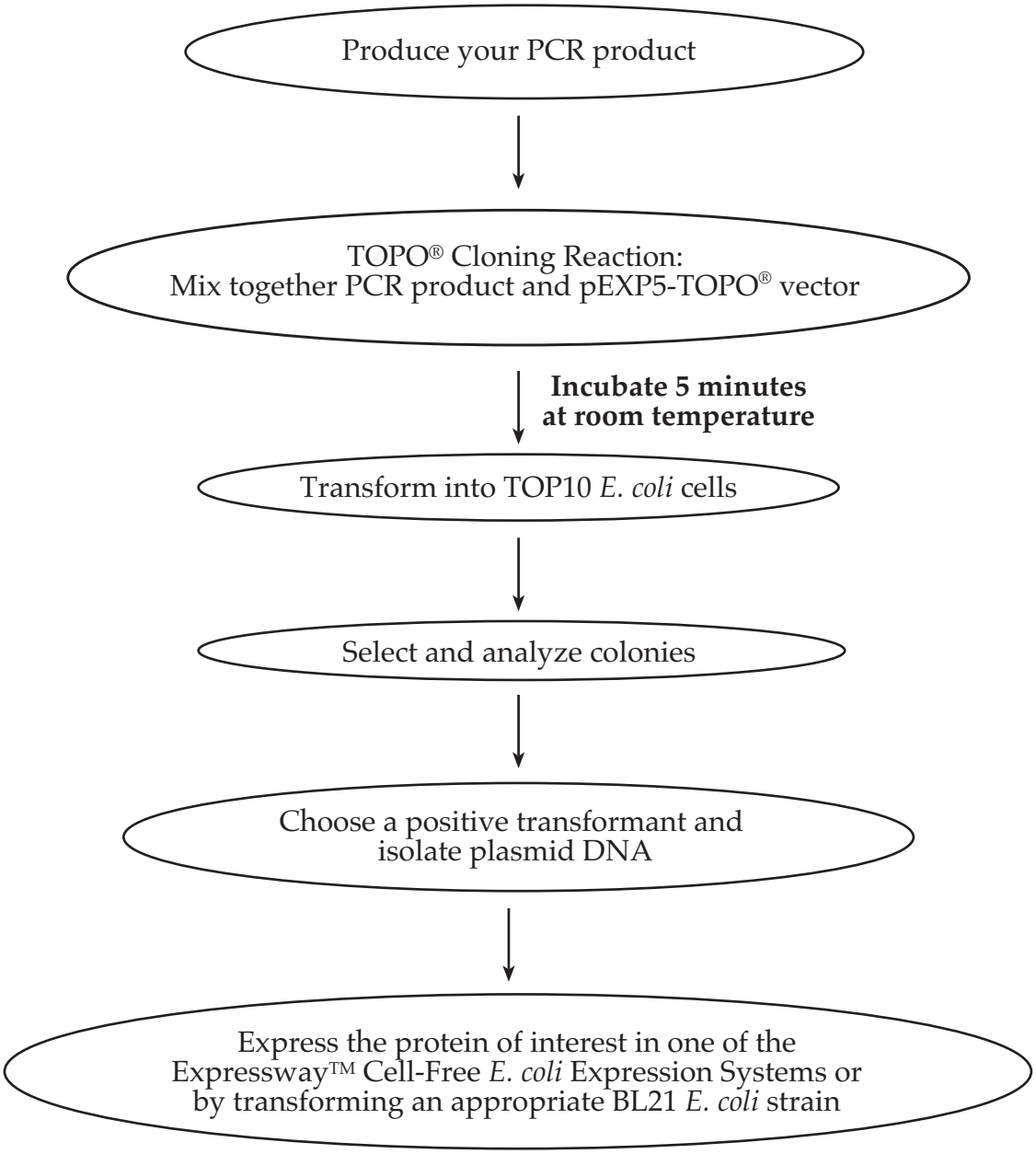
All BL21-derived *E. coli* cells available from Invitrogen are supplied chemically competent in One Shot® format for fast, easy, and efficient transformation. For more information about any of these strains, see www.invitrogen.com or contact Technical Service (page 36).

E. coli Strain	Features	Benefit	Catalog no.
BL21-AI™	Carries T7 RNA polymerase gene under the control of the *araBAD* promoter, allowing tightly regulated, inducible expression of T7 RNA polymerase	Tight regulation and high-level expression of toxic proteins	C6070-03
BL21 Star™(DE3)	• Carries a mutated RNase E gene (*rne131*), allowing increased mRNA stability and higher protein yields • Contains the λDE3 lysogen, allowing IPTG-inducible expression of T7 RNA polymerase	Extremely high expression of non-toxic proteins	C6010-03
BL21 Star™(DE3)pLysS	• Carries a mutated RNase E gene (*rne131*), allowing increased mRNA stability and higher protein yields • Contains the λDE3 lysogen, allowing IPTG-inducible expression of T7 RNA polymerase • Contains the pLysS plasmid, reducing basal expression of T7-driven genes	High expression of proteins that are slightly growth inhibitive to *E. coli*	C6020-03

Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and TOPO® Clone your *Taq*-amplified PCR product into pEXP5-NT/TOPO® or pEXP5-CT/TOPO®.



Methods

Designing PCR Primers

Introduction

Before using the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA Expression Kit, you must first design PCR primers and produce your PCR product. Use the guidelines and diagrams provided in this section to help you design PCR primers.

Cloning into pEXP5-NT/TOPO®

pEXP5-NT/TOPO® allows expression of your recombinant protein fused to an N-terminal peptide containing a polyhistidine (6xHis) tag and a TEV recognition site. The 6xHis tag enables detection of the recombinant protein with an Anti-HisG Antibody and purification using metal-chelating resin. The TEV recognition site allows removal of the N-terminal tag using TEV Protease. Consider the following when designing your PCR primers. Refer to the diagram on page 7 for more help.

If you wish to…	Then…
Include the N-terminal 6xHis tag	Design the forward PCR primer to place the gene of interest in frame with the N-terminal tag. Note that: • A Shine-Dalgarno ribosome binding site (RBS) is included upstream of the initiation ATG in the N-terminal tag and is optimally spaced to facilitate proper translation • At least two non-native amino acids will be present between the TEV cleavage site and the start of your gene **Recommendation:** If you plan to use the expression construct as a template for cell-free protein synthesis in the Expressway™ Cell-Free *E. coli* Expression System, design the forward PCR primer such that the first 3 nt of the PCR product encode the ATG initiation codon of your protein. This minimizes the number of non-native amino acids added to the N-terminus of your gene and maximizes the yield of recombinant protein obtained.
Express your protein with a native N-terminus, *i.e.* without the N-terminal tag	Design the forward PCR primer to include the following: • A stop codon to terminate the N-terminal tag • A second RBS (*e.g.* AGGAGA) 6-10 base pairs 5′ of the initiation ATG codon of your protein

When designing the reverse PCR primer, be sure to include a stop codon in the reverse primer or design the reverse PCR primer to hybridize downstream of the native stop codon.

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Designing PCR Primers, continued

Cloning into pEXP5-CT/TOPO®

pEXP5-CT/TOPO® allows expression of your recombinant protein fused to a C-terminal peptide containing a polyhistidine (6xHis) tag. The 6xHis tag enables detection with an Anti-His(C-term) Antibody and purification of the recombinant protein using metal-chelating resin. Consider the following when designing your PCR primers. Refer to the diagram on page 8 for more help.

Note: For maximal expression of native protein, design the forward PCR primer to place the initiation ATG codon of the desired protein 6-10 base pairs from the RBS (Gold, 1988; Miller, 1992). This ensures the optimal spacing necessary for proper translation to occur.

If you wish to…	Then…
Express your protein with a native N-terminus using the vector-encoded RBS	Design the forward PCR primer such that the first 3 nt of the PCR product encode the initiation ATG codon of your protein.
Include the C-terminal 6xHis tag	Design the reverse PCR primer to remove the native stop codon in the gene of interest and preserve the reading frame through the C-terminal tag.
Not include the C-terminal 6xHis tag	Include the native sequence encoding the stop codon in the reverse PCR primer or make sure the stop codon is upstream from the reverse PCR primer binding site.

Important

When synthesizing PCR primers, **do not** add 5´ phosphates to the primers as this will prevent the synthesized PCR product from ligating into the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector.

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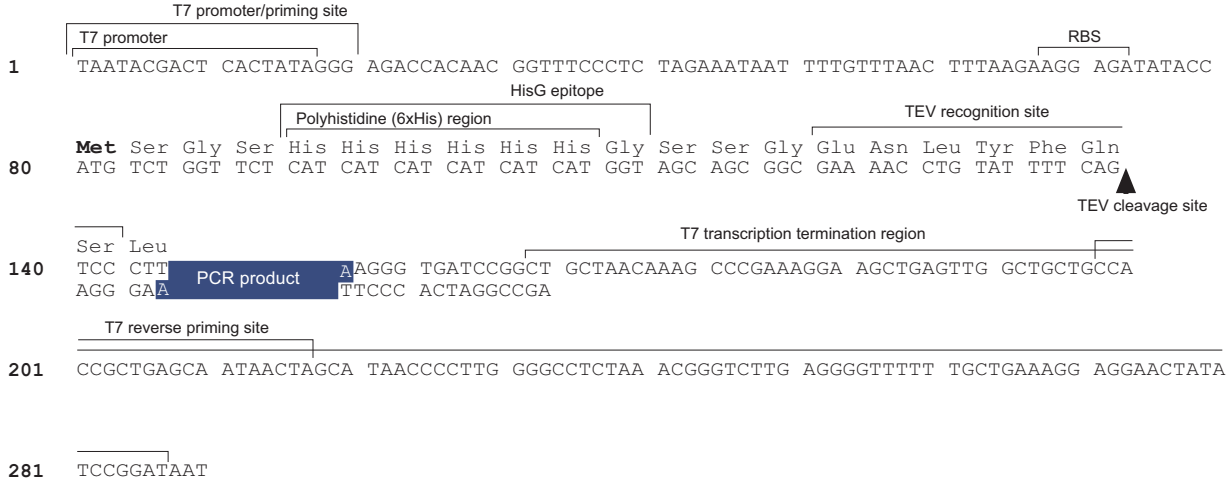
Designing PCR Primers, continued

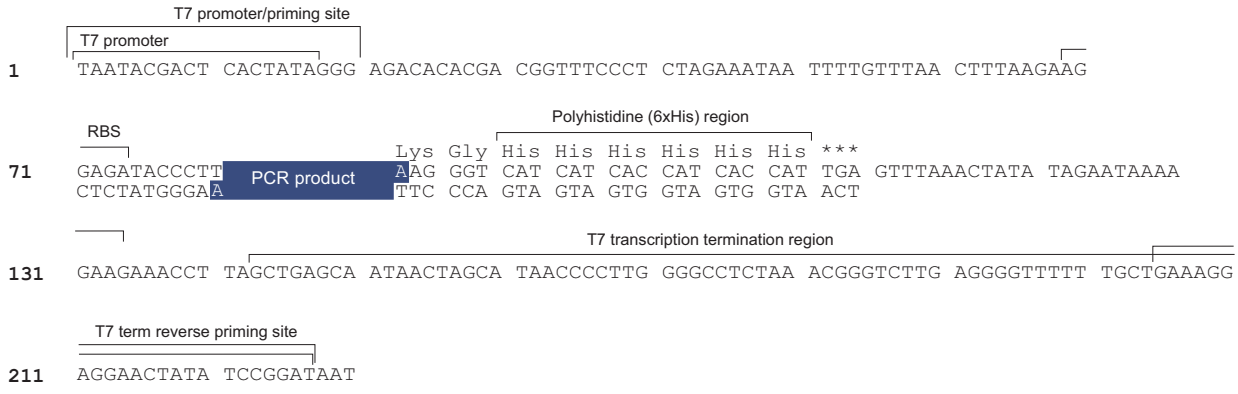
TOPO® Cloning Site for pEXP5-NT/TOPO®

Use the diagram below to help you design PCR primers and produce your PCR product for TOPO® Cloning into pEXP5-NT/TOPO®. **The sequence of pEXP5-NT/TOPO® is available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).** For more information about pEXP5-NT/TOPO®, see pages 30-33.



TOPO® Cloning Site for pEXP5-CT/TOPO®

Use the diagram below to help you design PCR primers and produce your PCR product for TOPO® Cloning into pEXP5-CT/TOPO®. **The sequence of pEXP5-CT/TOPO® is available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).** For more information about pEXP5-CT/TOPO®, see pages 34-35.



Producing PCR Products

Introduction

Once you have synthesized appropriate PCR primers, you may use the primers and a suitable DNA polymerase to produce your PCR product. **Remember that your PCR product must have single 3′ A-overhangs.**

Materials Supplied by the User

You will need the following reagents and equipment for PCR. **Note:** dNTPs (adjusted to pH 8) are provided in the kit.

- *Taq* polymerase or other suitable DNA polymerase

  Note: For improved specificity and higher yields, we recommend using Platinum® *Taq* DNA Polymerase available from Invitrogen (see page x for ordering information) to generate your PCR product.
- Thermocycler
- DNA template and primers to produce the PCR product

Polymerase Mixtures

You may use a polymerase mixture containing *Taq* polymerase and a proofreading polymerase to produce your PCR product; however, the mixture must contain a ratio of *Taq* polymerase:proofreading polymerase in excess of 10:1 to ensure the presence of 3′ A-overhangs on the PCR product. We recommend using Platinum® *Taq* DNA Polymerase High Fidelity available from Invitrogen (see page x for ordering information).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you may add 3′ A-overhangs to your PCR product 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 step 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
dNTP Mix (50 mM)	0.5 µl
PCR primers (100-200 ng each)	1 µM each
Water	add to a final volume of 49 µl
Taq Polymerase (1 U/µl)	1 µl
Total volume	50 µl

2. Use agarose gel electrophoresis to verify the quality of your PCR product. You should see a single, discrete band of the correct size. If you do not see a single band, refer to the **Note** on the next page.

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Producing PCR Products, continued

Note

If you do not obtain a single, discrete band from your PCR, try the following:

- Optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit available from Invitrogen (Catalog no. K1220-01) incorporates many of the recommendations found in this reference. For more information, refer to www.invitrogen.com or contact Technical Service (see page 36).
- Gel-purify your fragment using one of the methods on pages 27-28. Take care to avoid sources of nuclease contamination.

Setting Up the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector and transform the recombinant vector into One Shot® TOP10 competent *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® TOP10 Competent *E. coli*** (pages 14-16) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 25-26 in parallel with your samples.

Note

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction can increase the number of transformants 2- to 3-fold. In addition, incubating the reaction mixture for greater than 5 minutes in the presence of salt 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.

Including salt in the TOPO® Cloning reaction 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.

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **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 page x for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed on the next page.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed on the next page.

continued on next page

Setting Up the TOPO® Cloning Reaction, continued

Materials Needed

You should have the following materials on hand before beginning:

- Your PCR product (freshly prepared)
- pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector (supplied with the kit; keep at -20°C until use)
- Salt Solution (supplied with the kit) or Dilute Salt Solution as appropriate
- Water (supplied with the kit)

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction using the reagents in the order shown, and depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.*

Note: The red 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
Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number 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 may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent *E. coli***, next page.

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

Transforming One Shot® TOP10 Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pEXP5-NT/TOPO® or pEXP5-CT/TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit to facilitate transformation. You may also transform electrocompetent cells, if desired (see page x for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Selecting a One Shot® Chemical Transformation Protocol

Two protocols are provided to transform One Shot® TOP10 chemically competent *E. coli*. Consider the following factors and choose the protocol that best suits your needs.

If you wish to…	Then use the…
maximize the number of transformants	regular chemical transformation protocol, page 15
clone large PCR products (>1000 bp)	
obtain transformants as quickly as possible	rapid chemical transformation protocol, page 16 **Note:** This procedure is less efficient; the total number of transformants obtained may be lower than that obtained with the regular chemical transformation protocol

Materials Needed

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

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot® TOP10 chemically competent *E. coli* (supplied with the kit)
- S.O.C. Medium (supplied with the kit)
- pUC19 positive control (to verify transformation efficiency, if desired)
- 42°C water bath (or electroporator with cuvettes, optional)
- 15 ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 100 µg/ml ampicillin (two for each transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned into the vector. Sequencing primers are included in the kit to allow you to sequence across an insert in the TOPO® Cloning site to confirm orientation and reading frame.

continued on next page

Transforming One Shot® TOP10 Competent *E. coli*, continued

Preparing for Transformation

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

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. Medium to room temperature.
- Warm LB plates containing 100 µg/ml ampicillin at 37°C for 30 minutes (see **Important Note** below).
- Thaw **on ice** one vial of One Shot® cells for each transformation.

Important

If you are performing the rapid chemical transformation protocol, it is **essential** that you prewarm your LB plates containing 100 µg/ml ampicillin prior to spreading.

One Shot® TOP10 Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 13 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

   Note: If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
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 S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. 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 S.O.C. Medium. 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 should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 17).

continued on next page

Transforming One Shot® TOP10 Competent *E. coli*, continued

Rapid One Shot® Chemical Transformation Protocol

Use the alternative protocol below to rapidly transform One Shot® TOP10 chemically competent *E. coli*. Before beginning, make sure to pre-warm LB agar plates containing 100 µg/ml ampicillin at 37°C for 30 minutes.

1. Add **4 µl** of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 13 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µl** of cells on a prewarmed selective plate and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 17).

One Shot® Electroporation Protocol

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 13 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see recommendation below.
3. Immediately add 250 µl of room temperature S.O.C. Medium.
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 expression of the ampicillin 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 S.O.C. Medium. 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 should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 17).

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.

Analyzing Transformants

Analyzing Positive Clones

1. Pick 10 colonies and culture them overnight in LB or SOB medium containing 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 using Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
3. Analyze the plasmids by restriction analysis, sequencing, or PCR to confirm the presence and correct orientation of the insert.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation and in frame with the appropriate N- or C-terminal tag. Use the sequencing primers supplied with the kit to help you sequence your insert (see table). For the location of the priming sites in pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, see the diagrams on page 9.

Vector	Forward Primer	Reverse Primer
pEXP5-NT/TOPO®	T7 forward	T7 reverse
pEXP5-CT/TOPO®	T7 forward	T7 term reverse

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the Forward sequencing primer or the Reverse sequencing primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above.
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

continued on next page

Analyzing Transformants, continued

Long-Term Storage

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

1. Streak the original colony out for single colonies on an LB plate containing 100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 µg/ml ampicillin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Expression and Analysis

Introduction

Once you have obtained purified plasmid DNA of your pEXP5-NT/TOPO® or pEXP5-CT/TOPO® expression construct, you may express your recombinant fusion protein by:

- Performing a cell-free protein synthesis reaction using one of the Expressway™ Cell-Free *E. coli* Expression Systems (*e.g.* Expressway™ Maxi or NMR Cell-Free *E. coli* Expression System)
- Transforming a suitable BL21 *E. coli* strain

General guidelines are provided in this section.

Plasmid Preparation

You may prepare plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) available from Invitrogen.

Using the Expressway™ Cell-Free *E. coli* Expression System

Your pEXP5-NT/TOPO® or pEXP5-CT/TOPO® expression construct provides an optimal template for recombinant protein production in one of the Expressway™ Cell-Free *E. coli* Expression Systems (*e.g.* Expressway™ Maxi or NMR Cell-Free *E. coli* Expression Systems). To synthesize and analyze your recombinant fusion protein from pEXP5-NT/TOPO® or pEXP5-CT/TOPO® using one of the Expressway™ Cell-Free *E. coli* Expression Systems, refer to the manual supplied with the kit you are using. Manuals are also available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).

BL21 *E. coli* Expression Strain

To facilitate expression of your recombinant fusion protein in *E. coli*, you must use a strain that permits expression of T7-regulated genes. We recommend using one of the BL21 *E. coli* strains available from Invitrogen (*e.g.* BL21-AI™, BL21 Star™(DE3), or BL21 Star™(DE3)pLysS) as a host for your pEXP5-NT/TOPO® or pEXP5-CT/TOPO® construct. For more information about the BL21-AI™, BL21 Star™(DE3), and BL21 Star™(DE3)pLysS strains, see page 5 or the manual for each strain. All manuals are available for downloading from www.invitrogen.com or by contacting Technical Service (page 36).

Performing Expression in *E. coli*

If you use a BL21-derived *E. coli* strain for expression, you will transform the cells and induce expression of your recombinant protein with IPTG. For guidelines to transform *E. coli* and perform expression studies, see the **Appendix**, pages 30-31.

continued on next page

Detecting Recombinant Fusion Proteins

You may detect expression of your recombinant fusion protein by Western blot analysis using antibodies against the appropriate epitope available from Invitrogen (see table below and page xi for ordering information) or an antibody to your protein of interest.

Vector	Epitope	Antibody
pEXP5-NT/TOPO®	HisG (HHHHHHG)	• Anti-HisG Antibody • Anti-HisG-HRP Antibody • Anti-HisG-AP Antibody
pEXP5-CT/TOPO®	C-terminal 6xHis (HHHHH-COOH)	• Anti-His(C-term) Antibody • Anti-His(C-term)-HRP Antibody • Anti-His(C-term)-AP Antibody

In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a HisG or C-terminal 6xHis epitope. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescence methods. For more information, refer to www.invitrogen.com or call Technical Service (see page 36).

Note

Expression of your protein with the N- or C-terminal tags in pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, respectively, will increase the size of your recombinant protein. The table below lists the increase in the molecular weight of your recombinant fusion protein that you should see from the particular tag in each vector. Be sure to account for any additional amino acids between the tag and your fusion protein.

Vector	Peptide Tag	Expected Size Increase (kDa)
pEXP5-NT/TOPO®	N-terminal	2.5 kDa
pEXP5-CT/TOPO®	C-terminal	1.1 kDa

Purifying Recombinant Fusion Proteins

The presence of the N-terminal or C-terminal polyhistidine (6xHis) tag in your recombinant fusion protein allows use of a metal-chelating resin such as ProBond™ or Ni-NTA to purify your fusion protein. ProBond™ and Ni-NTA are available from Invitrogen (see page xi for ordering information). Refer to the manual included with each product for instructions to purify your 6xHis-tagged fusion protein.

Note: Other metal-chelating resins and purification methods are suitable.

continued on next page

Expression and Analysis, continued

Cleavage of the N-terminal Tag in pEXP5-NT/TOPO®

The pEXP5-NT/TOPO® vector contains a Tobacco Etch Virus (TEV) recognition site (Carrington and Dougherty, 1988; Dougherty *et al.*, 1988) to allow removal of the N-terminal tag from your recombinant fusion protein using TEV protease, if desired. **Cleavage with TEV protease generates nearly native protein since only 2 amino acids will remain at the N-terminus of your protein (assuming that the first codon of your protein directly follows the TOPO® Cloning site; see diagram on page 9 for reference).**

Obtaining TEV Protease

For highly efficient TEV protease-directed cleavage, we recommend using AcTEV™ Protease available from Invitrogen (Catalog nos. 12575-015 and 12575-023). AcTEV™ Protease is an enhanced form of TEV protease that is highly site-specific, active, and more stable than native TEV protease (Nayak *et al.*, 2003). Following digestion, AcTEV™ Protease may be easily removed from the cleavage reaction by affinity chromatography using the 6xHis tag at the N-terminus of the protease. For instructions and guidelines to perform AcTEV™ Protease-directed cleavage, refer to the manual included with the product. The manual is also available from www.invitrogen.com or by contacting Technical Service (page 36).

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 25-26) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incomplete extension during PCR	Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Excess (or overly dilute) PCR product used in the TOPO® Cloning reaction	Reduce (or concentrate) the amount of PCR product.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Used a proofreading polymerase or a *Taq*/proofreading polymerase mixture for PCR	• Use *Taq* polymerase or another DNA polymerase that leaves 3′ A-overhangs to produce your PCR product. • Add 3′ A-overhangs to your blunt PCR product by incubating with *Taq* polymerase (see page 29).
	Large PCR product	• Increase the amount of PCR product used in the TOPO® Cloning reaction. • Increase the incubation time of the TOPO® Cloning reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single band on an agarose gel)	• Optimize your PCR conditions. • Gel-purify your PCR product.
	Cloning large pool of PCR products or a toxic gene	Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes.

continued on next page

Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies, continued	PCR product does not contain sufficient 3′ A-overhangs even though you used *Taq* polymerase	• Increase the final extension time to ensure that all 3′ ends are adenylated. • *Taq* polymerase is most efficient at adding a non-template 3′ A next to a C, and less efficient at adding a nontemplate 3′ A next to another A. You may have to re-design your primers so that they contain a 5′ G instead of a 5′ T (Brownstein *et al.*, 1996).
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and smaller PCR products. • Optimize your PCR conditions. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® TOP10 competent *E. coli* stored incorrectly	Store One Shot® TOP10 competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C before plating.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

continued on next page

Troubleshooting, continued

Cell-Free Expression

If you are using an Expressway™ Cell-Free *E. coli* Expression System to express your recombinant protein and need assistance to troubleshoot your expression experiment, refer to the manual supplied with the product. If you are using another cell-free system, follow manufacturer's instructions and recommendations.

Prokaryotic Expression

If you are using a BL21-derived *E. coli* strain as a host to express your recombinant protein, refer to the table below for general solutions to troubleshoot your expression experiment. For solutions related to specific features of each BL21-derived *E. coli* strain, see the manual supplied with the strain.

Problem	Reason	Solution
No expression of recombinant protein	Gene of interest not in frame with the epitope tag	Sequence your construct to verify if the insert is cloned in frame with the epitope tag. If not in frame, redesign your PCR primers.
	Incorrect antibody used for detection	Use an antibody to your protein or one of the antibodies listed on page xi, as appropriate.
Low expression of recombinant protein	Gene of interest is toxic to *E. coli* **Note:** Evidence of toxicity includes loss of plasmid or slow growth relative to a control.	• Transform your expression construct into an *E. coli* strain in which T7 RNA polymerase expression is tightly regulated (*e.g.* BL21-AI™; see page 5). • Transform your expression construct into a BL21 strain containing pLysS or pLysE (*e.g.* BL21 Star™(DE3) pLysS; see page 5). • Transform the BL21 *E. coli* strain, then perform the expression at room temperature rather than 37°C.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using reagents included in the kit and using it directly in a TOPO® cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 100 µg/ml ampicillin.

Producing the Control PCR Product

Use the procedure below to produce the 750 bp control PCR product using *Taq* polymerase.

1. In a 0.5 ml microcentrifuge tube, set up the following 50 µl PCR:

Reagent	Amount
Control DNA Template (100 ng)	1 µl
10X PCR Buffer	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Water	41.5 µl
Taq polymerase (1 U/µl)	1 µl
Total volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
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	55°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 750 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pEXP5-NT/TOPO® or pEXP5-CT/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"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
TOPO® vector	1 µl	1 µl
Total volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10 competent cells using the procedure on page 15.
4. Spread 10-50 µl of each transformation mix onto LB plates containing 100 µg/ml ampicillin. When plating small volumes, add 20 µl of S.O.C. Medium to ensure even spreading. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

What You Should See

The "vector + PCR insert" reaction should produce hundreds of colonies. Greater than 85% of these will contain the 750 bp insert when analyzed by *Ava*I digestion. The table below lists the expected digestion patterns for inserts cloned in either direction:

Vector	Restriction Enzyme	Expected Digestion Pattern (bp)	
pEXP5-NT TOPO®	*Ava*I	Orientation 1:	2811 bp and 683 bp
		Orientation 2:	3386 bp and 108 bp
		Empty Vector:	2745 bp
pEXP5-CT TOPO®	*Ava*I	Orientation 1:	3326 bp and 107 bp
		Orientation 2:	2751 bp and 683 bp
		Empty Vector:	2685 bp

The "vector only" reaction should yield very few colonies (< 15% of the vector + PCR insert plate).

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 15. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. Medium on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥ 1 x 10^9 cfu/µg DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish 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

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 10).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

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 the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge for 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.
7. Centrifuge for 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
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 13.

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 (see page 13) Be sure to make the gel slice as small as possible for best results.

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Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result 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 13.
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 One Shot® competent cells using the protocol on page 15.

Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by proofreading polymerases into TOPO TA Cloning® vectors is often difficult because proofreading polymerases remove the 3´ A-overhangs necessary for TA 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, 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. A sufficient number of PCR products will retain the 3´ A-overhangs.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place on ice and use immediately in the TOPO® Cloning reaction.

Note: If you plan to store your sample overnight before proceeding with TOPO® Cloning, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR reaction.

Note

You may also gel-purify your PCR product after amplification with a proofreading polymerase. After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase. Incubate the reaction for 10-15 minutes at 72°C and use in the TOPO® Cloning reaction.

Expressing Recombinant Protein in *E. coli*

Introduction

Follow the guidelines provided in this section or your own protocol to express your recombinant fusion protein from your pEXP5-NT/TOPO® or pEXP5-CT/TOPO® construct in *E. coli*. You will need purified plasmid DNA of your expression construct and a suitable BL21-derived *E. coli* strain. Since each recombinant protein has different characteristics that may affect expression levels, we recommend performing a time course experiment to determine the conditions needed for optimal expression of your recombinant protein.

Expression Guidelines

Follow these general guidelines to perform expression your recombinant fusion protein. These guidelines assume that you are using a BL21-derived *E. coli* strain in which the expression of T7 RNA polymerase is inducible.

1. Transform your pEXP5-NT/TOPO® or pEXP5-CT/TOPO® expression construct into suitable, competent BL21-derived *E. coli* (see the manual supplied with the cells for instructions to perform transformation).
2. Select an appropriate transformant and initiate a culture in LB medium containing 100 µg/ml ampicillin. Grow at 37°C with shaking until the OD_{600} reaches 0.6-1.0.
3. Inoculate 10 ml of fresh LB medium containing 100 µg/ml ampicillin to an OD_{600} of 0.05-0.1 and grow at 37°C until the culture reaches mid-log phase (OD_{600} is ~ 0.5-0.8).
4. Split the culture into two 5 ml cultures. Add inducer to one of the cultures. You will now have two cultures: one induced, one uninduced.
5. Remove a 500 µl aliquot from **each** culture, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
6. Freeze the cell pellets at -20°C. These are the zero time point samples.
7. Continue to incubate the cultures at 37°C with shaking. Take time points for each culture every hour for 4 to 6 hours.
8. For each time point, remove 500 µl from the induced and uninduced cultures and process as described in Steps 5 and 6. Proceed to **Analyzing Samples**, below.

Preparing Samples

Once you have finished your pilot expression, analyze the samples you have collected using SDS-PAGE. Before starting, prepare a polyacrylamide gel or use one of the pre-cast polyacrylamide gels available from Invitrogen (see the next page).

1. Thaw the samples (Steps 6-8, above) and resuspend each cell pellet in 80 µl of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5-10 µl of each sample on a polyacrylamide gel and electrophorese. Save your samples by storing them at -20°C.

continued on next page

Expressing Recombinant Protein in *E. coli*, continued

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, see www.invitrogen.com or call Technical Service (page 36).

Analyzing Samples

To determine the success of your expression experiment, you may want to perform the following types of analyses. Other types of analyses are suitable.

- Stain the polyacrylamide gel with Coomassie Brilliant Blue R®-250 stain and look for a band of increasing intensity in the expected size range for the recombinant protein. Use the uninduced culture as a negative control.
- Perform a western blot to confirm that the overexpressed band is your desired protein (see page 20).

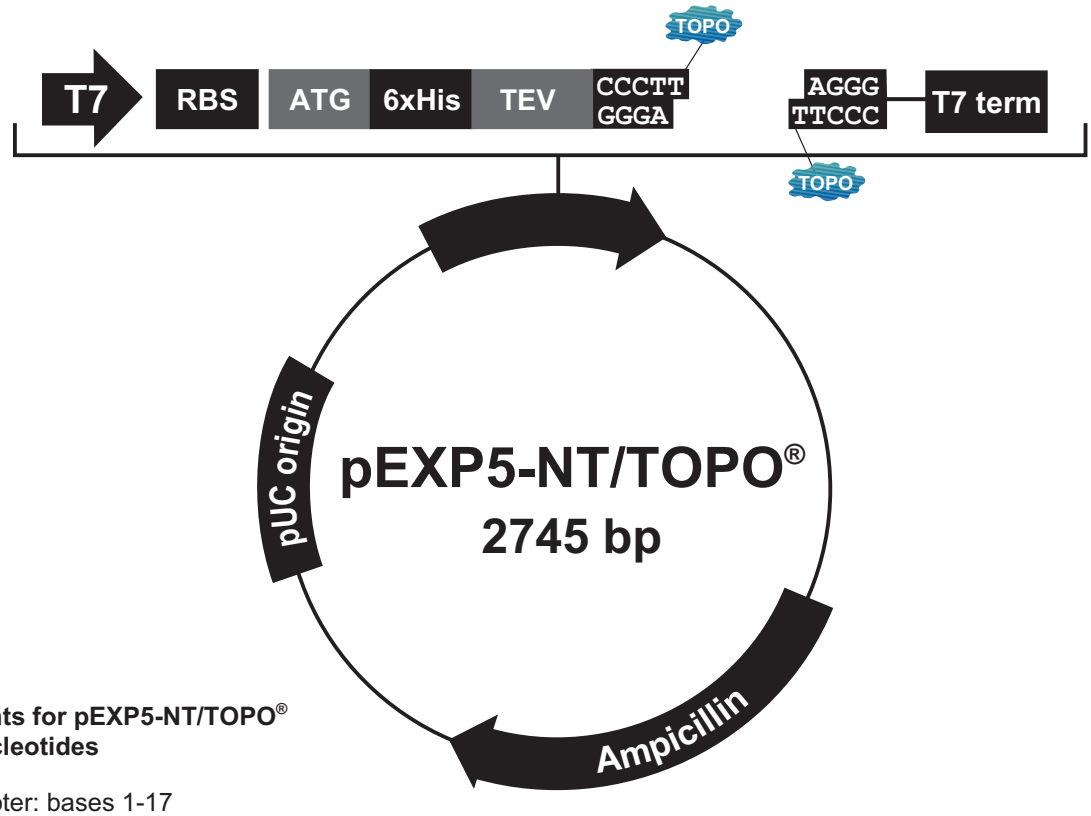
Once you have obtained a suitable amount of recombinant fusion protein, you may purify the recombinant protein (see page 20) and remove the N-terminal tag (pEXP5-NT/TOPO®-expressed proteins only; see page 21).

Coomassie Brilliant Blue R® is a registered trademark of Imperial Chemical Industries PLC

Map and Features of pEXP5-NT/TOPO®

pEXP5-NT/TOPO® Map

The figure below shows the features of the pEXP5-NT/TOPO® vector. **The complete sequence of pEXP5-NT/TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 36).**



Comments for pEXP5-NT/TOPO®
2745 nucleotides

T7 promoter: bases 1-17
T7 forward priming site: bases 1-20
Ribosome binding site (RBS): bases 68-73
Initiation ATG: bases 80-82
Polyhistidine (6xHis) region: bases 92-109
HisG epitope: bases 92-112
TEV recognition site: bases 122-142
TOPO® recognition site 1: bases 141-145
TOPO® recognition site 2: bases 146-150
T7 reverse priming site: bases 198-217
T7 transcription terminator: bases 159-287
bla promoter: bases 399-497
Ampicillin resistance gene: bases 498-1358
pUC origin: 1503-2176

continued on next page

Map and Features of pEXP5-NT/TOPO®, continued

Features of pEXP5-NT/TOPO®

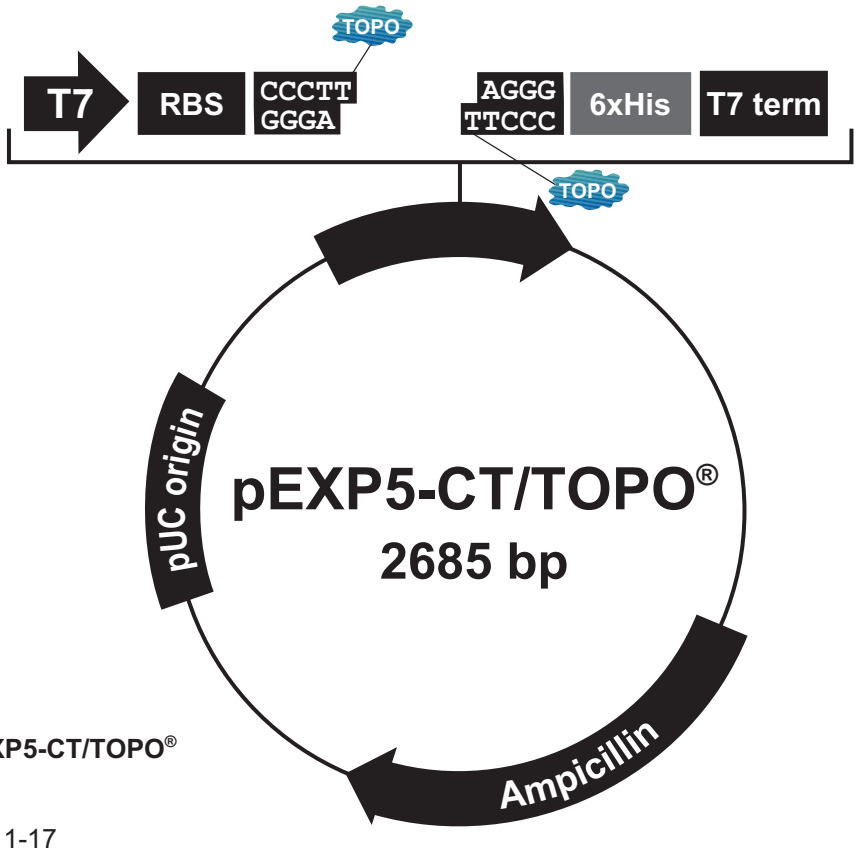
pEXP5-NT/TOPO® (2745 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Allows high-level expression of your recombinant protein in the Expressway™ Cell-Free *E. coli* Expression System or in *E. coli* strains expressing the T7 RNA polymerase.
T7 forward priming site	Allows sequencing in the sense orientation.
Ribosome binding site (RBS)	Optimally spaced from the initiation ATG for efficient translation of the PCR product.
Initiation ATG	Allows translation initiation of the recombinant fusion protein.
N-terminal polyhistidine (6xHis) tag	Allows detection of the recombinant fusion protein with an Anti-HisG Antibody and purification with metal-chelating resin (*i.e.* ProBond™ or Ni-NTA).
TEV recognition site (Glu-X-X-Tyr-X-Gln-Ser)	Allows removal of the N-terminal tag from your recombinant fusion protein using TEV protease (Carrington and Dougherty, 1988; Dougherty *et al.*, 1988; Dougherty *et al.*, 1989).
TOPO® Cloning site	Allows rapid cloning of your *Taq*-amplified PCR product.
T7 transcription terminator	Sequence from bacteriophage T7 that allows efficient transcription termination.
T7 reverse priming site	Allows sequencing of the insert.
bla promoter	Allows expression of the ampicillin resistance gene in *E. coli*.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Map and Features of pEXP5-CT/TOPO®

pEXP5-CT/TOPO® Map

The figure below shows the features of the pEXP5-CT/TOPO® vector. **The complete sequence of pEXP5-CT/TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 36).**



Comments for pEXP5-CT/TOPO®
2685 nucleotides

T7 promoter: bases 1-17
T7 forward priming site: bases 1-20
Ribosome binding site (RBS): bases 69-74
TOPO® recognition site 1: bases 77-81
TOPO® recognition site 2: bases 82-86
Polyhistidine (6xHis) region: bases 88-105
T7 term reverse priming site: bases 205-227
T7 transcription terminator: bases 143-227
bla promoter: bases 339-437
Ampicillin resistance gene: bases 438-1298
pUC origin: 1443-2116

continued on next page

Map and Features of pEXP5-CT/TOPO®, continued

Features of pEXP5-CT/TOPO®

pEXP5-CT/TOPO® (2685 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Allows high-level expression of your recombinant protein in the Expressway™ Cell-Free *E. coli* Expression System or in *E. coli* strains expressing the T7 RNA polymerase.
T7 forward priming site	Allows sequencing in the sense orientation.
Ribosome binding site (RBS)	Optimally spaced from the initiation ATG for efficient translation of the PCR product.
TOPO® Cloning site	Allows rapid cloning of your *Taq*-amplified PCR product.
C-terminal polyhistidine (6xHis) tag	Allows detection of the recombinant fusion protein with an Anti-His(C-term) Antibody and purification with metal-chelating resin (*i.e.* ProBond™ or Ni-NTA).
T7 transcription terminator	Sequence from bacteriophage T7 that allows efficient transcription termination.
T7 reverse priming site	Allows sequencing of the insert.
bla promoter	Allows expression of the ampicillin resistance gene in *E. coli*.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

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

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Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Support** and then **Technical Resources** and follow instructions on the page to download the MSDS for your product.

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Introduction

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pEXP5-NT/TOPO® and the pEXP5-CT/TOPO® TA Expression Kits.

pEXP5-NT and pEXP5-CT Vectors

Prior to adaptation with topoisomerase I, the parental supercoiled pEXP5-NT and pEXP5-CT vectors are qualified by restriction enzyme digestion to verify identity and structure.

TOPO® Cloning Efficiency

After adaptation with topoisomerase I, each lot of pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector is functionally qualified using the control reagents included in the kit. Under conditions described on pages 25-26, a 750 bp control PCR product is amplified, TOPO® Cloned into the appropriate pEXP5-TOPO® vector, and transformed into the One Shot® TOP10 chemically competent *E. coli* included with the kit.

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

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® TOP10 Chemically Competent *E. coli*

One Shot® TOP10 chemically competent 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 greater than 1×10^9 cfu/µg plasmid DNA.

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

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