invitrogen™
by life technologies™

Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology

Five-minute, directional TOPO® Cloning of blunt-end PCR products for inducible expression and specific detection of Lumio™ fusion proteins in *E. coli*

Catalog nos. K160-01 and K161-01

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User Manual

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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.

Step	Action
Design PCR Primers	• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer. • Design the primers such that your gene of interest will be optimally expressed and fused in frame with the Lumio™ tag, if desired.
Amplify Your Gene of Interest	1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product. 2. Use agarose gel electrophoresis to check the integrity and yield of your PCR product.
Perform the TOPO® Cloning Reaction	1. Set up the following TOPO® Cloning reaction. Be sure that you use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in the reaction. **Note:** If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in the TOPO® Cloning reaction. Fresh PCR product — 0.5 to 4 µl Salt Solution — 1 µl Sterile water — add to a final volume of 5 µl TOPO® vector — 1 µl Total volume — 6 µl 2. Mix gently and incubate for 5 minutes at room temperature. 3. Place on ice and proceed to transform One Shot® TOP10 chemically competent *E. coli*, below.
Transform TOP10 Chemically Competent *E. coli*	1. Add 3 µl of the TOPO® Cloning reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µl of room temperature SOC medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 100-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers supplied with the kit to perform the control reaction. See the protocol on pages 42-43 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Quantity	Catalog no.
Champion™ pET160 Directional TOPO® Expression Kit with Lumio™ Technology	20 reactions	K160-01
Champion™ pET161 Directional TOPO® Expression Kit with Lumio™ Technology	20 reactions	K161-01

Shipping/Storage

The Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology are shipped on dry ice. Each kit contains five boxes (see below). Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pET TOPO® Reagents	-20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	-80°C
3	BL21 Star™(DE3) One Shot® Chemically Competent *E. coli*	-80°C
4	Lumio™ Green Detection Kit	-20°C, protected from light
5	BenchMark™ Fluorescent Protein Standard	-20°C, protected from light

continued on next page

Kit Contents and Storage, continued

pET TOPO® Reagents

pET TOPO® Reagents (Box 1) are listed below. Each box includes PCR reagents and the appropriate vectors and primers. **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pET vector, TOPO®-adapted (pET160/GW/D-TOPO® or pET161/GW/D-TOPO®)	15-20 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	--	1 ml
T7 Promoter Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
T7 Reverse Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl
Expression Control Plasmid (pET160-GW/CAT or pET161-GW/CAT)	lyophilized in TE Buffer, pH 8	100 ng

Sequences of the Primers

The table below provides the sequences and amounts supplied of the T7 Promoter and T7 Reverse sequencing primers. Two micrograms of each primer are supplied.

Primer	Sequence	pMoles Supplied
T7 Promoter Primer	5´-TAATACGACTCACTATAGGG-3´	327
T7 Reverse Primer	5´-TAGTTATTGCTCAGCGGTGG-3´	325

continued on next page

Kit Contents and Storage, continued

One Shot® TOP10 Reagents

The table below lists the items included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency should be greater than 1 x 10^9 cfu/µg DNA.

Store Box 2 at -80°C.

Item	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	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

BL21 Star™(DE3) One Shot® Reagents

The table below describes the items included in the BL21 Star™(DE3) One Shot® Chemically Competent *E. coli* kit (Box 3). Transformation efficiency is at least 1 x 10^8 cfu/µg DNA.

Store Box 3 at -80°C.

Item	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
BL21 Star™(DE3)	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

continued on next page

Kit Contents and Storage, continued

Genotype of TOP10

Use this *E. coli* strain for general cloning of blunt-end PCR products into the pET TOPO® vectors.

Genotype: 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

Genotype of BL21 Star™ (DE3)

Use this *E. coli* strain for expression only. Do not use these cells to propagate or maintain your construct.

Genotype: F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm rne131* (DE3)

The DE3 designation means this strain contains the lambda DE3 lysogen which carries the gene for T7 RNA polymerase under the control of the *lac*UV5 promoter. IPTG is required to induce expression of the T7 RNA polymerase.

The strain is an *E. coli* B/r strain and does not contain the *lon* protease. It also has a mutation in the outer membrane protease, OmpT. The lack of these two key proteases reduces degradation of heterologous proteins expressed in the strain.

The strain carries a mutated *rne* gene (*rne131*) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA, resulting in an increase in mRNA stability (see page 6).

Lumio™ Green Detection Kit and BenchMark™ Fluorescent Protein Standard

The Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology are supplied with the Lumio™ Green Detection Kit (Box 4) and the BenchMark™ Fluorescent Protein Standard (Box 5). Refer to their corresponding manuals for detailed information pertaining to each item and a description of the reagents provided.

Accessory Products

Additional Products

Many of the reagents supplied in the Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
Lumio™ Green Detection Kit	100 reactions	LC6090
BenchMark™ Fluorescent Protein Standard	250 µl	LC5928
One Shot® TOP10 Chemically Competent *E coli*	10 x 50 µl	C4040-10
	20 x 50 µl	C4040-03
BL21 Star™(DE3) One Shot® Chemically Competent *E. coli*	20 x 50 µl	C6010-03
BL21 Star™(DE3)pLysS One Shot® Chemically Competent *E. coli*	20 x 50 µl	C6020-03
PureLink™ HQ Plasmid Purification Kit	100 reactions	K2100-01
PureLink™ Quick Gel Extraction Kit	50 preps	K2100-12
Ampicillin	200 mg	11593-019
Isopropylthio-β-galactoside (IPTG)	1 g	15529-019
AcTEV™ Protease	1000 units	12575-015
CAT Antiserum	50 µl	R902-25
Gateway® BP Clonase™ Enzyme Mix	20 reactions	11789-013
	100 reactions	11789-021
pDONR™221	6 µg	12213-013
pDONR™/Zeo	6 µg	12536-017

Purification of Recombinant Protein

If your gene of interest in is frame with a C-terminal or N-terminal peptide containing a polyhistidine (6xHis) tag, you may use Invitrogen's ProBond™ or Ni-NTA Purification System to purify your recombinant fusion protein. See the table below for ordering information.

Product	Amount	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Chelating Resin	50 ml	R801-01
	150 ml	R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The Champion™ pET Directional TOPO® Expression Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for high-level, T7-regulated expression in *E. coli*. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

Using the Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology facilitates sensitive and specific in-gel detection of Lumio™ fusion proteins in polyacrylamide gels without the need for staining or western blotting. In addition, the BenchMark™ Fluorescent Protein Standard consists of molecular weight range proteins conjugated to a fluorescent dye to allow you to easily determine the molecular weight of your Lumio™ fusion protein.

Features of the pET160 and pET161 Directional TOPO® Vectors

The pET TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for regulated expression in *E. coli*. Features of the vectors include:

- T7*lac* promoter for high-level, IPTG-inducible expression of the gene of interest in *E. coli* (Dubendorff and Studier, 1991; Studier *et al.*, 1990)
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- N-terminal or C-terminal Lumio™ tag for specific detection of recombinant proteins using the Lumio™ Green Detection Kit
- N-terminal or C-terminal 6xHis tag for purification of recombinant fusion proteins
- TEV protease recognition site for cleavage of the fusion tag from the recombinant protein of interest (pET160/GW/D-TOPO® only)
- *lacI* gene encoding the lac repressor to reduce basal transcription from the T7*lac* promoter in the pET TOPO® vector and from the *lacUV5* promoter in the *E. coli* host chromosome (see page 4 for more information)
- Ampicillin resistance marker for selection in *E. coli*
- pBR322 origin for low-copy replication and maintenance in *E. coli*

The Champion™ pET Expression System

The Champion™ pET Expression System is based on expression vectors originally developed by Studier and colleagues, and takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase to allow regulated expression of heterologous genes in *E. coli* from the T7 promoter (Rosenberg *et al.*, 1987; Studier and Moffatt, 1986; Studier *et al.*, 1990). For more information about the Champion™ pET Expression System, see page 4.

continued on next page

Overview, continued

The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest, simply TOPO® Clone your blunt-end PCR product into pET160/GW/D-TOPO® or pET161/GW/D-TOPO® and transform BL21 Star™ *E. coli*.

To express your gene of interest in any other expression system:

1. Generate an entry clone by performing a BP recombination reaction between your expression clone and a Gateway® donor vector.
2. Perform an LR recombination reaction between the entry clone and a variety of Gateway® destination vectors to generate an expression construct to express your protein of interest in virtually any expression system.

For more information about the Gateway® Technology, refer to the Gateway® Technology manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support (page 54).

Lumio™ Technology

The Lumio™ System is based on the FlAsH (Fluorescein Arsenical Hairpin) technology which uses a biarsenical reagent to bind and detect proteins containing a tetracysteine motif (*i.e.* Lumio™ tag) (Griffin *et al.*, 1998). The biarsenical reagent becomes strongly fluorescent only upon binding to the tetracysteine motif, allowing specific detection of Lumio™ fusion proteins directly in gels. For more information about the Lumio™ Technology, see page 7.

How Directional TOPO® Cloning Works

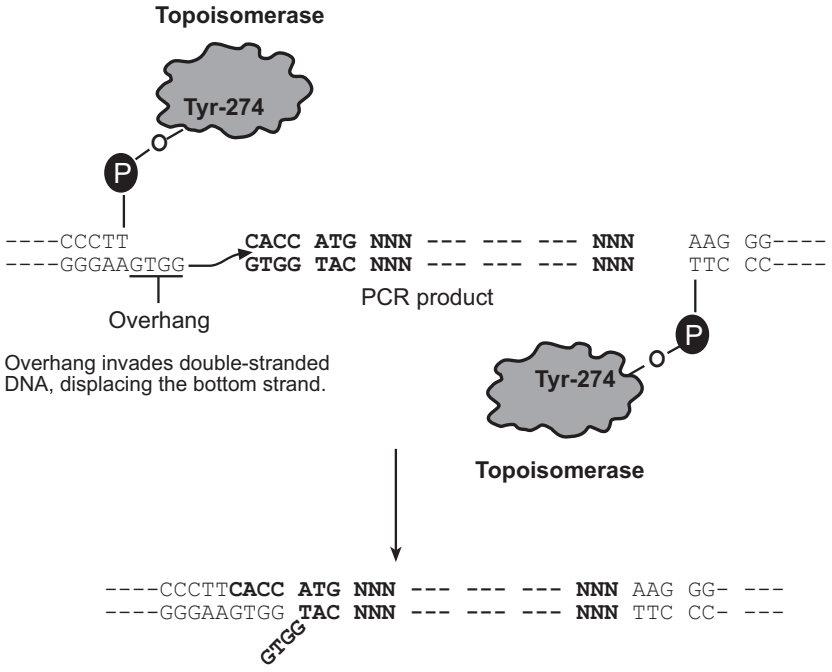
How Topoisomerase I Works

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

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



T7-Regulated Expression

The Basis of T7-Regulated Expression

The Champion™ pET Expression System uses elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*. In pET160/GW/D-TOPO® and pET161/GW/D-TOPO®, expression of the gene of interest is controlled by a strong bacteriophage T7 promoter that has been modified to contain a *lac* operator sequence (see below). 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, it is necessary to deliver T7 RNA polymerase to the cells by inducing expression of the polymerase or infecting the cell with phage expressing the polymerase. In the Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology, T7 RNA polymerase is supplied by the BL21 Star™(DE3) host *E. coli* strain in a regulated manner (see below). When sufficient T7 RNA polymerase is produced, it binds to the T7 promoter and transcribes the gene of interest.

Regulating Expression of T7 RNA Polymerase

The BL21 Star™(DE3) *E. coli* strain is specifically included in each Champion™ pET Directional TOPO® Expression Kit with Lumio™ Technology for expression of T7-regulated genes. This strain carries the DE3 bacteriophage lambda lysogen. This λDE3 lysogen contains a *lac* construct consisting of the following elements:

- the *lacI* gene encoding the lac repressor
- the T7 RNA polymerase gene under control of the *lacUV5* promoter
- a small portion of the *lacZ* gene

This *lac* construct is inserted into the *int* gene such that it inactivates the *int* gene. Disruption of the *int* gene prevents excision of the phage (*i.e.* lysis) in the absence of helper phage. The *lac* repressor (encoded by *lacI*) represses expression of T7 RNA polymerase. Addition of the gratuitous inducer, isopropyl β–D-thiogalactoside (IPTG), allows expression of T7 RNA polymerase from the *lacUV5* promoter.

The BL21 Star™(DE3) strain also contains other features which facilitate high-level expression of heterologous genes. For more information, see page 6.

T7*lac* Promoter

Studies have shown that there is always some basal expression of T7 RNA polymerase from the *lacUV5* promoter in λDE3 lysogens even in the absence of inducer (Studier and Moffatt, 1986). In general, this is not a problem, but if the gene of interest is toxic to the *E. coli* host, basal expression of the gene of interest may lead to plasmid instability and/or cell death.

To address this problem, the pET160/GW/D-TOPO® and pET161/GW/D-TOPO® vectors have been designed to contain a T7*lac* promoter to drive expression of the gene of interest. The T7*lac* promoter consists of a *lac* operator sequence placed downstream of the T7 promoter. The *lac* operator serves as a binding site for the lac repressor (encoded by the *lacI* gene) and functions to further repress T7 RNA polymerase-induced basal transcription of the gene of interest in BL21 Star™(DE3) cells.

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T7-Regulated Expression, continued

Expressing Toxic Genes

In some cases, the gene of interest is so toxic to BL21 Star™(DE3) cells that other *E. coli* host strains may be required for expression. For a discussion of other alternative strains that may be used, see page 6.

Using TOP10 Cells

One Shot® TOP10 competent *E. coli*, which do not contain T7 RNA polymerase, are included in each Champion™ pET Directional TOPO® Expression Kits with Lumio™ Technology to provide a host for stable propagation and maintenance of recombinant plasmids. As mentioned on the previous page, the presence of T7 RNA polymerase, even at basal levels, can lead to expression of the desired gene even in the absence of inducer. If the gene of interest is toxic to the *E. coli* host, plasmid instability and/or cell death may result. **We recommend that you transform your TOPO® Cloning reaction into TOP10 cells for characterization of the construct, propagation, and maintenance.** When you are ready to perform an expression experiment, transform your construct into BL21 Star™(DE3) *E. coli*.

BL21 Star™ *E. coli* Strains

BL21 Star™ Strains

The BL21 Star™(DE3) *E. coli* strain is included in each Champion™ pET Directional TOPO® Expression Kit with Lumio™ Technology for use as a host for expression. Other BL21 Star™ strains are also available from Invitrogen (see below). In addition to the λDE3 lysogen which allows high-level expression of T7-regulated genes (see page 3), the BL21 Star™ strains also contain the *rne131* mutation. This particular mutation further enhances the expression capabilities of BL21 Star™.

rne131 Mutation

The *rne* gene encodes the RNase E enzyme, an essential, 1061 amino acid *E. coli* endonuclease which is involved in rRNA maturation and mRNA degradation as a component of a protein complex known as a "degradosome" (Grunberg-Manago, 1999; Lopez *et al.*, 1999). Various studies have shown that the N-terminal portion of RNase E (approximately 584 amino acids) is required for rRNA processing and cell growth while the C-terminal portion of the enzyme (approximately 477 amino acids) is required for mRNA degradation (Kido *et al.*, 1996; Lopez *et al.*, 1999). The *rne131* mutation (present in the BL21 Star™ strains) encodes a truncated RNase E which lacks the C-terminal 477 amino acids of the enzyme required for mRNA degradation (Kido *et al.*, 1996; Lopez *et al.*, 1999). Thus, mRNAs expressed in the RNase E-defective BL21 Star™ strains exhibit increased stability when compared to other BL21 strains. When heterologous genes are expressed in the BL21 Star™ strains from T7-based expression vectors, the yields of recombinant proteins generally increase.

BL21 Star™(DE3)pLysS Strain

If you discover that your gene is toxic to BL21 Star™(DE3) cells, you may want to perform your expression experiments in the BL21 Star™(DE3)pLysS strain (see page x for ordering information). The BL21 Star™(DE3)pLysS strain contains the pLysS plasmid, which produces T7 lysozyme. T7 lysozyme binds to T7 RNA polymerase and inhibits transcription. This activity results in reduced basal levels of T7 RNA polymerase, leading to reduced basal expression of T7-driven heterologous genes. For more information about BL21 Star™(DE3)pLysS, refer to our Web site (www.invitrogen.com) or contact Technical Support (page 54).

Note

Note that while BL21 Star™(DE3)pLysS reduces basal expression from the gene of interest when compared to BL21 Star™(DE3), it also generally reduces the overall induced level of expression of recombinant protein.

The Lumio™ Technology

Advantages of the Lumio™ Technology

Using the Champion™ pET Expression Kits with Lumio™ Technology provides the following advantages:

- Lumio™ fusion protein sensitivity at nanogram levels
- Rapid detection of Lumio™ fusion proteins directly in the gel without the need for staining or western blotting
- Capable of detecting N-terminal and C-terminal Lumio™ fusion proteins
- Detection compatible with downstream applications such as Coomassie® staining, silver staining, fluorescent staining, western blotting, or mass spectrometry analysis

For more information about the Lumio™ Technology and the Lumio™ Green Detection Kit, refer to the Lumio™ Green Detection Kit manual.

Components of the Lumio™ System

The Lumio™ System consists of two major components:

- The tetracysteine Lumio™ tag (Cys-Cys-Pro-Gly-Cys-Cys). When fused to a gene of interest, the Lumio™ tag allows the expressed fusion protein from the pET Directional TOPO® vector construct to be specifically recognized by a biarsenical labeling reagent. For more information on the tetracysteine motif, see below.
- The biarsenical Lumio™ Green Detection Reagent which becomes fluorescent upon binding to recombinant proteins containing the Lumio™ tag. The Lumio™ Green Reagent is supplied pre-complexed to EDT (1,2-ethanedithiol) which stabilizes and solubilizes the biarsenic reagent.

Tetracysteine Motif

The Lumio™ Green Detection Reagent binds a tetracysteine motif consisting of Cys-Cys-Xaa-Xaa-Cys-Cys where Cys equals cysteine and Xaa equals any amino acid other than cysteine. This motif is rarely seen in naturally occurring proteins allowing specific fluorescence labeling and detection of recombinant proteins fused to the Lumio™ tag. In the Lumio™ System, the optimized Cys-Cys-Pro-Gly-Cys-Cys tetracysteine motif is used as this motif has been shown to have a higher affinity for and more rapid binding to biarsenic compounds as well as enhanced stability compared to other characterized motifs (Adams *et al.*, 2002).

Working with Arsenic Compounds

Introduction

The Lumio™ Green Detection Reagent supplied with the Lumio™ Green Detection Kit is a biarsenical compound and should be handled with care. Information on handling and disposing the Lumio™ Green Detection Reagent is described below.

Exercise caution when handling the Lumio™ Green Reagent. Wear protective clothing, eyewear, and gloves suitable for use with dimethyl sulfoxide (*e.g.* nitrile gloves) when handling the Lumio™ Green Detection Reagent. Review the Material Safety Data Sheet (MSDS) before handling

Dermal Toxicity Evaluation

A dermal toxicity evaluation of the Lumio™ Green Detection Reagent was independently performed by MB Research Laboratories, Spinnerstown, PA, USA by applying a full vial of material to the mouse skin. In this study, no adverse reaction or toxicity was noted. Although arsenic compounds are toxic, this product contains <0.2% of an organic arsenic compound that shows no toxicity at a maximum dose level likely to be handled. The toxicology of this material, however, has not been fully investigated. Handle according to your chemical hygiene plan and prevent contact with this material.

Accidental Spills and Accidental Contact

Treat accidental spills of the Lumio™ Green Detection Reagent on surfaces with 10% bleach for 10 minutes and then carefully clean up. Discard arsenic-containing waste according to your institution's guidelines.

Treat accidental contact of the Lumio™ Green Detection Reagent with human skin by washing excess reagent with soap and water as soon as possible. Consult a physician following contact with Lumio™ Green Reagent. Do not treat arsenic skin exposure with EDT (1,2-ethanedithiol) as this may promote uptake of the Lumio™ Green Reagent into the body.

Disposing the Lumio™ Green Reagent

All excess reagents that contain or have come in contact with arsenic compounds should be discarded according to your institution's guidelines and all applicable local, state, and federal requirements.

In general, we recommend disposing of protein samples labeled with the Lumio™ Green Detection Reagent and polyacrylamide gels containing protein samples labeled with the Lumio™ Green Detection Reagent as hazardous waste. For specific disposal requirements in your area, consult your safety officer.

Experimental Outline

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.

Step	Action	Page
1	Design PCR primers to clone your gene of interest in frame with the Lumio™ tag. Consult the diagram on page 13 or page 15 to help you design your PCR primers.	10-15
2	Produce your blunt-end PCR product.	16
3	TOPO® Clone your PCR product into pET160/GW/D-TOPO® or pET161/GW/D-TOPO® and transform into One Shot® TOP10 *E. coli*. Select for transformants on LB agar plates containing 100 µg/ml ampicillin.	17-21
4	Analyze transformants for the presence and orientation of the insert by restriction digestion, PCR, or sequencing.	22-23
5	Select positive transformant and isolate DNA. Transform BL21 Star™(DE3) *E. coli* and induce expression with IPTG.	24-26
6	Perform in-gel detection of Lumio™ fusion proteins using the Lumio™ Green Detection Kit.	27-32

Methods

General Requirements for Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Depending on the pET TOPO® vector you are using, consider the following when designing your PCR primers:

- Sequences required to facilitate directional cloning (see below)
- Sequences required to fuse you PCR product in frame with the Lumio™ tag

General Requirements for the Forward Primer

To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pET TOPO® vector.

For example, below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer:

DNA sequence: `5′-ATG GGA TCT GAT AAA`
Proposed Forward PCR primer: `5′-C ACC ATG GGA TCT GAT AAA`

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

General Requirements for the Reverse Primer

In general, design the reverse PCR primer to allow you to clone your PCR product in frame with any C-terminal fusions, if desired. **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 75%, and may increase the chances of your ORF cloning in the opposite orientation.** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch, but this has not been tested thoroughly.

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General Requirements for Designing PCR Primers, continued

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag. The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3′

One possibility is to design the reverse PCR primer to start with the codon just up-stream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TGA-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another possibility is to design the reverse primer so that it hybridizes just down-stream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine (see below).

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

…GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TGA-3′

- To fuse the ORF in frame with a C-terminal tag, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.
- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-TCA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that pET160/GW/D-TOPO® and pET161/GW/D-TOPO® accept blunt-end PCR products.
- Do not add 5′ phosphates to your primers for PCR. This will prevent ligation into the pET/GW/D-TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

Cloning into pET160/GW/D-TOPO®

Introduction

pET160/GW/D-TOPO® allows expression of recombinant proteins with an N-terminal peptide containing the 6xHis and Lumio™ tags. The N-terminal peptide also includes a TEV protease cleavage site to enable removal of the tag after protein purification using TEV protease.

Additional Cloning Considerations

In addition to the guidelines on pages 10-11, consider the following when designing PCR primers to clone your DNA into pET160/GW/D-TOPO®.

Be sure to include a stop codon in the reverse primer or design the reverse primer to hybridize downstream of the native stop codon.

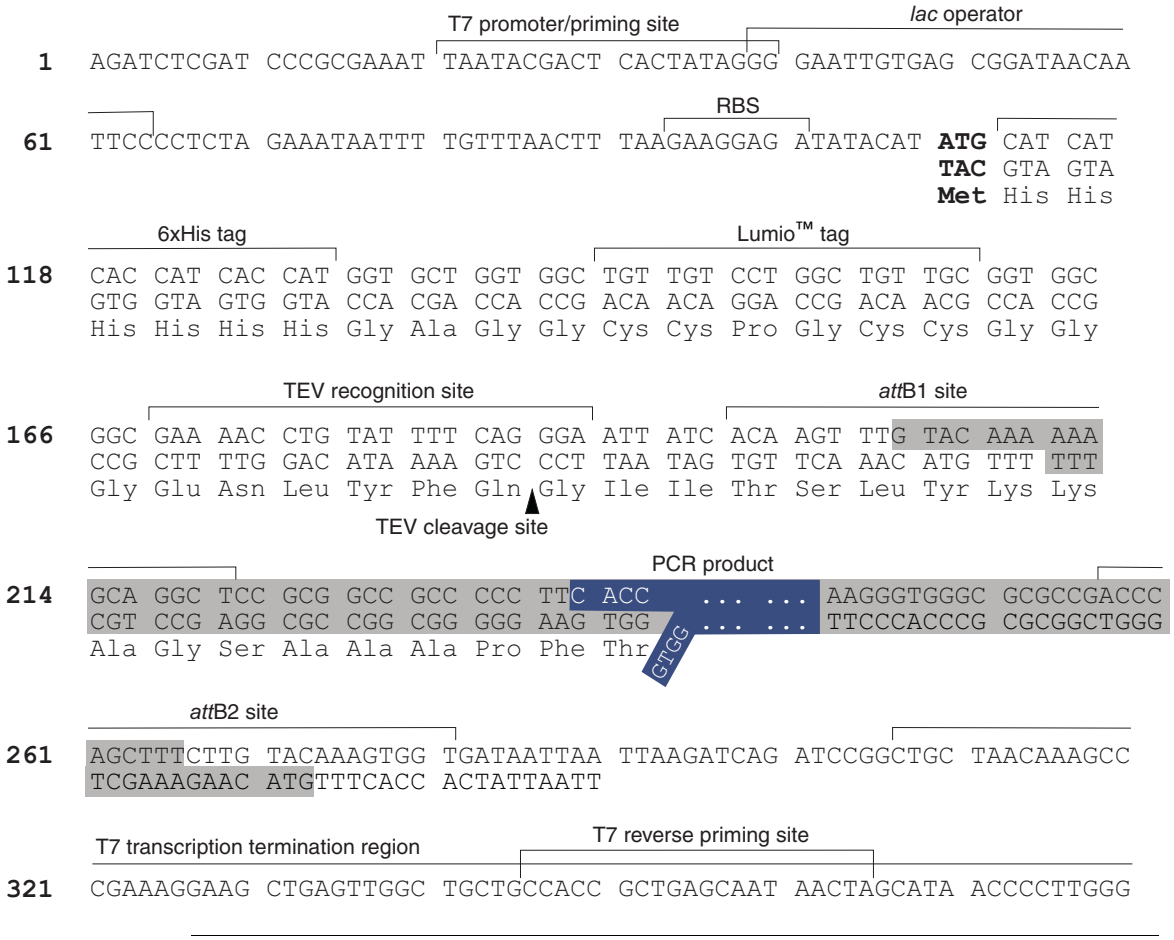
If you wish to...	Then...
include the 6xHis and Lumio™ tags	design the forward PCR primer to place the gene of interest in frame with the N-terminal tag. Note that: • a ribosome binding site (RBS) is included upstream of the initiation ATG in the N-terminal tag to ensure optimal spacing for proper translation • at least eighteen nonnative amino acids will be present between the TEV cleavage site and the start of your gene
express your protein with a native N-terminus, *i.e.* without the N-terminal peptide	design the forward PCR primer to include the following: • a stop codon to terminate the N-terminal peptide • a second ribosome binding site 9-10 base pairs 5′ of the ATG initiation codon of your protein

continued on next page

Cloning into pET160/GW/D-TOPO®, continued

TOPO® Cloning Site of pET160/GW/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pET160/GW/D-TOPO®. The shaded region corresponds to sequences that will be transferred from the pET160/GW/D-TOPO® vector into the entry clone following the BP recombination reaction. **The sequence of the vector is available for downloading from our Web site or by contacting Technical Support (page 54).**



Cloning into pET161/GW/D-TOPO®

Introduction

pET161/GW/D-TOPO® allows expression of recombinant proteins with a C-terminal peptide containing the Lumio™ and 6xHis tags and contains a ribosome binding site and ATG initiation codon. If you express your protein using the vector-encoded ribosome binding site and ATG initiation codon, at least 30 additional amino acids will be present at the N-terminus of your protein (see diagram on the next page).

Note: If you wish to express the native N-terminus without the additional amino acids, include a stop codon and a second ribosome binding site upstream of the ATG initiation codon in your sequence of interest (see below).

Additional Cloning Considerations

In addition to the guidelines on pages 10-11, consider the following when designing PCR primers to clone your DNA into pET161/GW/D-TOPO®.

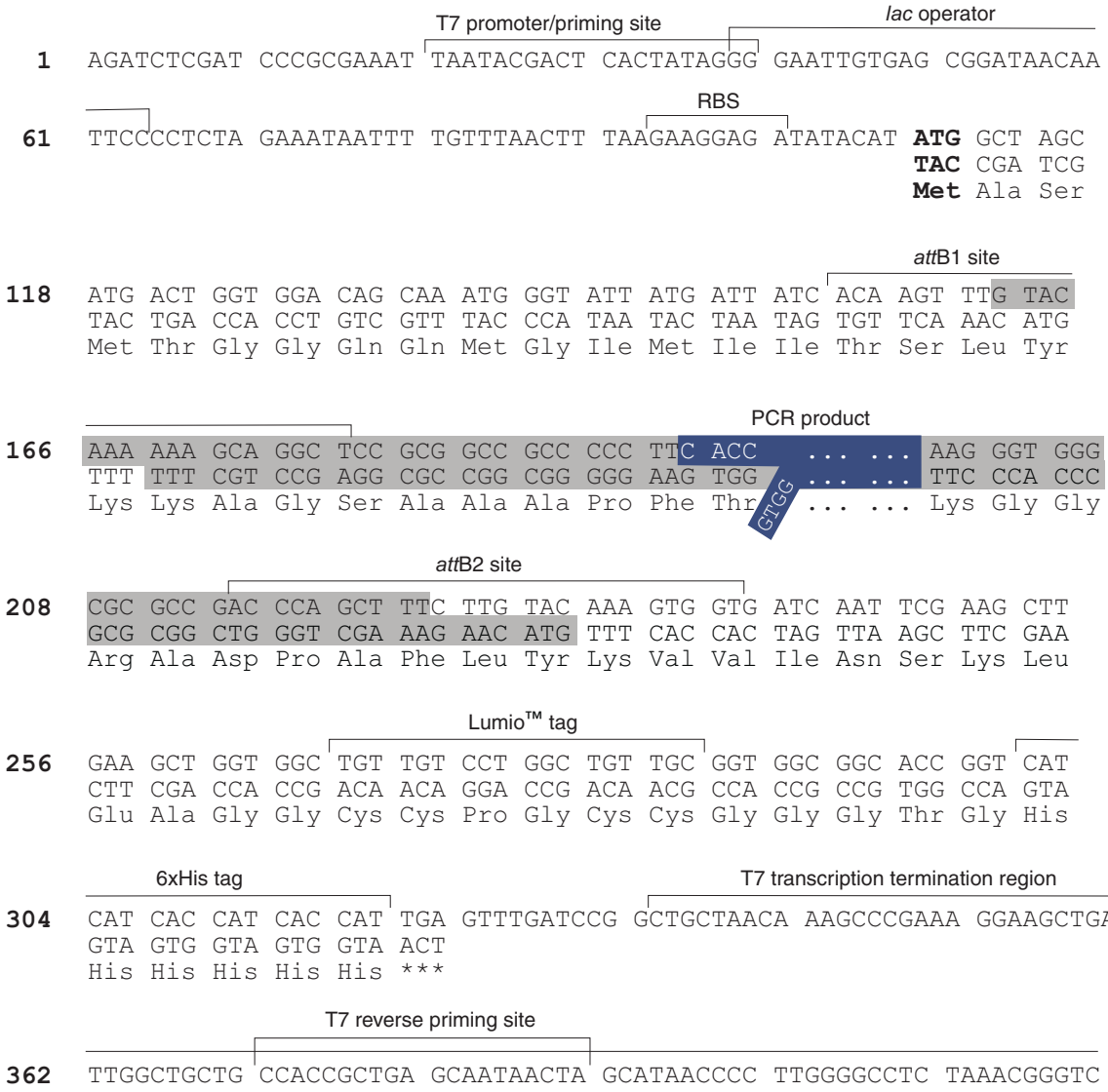
If you wish to...	Then...
express your protein with a native N-terminus, *i.e.* without the additional N-terminal amino acids	design the forward PCR primer to include the following: • a stop codon to terminate the N-terminal peptide • a second ribosome binding site 9-10 base pairs 5′ of the initiation ATG codon of your protein
include the C-terminal Lumio™ and 6xHis tags	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 Lumio™ and 6xHis tags	design the reverse primer to include the native stop codon or make sure the stop codon is upstream from the reverse PCR primer binding site

continued on next page

Cloning into pET161/GW/D-TOPO®, continued

TOPO® Cloning Site of pET161/GW/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pET161/GW/D-TOPO®. The shaded region corresponds to sequences that will be transferred from the pET161/GW/D-TOPO® vector into the entry clone following the BP recombination reaction. **The sequence of the vector is available for downloading from our Web site or from Technical Support (see page 54).**



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Needed

You should have the following materials on hand before beginning.

Note: dNTPs (adjusted to pH 8) are provided in the kit.

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

Producing PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below:

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at -20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 44-45).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Performing the TOPO® Cloning Reaction

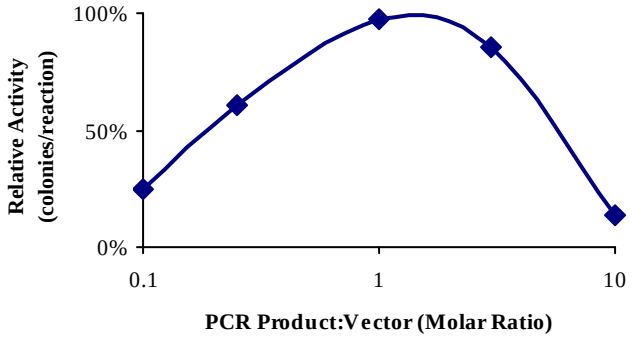
Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into pET160/GW/D-TOPO® or pET161/GW/D-TOPO® and transform the recombinant vector into One Shot® TOP10 *E. coli*. To ensure that you obtain the best possible results, we recommend that you read this section and the section entitled **Transforming One Shot® TOP10 Competent Cells** (pages 19-21) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 42-43 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For the pET160/GW/D-TOPO® and pET161/GW/D-TOPO® vectors, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Performing the TOPO® Cloning Reaction, continued

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 below.
- 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 below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

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

Reagents*	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 (1:4)	--	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
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 Cells**, next page.

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

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pET TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are supplied with the kit to facilitate transformation, however, you may also transform electrocompetent cells. Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

To maintain the stability of your construct, we recommend that you transform your TOPO® Cloning reaction into TOP10 cells and characterize transformants in TOP10 before proceeding to expression studies using BL21 Star™(DE3). Expression of T7 RNA polymerase in BL21 Star™(DE3) may be leaky and may lead to rearrangement or loss of your plasmid.

Materials Needed

You should have the following materials on hand before beginning:

- TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2 (previous page)
- S.O.C. medium (supplied with the kit)
- 42°C water bath (or electroporator with cuvettes, optional)
- 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 in the correct orientation, reducing the number of colonies to be analyzed. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

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

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm LB plates containing 100 µg/ml ampicillin at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® TOP10 cells from Box 2 for each transformation.

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Transforming One Shot® TOP10 Competent Cells, continued

Important

The number of colonies obtained after transforming pET160/GW/D-TOPO® or pET161/GW/D-TOPO® into One Shot® TOP10 cells is generally lower when compared to the number of colonies obtained after transforming other prokaryotic TOPO® vectors (*e.g.* pCR®T7 TOPO®, pBAD/Thio-TOPO®). This is due to the following:

- Directional TOPO® Cloning generally yields 2 to 5-fold fewer colonies than traditional bidirectional TOPO TA Cloning®
- Transforming low-copy number TOPO® plasmids generally yields 2 to 5-fold fewer colonies than transforming high-copy number TOPO® plasmids

If you have TOPO® Cloned previously, note that we have slightly modified the One Shot® TOP10 transformation protocols (see below and the next page) to address this issue. Briefly, we recommend the following:

- Increase the amount of TOPO® Cloning reaction that you transform into TOP10 cells (use 3 µl) **and**
- Increase the amount of transformed cells that you plate (use 100-200 µl for chemically competent cells and 50-100 µl for electrocompetent cells)

Example: When directionally TOPO® Cloning a 750 bp test insert into pET160/GW/D-TOPO® or pET161/GW/D-TOPO®, we generally obtain 500-1500 total colonies. Although fewer total colonies are obtained, greater than 90% of the colonies will contain plasmid with your PCR insert in the correct orientation.

One Shot® TOP10 Chemical Transformation Protocol

1. Add 3 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 18 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
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 100-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Positive Clones**, page 22).

   Note: If you see few transformants, refer to the **Troubleshooting** section, page 39 for tips to optimize your TOPO® Cloning and transformation reactions.

continued on next page

Transforming Competent Cells, continued

Transformation by Electroporation

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

1. Add 3 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 18 into 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the electrocompetent cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and an electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µl of room temperature S.O.C. 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 marker.
5. Spread 50-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Positive Clones**, page 22).

   Note: If you see few transformants, refer to the **Troubleshooting** section, page 39 for tips to optimize your TOPO® Cloning and transformation reactions.

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 5 colonies and culture them overnight in LB or S.O.B. medium containing 100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).

   Note: Since pET160/GW/D-TOPO® and pET161/GW/D-TOPO® are low-copy number plasmids, you may need to increase the amount of bacterial culture to obtain enough plasmid DNA for sequencing or analysis purposes. Use extra care during purification to obtain plasmid DNA of sufficiently pure quality for sequencing.
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

We recommend sequencing your construct to confirm that your gene is in frame with the appropriate N-terminal or C-terminal fusion tag, if desired. The T7 Promoter Primer and T7 Reverse Primer are included in each kit to help you sequence your insert. Refer to the diagram on page 13 or page 15 for the location of the primer binding sites.

Important

If you download the sequence for pET160/GW/D-TOPO® or pET161/GW/D-TOPO® from our Web site, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the T7 Promoter sequencing primer or the T7 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

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 42-43. These reactions will help you troubleshoot your experiment. Refer to the **Troubleshooting** section, page 34 for additional tips.

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 colony on LB plates 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.

Expressing Recombinant Proteins

Introduction

BL21 Star™(DE3) One Shot® *E. coli* (Box 3) are supplied with each Champion™ pET Directional TOPO® Expression Kit with Lumio™ Technology for use as the host for expression. You will need pure plasmid DNA of your pET TOPO® construct to transform into BL21 Star™(DE3) for expression studies. Since each recombinant protein has different characteristics that may affect optimal expression, we recommend performing a time course of expression to determine the best conditions for expression of your protein.

BL21 Star™ Strains

The BL21 Star™(DE3) *E. coli* strain is specifically designed for expression of genes regulated by the T7 promoter. Each time you perform an expression experiment, you will transform your plasmid into BL21 Star™(DE3). **Use the TOP10 strain, not the BL21 Star™(DE3) strain, for propagation and maintenance of your plasmid.** Basal level expression of T7 polymerase, particularly in BL21 Star™(DE3) cells, may lead to plasmid instability if your gene of interest is toxic to *E. coli*.

Note: If you are expressing a highly toxic gene, the BL21 Star™(DE3)pLysS strain is also available from Invitrogen for expression purposes. The BL21 Star™(DE3)pLysS strain contains the pLysS plasmid to further reduce basal level expression of the gene of interest. For more information, see page 6.

Positive Controls

pET160-GW/CAT or pET161-GW/CAT is provided for use as a positive control for expression. The control vectors allow expression of the chloramphenicol acetyltransferase (CAT) protein fused to either an N-terminal or C-terminal Lumio™ tag. To propagate and maintain each plasmid:

1. Resuspend the vector in 20 µl of sterile water to prepare a 5 ng/µl stock solution.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™-T1^R, or equivalent.
3. Select transformants on LB plates containing 100 µg/ml ampicillin.
4. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Basic Strategy

The basic steps needed to induce expression of your gene in BL21 Star™(DE3) *E. coli* are outlined below.

1. Isolate plasmid DNA using standard procedures and transform your construct and the positive control separately into BL21 Star™(DE3) One Shot® cells.
2. Grow the transformants and induce expression with IPTG over several hours. Take several time points to determine the optimal time of expression.
3. Optimize expression to maximize the yield of protein.

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Expressing Recombinant Proteins, continued

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) for isolation of pure plasmid DNA. Note that since you are purifying a low-copy number plasmid, you may need to increase the amount of bacterial culture that you use to prepare your plasmid construct.

Ampicillin Selection

Ampicillin generally works well for selection of transformants and expression experiments. However, if you find that your expression levels are low, you may want to use carbenicillin instead (see below).

The resistance gene for ampicillin encodes the β-lactamase protein which is secreted into the medium where it hydrolyzes ampicillin, inactivating the antibiotic. Since β-lactamase is catalytic, ampicillin is rapidly removed from the medium resulting in non-selective conditions. If your plasmid is unstable, this may result in the loss of plasmid and low expression levels.

Using Carbenicillin

Carbenicillin is generally more stable than ampicillin, and studies have shown that using carbenicillin in place of ampicillin may help to increase expression levels by preventing loss of the pET160/GW/D-TOPO® or pET161/GW/D-TOPO® plasmid. If you wish to use carbenicillin, perform your transformation and expression experiments in LB containing 50 µg/ml carbenicillin.

Note: If your gene of interest is highly toxic, increasing the concentration of carbenicillin used from 50 µg/ml to 200 µg/ml may help to increase expression levels.

Note

Cyclic AMP-mediated derepression of the *lacUV5* promoter in λDE3 lysogens can result in an increase in basal expression of T7 RNA polymerase. If you are expressing an extremely toxic gene, the pET construct may be unstable in BL21 Star™(DE3) cells. Adding 1% glucose to the bacterial culture medium may help to repress basal expression of T7 RNA polymerase and stabilize your pET construct.

Materials Needed

You should have the following materials on hand before beginning:

- Your pET/D-TOPO® expression construct (>10 µg/ml)
- pET160-GW/CAT or pET161-GW/CAT positive control plasmid, optional
- BL21 Star™(DE3) One Shot® cells (Box 3 supplied with the kit)
- S.O.B. or LB containing the appropriate antibiotic for selection (plus 1% glucose, if desired)
- 37°C incubator (shaking and nonshaking)
- 42°C water bath
- 1 M isopropyl β-D-thiogalactoside (IPTG; Invitrogen Catalog no. 15529-019)
- Liquid nitrogen

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Transforming BL21 Star™(DE3) One Shot® Cells

To transform your construct or the positive control into BL21 Star™(DE3) One Shot® cells, follow the instructions below. You will need one vial of cells per transformation.

Note: You will not plate the transformation reaction but inoculate it into medium for growth and subsequent expression.

1. Thaw on ice one vial of BL21 Star™(DE3) One Shot® cells per transformation.
2. Add 5-10 ng plasmid DNA in a 1 to 5 µl volume into each vial of BL21 Star™(DE3) One Shot® cells and mix by stirring gently with the pipette tip. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 250 µl of room temperature S.O.C. medium.
7. Cap the tube tightly, tape the tube on its side (for better aeration), and incubate at 37°C for 1 hour with shaking (200 rpm).
8. Add the **entire** transformation reaction to 10 ml of LB containing the appropriate antibiotic (and 1% glucose, if desired).
9. Grow overnight at 37°C with shaking. Proceed to **Pilot Expression**, below.

Pilot Expression

1. Inoculate 10 ml of LB containing the appropriate antibiotic (and 1% glucose, if desired) with 500 µl of the overnight culture from Step 9, above.
2. Grow two hours at 37°C with shaking. OD_{600} should be about 0.5-0.8 (mid-log).
3. Split the culture into two 5 ml cultures. Add IPTG to a final concentration of 0.5-1 mM to one of the cultures. You will now have two cultures: one induced, one uninduced.
4. Remove a 500 µl aliquot from **each** culture, centrifuge at maximum speed in a microcentrifuge for 30 seconds, and aspirate the supernatant.
5. Freeze the cell pellets at -20°C. These are the zero time point samples.
6. Continue to incubate the cultures at 37°C with shaking. Take time points for each culture every hour for 4 to 6 hours.
7. For each time point, remove 500 µl from the induced and uninduced cultures and process as described in Steps 4 and 5. Proceed to **Using the Lumio™ Green Detection Kit**, next page.

Using the Lumio™ Green Detection Kit

Introduction

Once you have finished your pilot expression, you are ready to analyze the samples using the Lumio™ Green Detection Kit. To detect Lumio™ fusion proteins, you will add the Lumio™ Green Detection Reagent, Lumio™ Gel Sample Buffer, and Lumio™ In-Gel Detection Enhancer to your cell lysates prior to electrophoresis.

If you have used the Champion™ pET Expression System before, note that the protocols for preparing sample lysates have been optimized for use with the Lumio™ Green Detection Kit. Follow the guidelines and protocols provided in this section to prepare samples for in-gel detection using the Lumio™ Green Detection Kit. For more detailed information, refer to the Lumio™ Green Detection Kit manual.

Lumio™ Gel Sample Buffer

The Lumio™ Gel Sample Buffer (4X) supplied with the kit is a proprietary sample buffer containing protein denaturing and reducing agents. The buffer is specifically formulated to provide optimal results with the Lumio™ Green Detection Reagent. **Always use the Lumio™ Gel Sample Buffer (4X) to prepare samples for electrophoresis.**

To prevent oxidation of the reducing agent in the buffer, store the Lumio™ Gel Sample Buffer (4X) at -20°C and minimize exposure to air. Use the buffer immediately upon removal from -20°C and return the buffer to -20°C immediately after use.

Lumio™ In-Gel Detection Enhancer

The Lumio™ In-Gel Detection Enhancer is a proprietary solution and is designed to reduce the non-specific binding of Lumio™ Green Detection Reagent with endogenous proteins.

BenchMark™ Fluorescent Protein Standard

The BenchMark™ Fluorescent Protein Standard is supplied with the Champion™ pET Expression System with Lumio™ Technology to allow easy and direct visualization of molecular weight ranges of your Lumio™ fusion protein on a SDS-PAGE gel. The standard consists of 7 distinct protein bands in the range of ~11-155 kDa and is supplied in a ready-to-use format. For detailed information and specifications, refer to the BenchMark™ Fluorescent Protein Standard manual.

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. For more information about pre-cast gels available from Invitrogen, visit to our Web site (www.invitrogen.com) or contact Technical Support (see page 54).

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Using the Lumio™ Green Detection Kit, continued

For optimal results with the Lumio™ Green Detection Kit, follow these guidelines:

- Load at least 1 picomole of the Lumio™ fusion protein
- Use 5 µl of BenchMark™ Fluorescent Protein Standard on a mini-gel as a molecular weight marker
- **Always use the Lumio™ Gel Sample Buffer (4X) to prepare samples for electrophoresis**
- Wear protective clothing, eyewear, and gloves suitable for use with dimethyl sulfoxide (*e.g.* nitrile gloves) when handling the Lumio™ Green Reagent
- Use the Lumio™ Gel Sample Buffer (4X) in a certified fume hood
- Visualize the gel immediately after electrophoresis to prevent diffusion of proteins as the proteins are not fixed in the gel during Lumio™ detection
- Avoid storing the protein sample in the Lumio™ Gel Sample Buffer or Lumio™ Green Detection Reagent

Materials Needed

You should have the following materials on hand before beginning:

- Cell pellets from **Pilot Expression**, page 26
- Lysis Buffer (see page 47 for recipe)
- 8 M urea, optional
- 4X Lumio™ Gel Sample Buffer (supplied with the kit)
- Lumio™ Green Detection Reagent (supplied with the kit)
- Lumio™ In-Gel Detection Enhancer (supplied with the kit)
- Water bath set at 70°C
- Appropriate pre-cast gels and running buffer

Preparing Lysate Samples

Follow the protocol below to prepare cell lysates.

1. Thaw the cell pellets from the pilot expression (Steps 5 and 7, page 26) and resuspend each pellet in 50 µl of Lysis Buffer (see page 47 for a recipe).

   Note: To facilitate lysis, you may need to add lysozyme or sonicate the cells.

2. If you wish to analyze total cell lysates, transfer 15 µl of each sample from Step 1 to a fresh tube. Proceed to **Adding Lumio™ Detection Reagents**, next page.

   If you wish to prepare lysate fractions to analyze soluble and insoluble protein, proceed to Step 3.

3. Centrifuge samples at maximum speed in a microcentrifuge for 5 minute at +4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Wash pellets once with Lysis Buffer to remove any residual soluble proteins. Resuspend the pellets in 50 µl of 8 M urea.
5. Transfer 15 µl of each supernatant and pellet sample to a fresh tube. Proceed to **Adding Lumio™ Detection Reagents**, next page.

continued on next page

Using the Lumio™ Green Detection Kit, continued

Adding Lumio™ Detection Reagents

At this point, you should have 15 µl lysate samples for each time point. Follow the protocol below to prepare these samples for electrophoresis using the Lumio™ Detection Reagents.

1. To each 15 µl lysate sample, add 5 µl of 4X Lumio™ Gel Sample Buffer.
2. Thaw the Lumio™ Green Detection Reagent and mix well by pipetting up and down. Add 0.2 µl of the Lumio™ Green Detection Reagent to each sample using a 2 µl-pipettor (P2 pipettor). Return the Lumio™ Green Detection Reagent to -20ºC immediately after use.

   Alternative: If you do not have a 2µl-pipettor, make a fresh 1:5 dilution of the Lumio™ Green Detection Reagent using 1X Lumio™ Gel Sample Buffer. Add 1 µl of this diluted Lumio™ Green Detection Reagent to each sample.
3. Mix samples well by pipetting up and down and incubate samples at 70°C for 10 minutes.
4. Allow samples to cool for 1-2 minutes and centrifuge briefly at high speed in a microcentrifuge.
5. Thaw the Lumio™ In-Gel Detection Enhancer and mix well by pipetting up and down. Add 2 µl Lumio™ In-Gel Detection Enhancer to each sample. Return the Lumio™ In-Gel Detection Enhance to -20ºC immediately after use.
6. Mix samples well by pipetting up and down and incubate samples at room temperature for 5 minutes.
7. Load 5-20 µl of each sample on an appropriate gel and perform electrophoresis. Proceed to **Analyzing Lumio™ Fusion Proteins**, next page.

   Note: If you are using NuPAGE® Novex Gels, there is no need to add NuPAGE® Antioxidant in the running buffer during electrophoresis.

Analyzing Lumio™ Fusion Proteins

Introduction

Once you have performed electrophoresis, you will visualize Lumio™ fusion proteins directly in the gel. General guidelines are provided below. For more detailed information, refer to the Lumio™ Green Detection Kit manual.

After electrophoresis is complete, we recommend removing the gel from the cassette. The sensitivity of detection is much higher when the gel is imaged after removal from the cassette. Avoid touching the gel with bare hands while handling or imaging the gel.

Required Equipment to Visualize the Gel

For optimal visualization of the fluorescent protein bands you will need one of the following:

- UV transilluminator (302 nm or 365 nm)

  To photograph a gel on the UV transilluminator, use a standard video camera, CCD (Charged Couple Device) camera, or a cooled CCD camera with ethidium bromide filter, SYBR® Green filter, or band pass filter encompassing the emission maxima (535 nm) of the stain.

  Note: If you are using 365 nm UV transilluminator, you may have to expose the gel for a longer time, as the sensitivity is lower than a 302 nm UV transilluminator.

- Laser-based scanner with a laser line that falls within the excitation maxima of the stain (500 nm), a 535 nm long pass filter, or a band pass filter centered at the emission maxima of 535 nm. The sensitivity of detection is higher with laser-based scanners equipped with the appropriate filters than with UV transillumination.

Visualizing and Imaging the Gel

Be sure to adjust the settings on the camera **prior to turning on the UV light** on the UV transilluminator. The fluorescent dye of the Lumio™ Detection Reagent is sensitive to photobleaching. Avoid exposing the gel to UV light for a long time.

1. Place the gel on a UV transilluminator (302 nm) equipped with a standard camera and make sure the ethidium bromide or SYBR® Green filter is selected on the camera.

   You may also use a laser-based scanner with a laser line that falls within the appropriate excitation and emission spectra (see above).

2. Image the gel with a suitable camera with the appropriate filters using a 4-10 second exposure. You may need to adjust the brightness and contrast to reduce any faint non-specific bands.

   You should see fluorescent bands of Lumio™ fusion proteins and the gel should have minimal background. The Lumio™ fusion protein bands appear white or black depending on the type of imaging system used. For an example of expected results, refer to the Lumio™ Green Detection Kit manual.

continued on next page

Analyzing Lumio™ Fusion Proteins, continued

Note

- The fluorescent signal is stable for 10-15 minutes if the gel is not exposed to UV light.
- The fluoresence emission of the Lumio™ Green Detection Reagent is in the green light region. If you have a suitable imaging system with a colored camera and appropriate filters, you may visualize and image the emitted green fluorescence.
- Longer exposure times may produce a fluorescent dye front.

Important

Detection with the Lumio™ Green Detection Kit is not permanent and is lost by subsequent staining of the gel with other protein stains. It is extremely important to record a permanent image of the gel prior to staining the gel with protein stains and gel drying.

Note

Expression of your protein with the N- and/or C-terminal tags will increase the size of your recombinant protein. The table below lists the expected size increase in molecular weight from the particular N- or C-terminal fusion tag in each pET Directional TOPO® vector. Note that the expected sizes take into account any additional amino acids between the gene of interest and the fusion peptide (see page 13 or page 15 for a diagram).

Vector	Fusion	Expected Size Increase
pET160/GW/D-TOPO®	N-terminal	4.5 kDa
pET161/GW/D-TOPO®	N-terminal (if using the vector-encoded ATG initiation codon)	3 kDa
	C-terminal	4 kDa

Detecting the 6xHis Tag

You may detect your fusion protein by western blotting using antibodies available from Invitrogen, if desired. If you are using pET160/GW/D-TOPO®, detect expression of your N-terminally tagged protein using the Anti-HisG Antibody (Catalog no. R940-25), Anti-HisG-HRP Antibody (Catalog no. R941-25), or Anti-HisG-AP Antibody (Catalog no. R942-25).

If you are using pET161/GW/D-TOPO®, detect expression of your C-terminally tagged protein using the Anti-His(C-term) Antibody (Catalog no. R930-25), Anti-His(C-term)-HRP Antibody (Catalog no. R931-25), or the Anti-His(C-term)-AP Antibody (Catalog no. R932-25).

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Analyzing Lumio™ Fusion Proteins, continued

Assay for CAT

If you use the pET160-GW/CAT or pET161-GW/CAT positive control vector, you may assay for CAT protein using CAT Antiserum available from Invitrogen (see page x for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT fusion protein is approximately 32 kDa.

Removal of the N-terminal Fusion Tag Using TEV Protease

If you are expressing your recombinant fusion protein from pET160/GW/D-TOPO®, you may use recombinant TEV protease available from Invitrogen (see page x for ordering information) to remove the N-terminal fusion tag. Instructions for digestion are included with the product. For more information, contact Technical Support (page 54).

Note: After digestion with TEV protease, eighteen vector-encoded amino acids will remain at the N-terminus of your protein.

Purifying Recombinant Fusion Proteins

Introduction

The presence of the polyhistidine (6xHis) tag in pET160/GW/D-TOPO® and pET161/GW/D-TOPO® allows purification of your recombinant fusion protein with a metal-chelating resin such as ProBond™ or Ni-NTA.

ProBond™ and Ni-NTA

ProBond™ and Ni-NTA are nickel-charged agarose resins that can be used for affinity purification of fusion proteins containing the 6xHis tag. Proteins bound to the resin may be eluted with either low pH buffer or competition with imidazole or histidine.

- To scale up your pilot expression for purification, see below.
- To purify your fusion protein using ProBond™ or Ni-NTA, refer to the manual included with each product. You may download the manuals from our Web site (www.invitrogen.com).
- To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.

Scaling-up Expression for Purification

We generally scale-up expression to a 50 ml bacterial culture for purification using a 2 ml ProBond™ or Ni-NTA column. Depending on the expression level of your recombinant fusion protein, you may need to adjust the culture volume to bind the maximum amount of recombinant fusion protein to your column.

To grow and induce a 50 ml bacterial culture:

1. Inoculate 10 ml of S.O.B. or LB containing the appropriate antibiotic with a BL21 Star™(DE3) transformation reaction (see page 25).
2. Grow overnight at 37°C with shaking (225-250 rpm) to OD_{600} = 1-2.
3. The next day, inoculate 50 ml of S.O.B. or LB containing the appropriate antibiotic with 1 ml of the overnight culture.

   Note: You can scale up further and inoculate all of the 10 ml overnight culture into 500 ml of medium, but you will need to adjust the bed volume of your ProBond™ or Ni-NTA column accordingly.
4. Grow the culture at 37°C with shaking (225-250 rpm) to an OD_{600} = ~0.5 (2-3 hours). The cells should be in mid-log phase.
5. Add 0.5-1 mM IPTG to induce expression.
6. Grow at 37°C with shaking until the optimal time point determined by the pilot expression is reached. Harvest the cells by centrifugation (3000 x g for 10 minutes at +4°C).
7. Proceed to purification or store the cells at -80°C for future use.

Additional Purification Steps

There may be cases when your specific fusion protein may not be completely purified by metal affinity chromatography. Other protein purification techniques may be utilized in conjunction with ProBond™ or Ni-NTA to purify the fusion protein (see Deutscher, 1990 for more information).

Creating an Entry Clone

Introduction

After you have TOPO® Cloned your gene of interest into pET160/GW/D-TOPO® or pET161/GW/D-TOPO®, you may perform a BP recombination reaction between your expression construct and a Gateway® donor vector to generate an entry clone. Once you generate an entry clone, your gene of interest may then be easily shuttled into a large selection of destination vectors using the LR recombination reaction. To ensure that you obtain the best possible results, we recommend that you read this section and the next section entitled **Performing the BP Recombination Reaction** (pages 36-38) before beginning.

Recombining the Expression Clone with a Donor Vector

Before performing the BP recombination reaction, consider the following points:

- The N-terminal or C-terminal peptide containing the Lumio™ and 6xHis tags will not be recombined into the entry clone. If you wish to fuse your gene of interest to any N-terminal or C-terminal peptides, the peptides will need to be provided by the destination vector in the LR recombination reaction.
- If you cloned the gene of interest in frame with the N-terminal peptide in pET160/GW/D-TOPO® or the C-terminal peptide in pET161/GW/D-TOPO®, the gene will remain in frame with any N-terminal or C-terminal tags provided by the destination vector following the LR recombination reaction.
- Depending on the design of your forward and reverse primers, your gene in the entry clone may not contain a ribosome binding site, ATG initiation codon, or a stop codon. If any of these elements are required, they will need to be provided by the destination vector in the LR recombination reaction.

Experimental Outline

To generate an entry clone, you will:

1. Perform a BP recombination reaction between your pET160/GW/D-TOPO® or pET161/GW/D-TOPO® expression clone and an *att*P-containing donor vector (see below)
2. Transform the reaction mixture into a suitable *E. coli* host
3. Select for entry clones

Gateway® Donor Vectors

Invitrogen offers a variety of Gateway® donor vectors to help you generate an entry clone containing your gene of interest (see page x for ordering information). For more information about the vectors available, refer to our Web site (www.invitrogen.com) or contact Technical Support (page 54).

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Important

For optimal efficiency, perform the BP recombination reaction using:

- **Linear** pET160/GW/D-TOPO® or pET161/GW/D-TOPO® expression clone (see below for guidelines to linearize expression clones)
- **Supercoiled** *att*P-containing donor vector

Note: Supercoiled or relaxed *att*B expression clones may be used, but will react less efficiently than linear *att*B expression clones.

Linearizing Expression Clones

We recommend that you linearize your pET160/GW/D-TOPO® or pET161/GW/D-TOPO® expression clone using a suitable restriction enzyme (see the guidelines below).

1. Linearize 1 to 2 µg of the expression clone with a unique restriction enzyme that does not digest within the gene of interest and is located outside the *att*B region.
2. Ethanol precipitate the DNA after digestion by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
3. Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.
4. Dissolve the DNA in TE Buffer, pH 8.0 to a final concentration of 50-150 ng/µl.

Performing the BP Recombination Reaction

Introduction

General guidelines and instructions are provided in this section to perform a BP recombination reaction using your pET160/GW/D-TOPO® or pET161/GW/D-TOPO® expression clone and a donor vector, and to transform the reaction mixture into a suitable *E. coli* host to select for entry clones. We recommend that you include a positive control (see below) in your experiment to help you evaluate your results.

Positive Control

pEXP7-tet is provided with the BP Clonase™ enzyme mix as a positive control for the BP reaction. pEXP7-tet is an approximately 1.4 kb linear fragment and contains *att*B sites flanking the tetracycline resistance gene and its promoter (Tc^r). Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene. The efficiency of the BP recombination reaction can easily be determined by streaking entry clones onto LB plates containing 20 µg/ml tetracycline.

Determining How Much DNA to Use

For optimal efficiency, we recommend using the following amounts of linearized *att*B expression clone and donor vector in a 20 µl BP recombination reaction:

- An equimolar amount of linearized *att*B expression clone and the donor vector
- 100 femtomoles (fmol) **each** of linearized *att*B expression clone and donor vector is preferred, but the amount of *att*B expression clone used may range from 40-100 fmol

  Note: 100 fmol of donor vector (pDONR™221 or pDONR™/Zeo) is approximately 300 ng.

For a formula to convert fmol of DNA to nanograms (ng), see below. For an example, see the next page.

- Do not use more than 500 ng of donor vector in a 20 µl BP reaction as this will affect the efficiency of the reaction
- Do not exceed more than 1 µg of total DNA (donor vector plus *att*B expression clone) in a 20 µl BP reaction as excess DNA will inhibit the reaction

Converting Femtomoles (fmol) to Nanograms (ng)

Use the following formula to convert femtomoles (fmol) of DNA to nanograms (ng) of DNA:

$$ng = (fmol)(N)\left(\frac{660fg}{fmol}\right)\left(\frac{1ng}{10^6 fg}\right)$$

where N is the size of the DNA in bp. For an example, see the next page.

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Performing the BP Recombination Reaction, continued

Example of fmol to ng Conversion

In this example, you need to use 100 fmol of your pET160/GW/D-TOPO® expression clone which is 7.5 kb in size in the BP reaction. Calculate the amount of your pET160/GW/D-TOPO® expression clone required for the reaction (in ng) by using the equation on the previous page:

$$(100\text{fmol})(7500\text{ bp})(\frac{660\text{fg}}{\text{fmol}})(\frac{1\text{ng}}{10^6\text{fg}}) = 495\text{ng of expression clone required}$$

Materials Needed

You should have the following materials on hand before beginning:

- Linearized pET160/GW/D-TOPO® or pET161/GW/D-TOPO® expression clone (see previous page to determine the amount of DNA to use)
- pDONR™ vector (resuspended to 150 ng/µl)
- BP Clonase™ enzyme mix (see page x for ordering information; keep at -80ºC until immediately before use)
- 5X BP Clonase Reaction Buffer (supplied with the BP Clonase™ enzyme mix)
- pEXP7-tet positive control, optional (50 ng/µl; supplied with the BP Clonase™ enzyme mix)
- TE Buffer, pH 8.0 (10 mm Tris-HCl, pH 8.0; 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the BP Clonase™ enzyme mix; thaw and keep on ice until use)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. medium
- LB agar plates containing the appropriate antibiotic to select for entry clones

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Performing the BP Recombination Reaction, continued

Setting Up the BP Recombination Reaction

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.

   Note: To include a negative control, set up a second sample reaction and substitute TE Buffer, pH 8.0 for the BP Clonase™ enzyme mix (see Step 4).

Components	Sample	Positive Control
pET160/GW/D-TOPO® or pET161/GW/D-TOPO® expression clone (40-100 fmol)	1-10 µl	--
pDONR™ vector (150 ng/µl)	2 µl	2 µl
pEXP7-tet positive control (50 ng/µl)	--	2 µl
5X BP Clonase™ Reaction Buffer	4 µl	4 µl
TE Buffer, pH 8.0	to 16 µl	8 µl

2. Remove the BP Clonase™ enzyme mix from -80°C and thaw on ice (~ 2 minutes).
3. Vortex the BP Clonase™ enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 4 µl of BP Clonase™ enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return BP Clonase™ enzyme mix to -80°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: For most applications, a 1 hour incubation will yield a sufficient number of entry clones. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. An overnight incubation typically yields 5-10 times more colonies than a 1 hour incubation.
6. Add 2 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 µl of the BP recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for entry clones.

   Note: You may store the BP reaction at -20°C for up to 1 week before transformation, if desired.

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1 x 10^8 cfu/µg, the BP recombination reaction should give >1500 colonies if the entire BP reaction is transformed and plated.

Verifying pEXP7-tet Entry Clones

If you included the pEXP7-tet control in your experiments, you may access the efficiency of the BP reaction by streaking entry clones onto LB plates containing 20 µg/ml tetracycline. True entry clones should be tetracycline-resistant.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions to help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 42-43) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR product too dilute	• Concentrate the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Large PCR product	• Increase the incubation time of the TOPO® 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, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of your choice. • Gel-purify the PCR product to remove primer-dimers and other artifacts.

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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	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	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.
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
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 using your proofreading polymerase of choice. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence CACC at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence CACC at the 5′ end.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use LB plates containing 100 µg/ml ampicillin.

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Troubleshooting, continued

Expression

The table below lists some potential problems and possible solutions that may help you troubleshoot your expression experiment. To help evaluate your results, we recommend including the expression control supplied with kit in your experiment.

Problem	Reason	Solution
No expression of recombinant protein	Gene of interest not in frame with the N-terminal or C-terminal peptide tag	Sequence your construct to verify if the insert is in frame with the peptide tag. If not in frame, redesign your PCR primers.
Low expression	Plasmid instability observed when using ampicillin for selection	Substitute carbenicillin for ampicillin in your transformation and expression experiments (see page 25).
	Toxic gene **Note:** Evidence of toxicity includes loss of plasmid or slow growth relative to the control.	• Add 1% glucose to the bacterial culture medium during transformation and expression. • Transform BL21 Star™(DE3) cells using the protocol on page 26, then perform the expression by growing cells at room temperature rather than 37°C for 24-48 hours. • Transform your expression construct into a pLysS-containing strain (*e.g.* BL21 Star™(DE3)pLysS). • Transform your expression construct into an *E. coli* strain in which expression of T7 RNA polymerase is tightly regulated (*e.g.* BL21-AI™ available from Invitrogen; see our Web site for more information). • Infect TOP10F′ (or other suitable F′ episome-containing host strain) with M13 or lambda phage expressing T7 RNA polymerase.

Detection with Lumio™ Green Detection Kit

If you are experiencing problems detecting your fusion protein using the Lumio™ Green Detection Kit, refer to the Lumio™ Green Detection Kit manual for possible problems and solutions to help you troubleshoot your in-gel detection experiment.

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 the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 100 µg/ml ampicillin (see page 46 for a recipe).

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Thermostable polymerase (1-2.5 units/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.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

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Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and either the pET160/GW/D-TOPO® or pET161/GW/D-TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform *E. coli* using electroporation, **do not** include the salt solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pET/D-TOPO® vector	1 µl	1 µl
Final volume	6 µl	µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 3 µl of each reaction into separate vials of One Shot® TOP10 cells (page 20).
4. Spread 100-200 µl of each transformation mix onto LB plates containing 100 µg/ml ampicillin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. Refer to the table below for expected digestion patterns.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pET160/GW/D-TOPO®	*Pst* I	Correct orientation: 1772, 4816 Reverse orientation: 1265, 5323 Empty vector: 5839
pET161/GW/D-TOPO®	*Pst* I	Correct orientation: 1836, 4774 Reverse orientation: 1329, 5281 Empty vector: 5861

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 20. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. 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. Two simple protocols are provided below.

Note

Cloning efficiency may decrease with purification of the PCR product (*i.e.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 14).

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit (Catalog no. K2100-12) allows you to rapidly purify PCR products from regular agarose gels.

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

Procedure continued on next page

continued on next page

Gel Purifying PCR Products, continued

Using the PureLink™ Quick Gel Extraction Kit, continued

Procedure continued from previous page

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

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in 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 18.
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® TOP10 cells using the method on page 20.

Note

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

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.

S.O.B. Medium (with Antibiotic)

2% Tryptone
0.5% Yeast Extract
0.05% NaCl
2.5 mM KCl
10 mM $MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.5 with 5 M NaOH and add deionized water to 1 liter.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$. You may also add antibiotic, if needed.
5. Store at +4°C. **Medium is stable for only 1-2 weeks.**

continued on next page

Recipes, continued

Lysis Buffer

50 mM potassium phosphate, pH 7.8
400 mM NaCl
100 mM KCl
10% glycerol
0.5% Triton X-100
10 mM imidazole

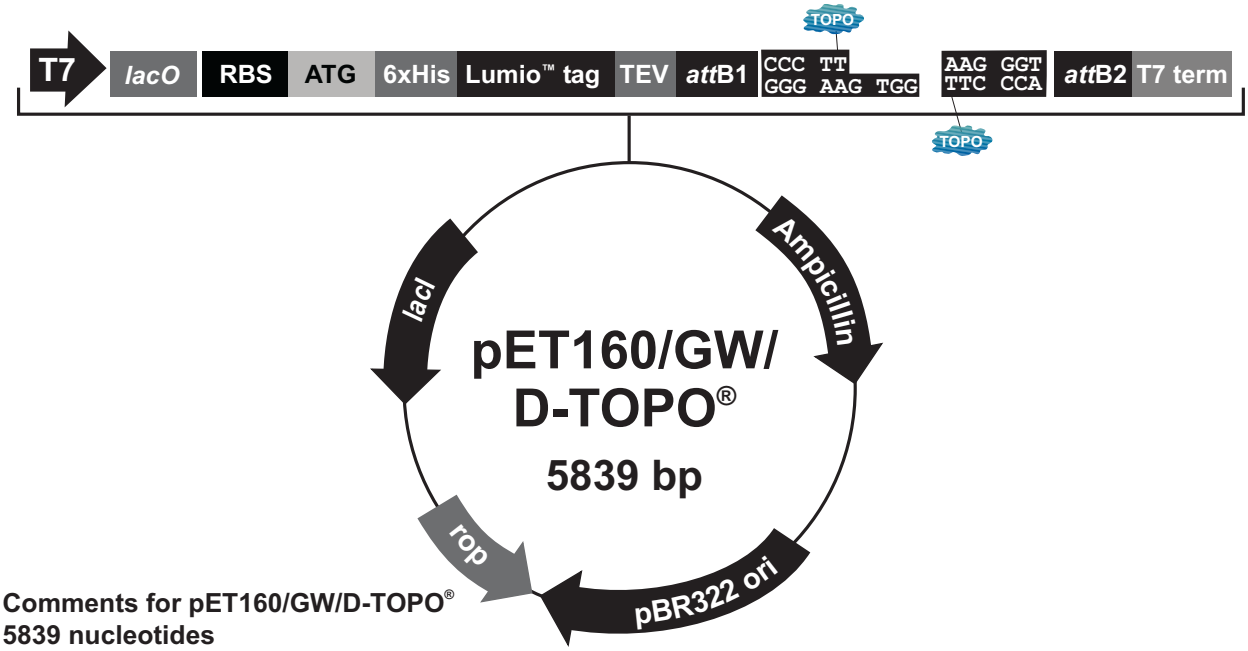
1. Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:

   0.3 ml KH_2PO_4
   4.7 ml K_2HPO_4
   2.3 g NaCl
   0.75 g KCl
   10 ml glycerol
   0.5 ml Triton X-100
   68 mg imidazole
3. Mix thoroughly and adjust pH to 7.8 with HCl. Bring the volume to 100 ml.
4. Store at +4°C.

Map and Features of pET160/GW/D-TOPO®

Map of pET160/GW/D-TOPO®

The figure below shows the elements of pET160/GW/D-TOPO® (5839 bp). **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support (page 54).**



Comments for pET160/GW/D-TOPO®
5839 nucleotides

T7 promoter/priming site: bases 21-40
lac operator (lacO): bases 40-64
Ribosome binding site (RBS): bases 94-101
Initiation ATG: bases 109-111
Polyhistidine (6xHis) region: bases 112-129
Lumio™ tag: bases 142-159
TEV recognition site: bases 169-189
*att*B1 site: bases 196-220
TOPO® recognition site1: bases 232-236
Overhang sequence (c): bases 237-240
TOPO® recognition site 2: bases 241-245
*att*B2 site: bases 257-281
T7 transcription termination region: bases 307-435
T7 reverse priming site: bases 346-365
bla promoter: bases 740-838
Ampicillin (*bla*) resistance gene: bases 839-1699
pBR322 origin: bases 1844-2517
ROP ORF (c): bases 2888-3079
*lac*I ORF (c): bases 4391-5482

(c) = complementary strand

continued on next page

Map and Features of pET160/GW/D-TOPO®, continued

Features of pET160/GW/D-TOPO®

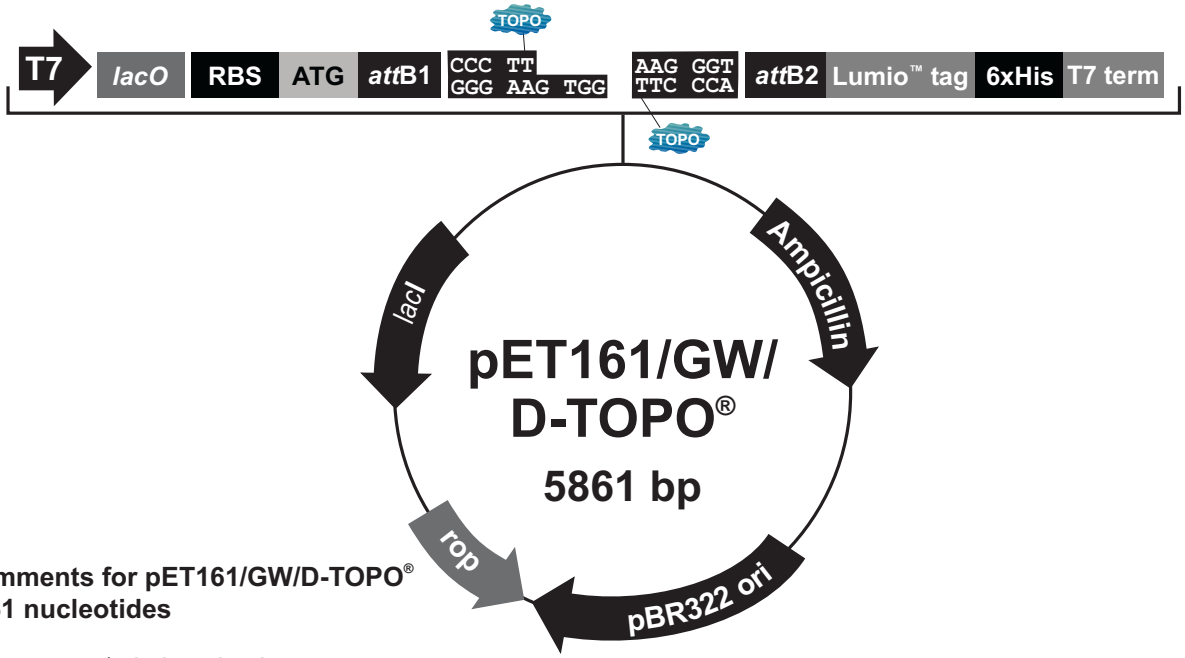
pET160/GW/D-TOPO® (5839 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Allows high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
T7 Promoter priming site	Allows sequencing of the insert.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the ATG initiation codon for efficient translation of PCR product.
N-terminal 6xHis tag	Allows purification of recombinant fusion protein on metal-chelating resin (*e.g.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-HisG Antibodies.
Lumio™ tag (Cys-Cys-Pro-Gly-Cys-Cys)	Allows binding of the Lumio™ Green Detection Reagent to facilitate in-gel detection of your recombinant fusion protein (Adams *et al.*, 2002).
TEV recognition site	Allows removal of the N-terminal tag from your recombinant protein using TEV protease (Carrington and Dougherty, 1988; Dougherty *et al.*, 1988).
*att*B1 and *att*B2 sites	Allows recombinational cloning of the gene of interest to generate a Gateway® entry clone.
TOPO® Cloning site (directional)	Allows directional cloning of your PCR product in frame with the N-terminal Lumio™ tag for expression in *E. coli.*
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from bacteriophage T7 which allows efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli.*
pBR322 origin of replication (*ori*)	Allows replication and maintenance in *E. coli.*
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*
lacI ORF	Encodes lac repressor which binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

Map and Features of pET161/GW/D-TOPO®

Map of pET161/GW/D-TOPO®

The figure below shows the elements of pET161/GW/D-TOPO® (5861 bp). **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support (page 54).**



Comments for pET161/GW/D-TOPO®
5861 nucleotides

T7 promoter/priming site: bases 21-40
lac operator (lacO): bases 40-64
Ribosome binding site (RBS): bases 94-101
Initiation ATG: bases 109-111
*att*B1 site: bases 154-178
TOPO® recognition site1: bases 190-194
Overhang sequence (c): bases 195-198
TOPO® recognition site 2: bases 199-203
*att*B2 site: bases 215-239
Lumio™ tag: bases 268-285
Polyhistidine (6xHis) region: bases 301-318
T7 transcription termination region: bases 333-461
T7 reverse priming site: bases 372-391
bla promoter: bases 762-860
Ampicillin (*bla*) resistance gene: bases 861-1721
pBR322 origin: bases 1866-2539
ROP ORF (c): bases 2910-3101
*lac*I ORF (c): bases 4413-5504

(c) = complementary strand

continued on next page

Map and Features of pET161/GW/D-TOPO®, continued

Features of pET161/GW/D-TOPO®

pET161/GW/D-TOPO® (5861 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
T7 promoter	Allows high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
T7 Promoter priming site	Allows sequencing of the insert.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the ATG initiation codon for efficient translation of PCR product.
*att*B1 and *att*B2 sites	Allows recombinational cloning of the gene of interest to generate a Gateway® entry clone.
TOPO® Cloning site (directional)	Allows directional cloning of your PCR product in frame with the C-terminal Lumio™ tag for expression in *E. coli.*
Lumio™ tag (Cys-Cys-Pro-Gly-Cys-Cys)	Allows binding of the Lumio™ Green Detection Reagent to facilitate in-gel detection of your recombinant fusion protein (Adams *et al.*, 2002).
C-terminal 6xHis tag	Allows purification of recombinant fusion protein on metal-chelating resin (*e.g.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-His(C-term) Antibodies.
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from bacteriophage T7 which allows efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Allows replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli.*
lacI ORF	Encodes lac repressor which binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

Map of pET160-GW/CAT

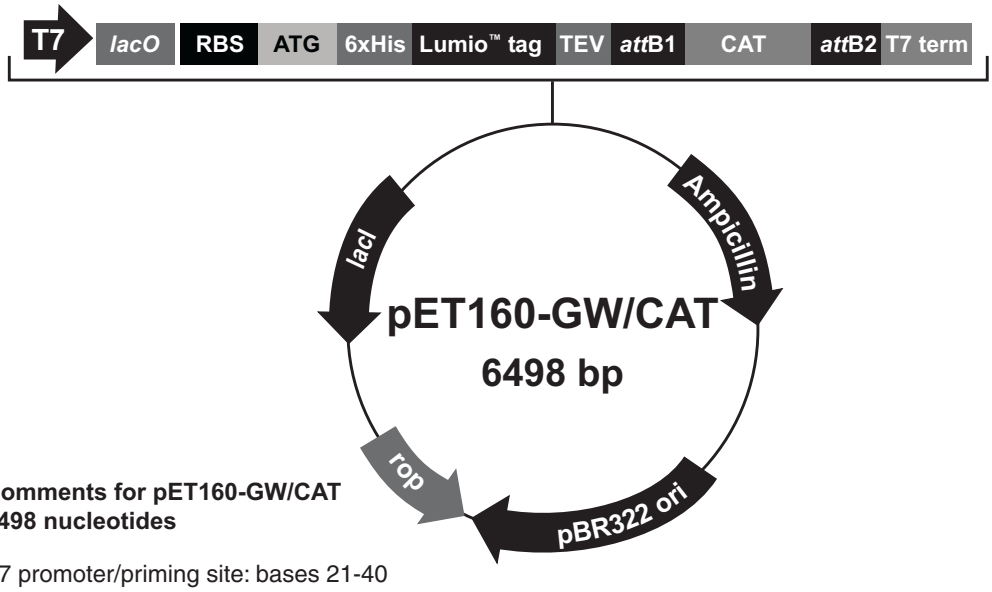
Description

pET160-GW/CAT (6498 bp) is a control vector containing the chloramphenicol acetyltransferase (CAT) gene and was constructed using the Gateway® LR recombination reaction between an entry clone containing the CAT gene and pET160-DEST. CAT is expressed as a fusion to the Lumio™ tag. The molecular weight of the CAT fusion protein is approximately 32 kDa.

For more information on the Gateway® Technology, refer to the Gateway® Technology manual. For more information on pET160-DEST, refer to the Champion™ pET Gateway® Expression Kits with Lumio™ Technology manual.

Map

The complete sequence of pET160-GW/CAT is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Support (page 54).



Comments for pET160-GW/CAT
6498 nucleotides

T7 promoter/priming site: bases 21-40
lac operator (lacO): bases 40-64
Ribosome binding site (RBS): bases 91-101
Initiation ATG: bases 109-111
Polyhistidine (6xHis) region: bases 112-129
Lumio™ tag: bases 142-159
TEV recognition site: bases 169-189
*att*B1 site: bases 196-220
CAT gene: bases 241-942
*att*B2 site: bases 916-940
T7 transcription termination region: bases 966-1094
T7 reverse priming site: bases 1005-1024
bla promoter: bases 1399-1497
Ampicillin (*bla*) resistance gene: bases 1498-2358
pBR322 origin: bases 2503-3176
ROP ORF (c): bases 3547-3738
*lac*I ORF (c): bases 5050-6141

(c) = complementary strand

Map of pET161-GW/CAT

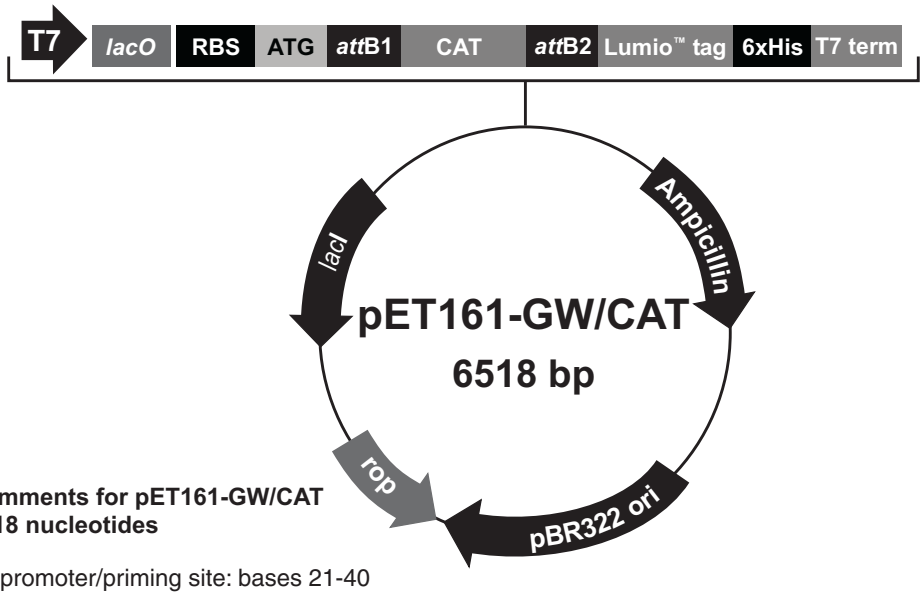
Description

pET161-GW/CAT (6518 bp) is a control vector containing the chloramphenicol acetyltransferase (CAT) gene and was constructed using the Gateway® LR recombination reaction between an entry clone containing the CAT gene and pET161-DEST. CAT is expressed as a fusion to the Lumio™ tag. The molecular weight of the CAT fusion protein is approximately 32 kDa.

For more information on the Gateway® Technology, refer to the Gateway® Technology manual. For more information on pET161-DEST, refer to the Champion™ pET Gateway® Expression Kits with Lumio™ Technology manual.

Map

The complete sequence of pET161-GW/CAT is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Support (page 54).



Comments for pET161-GW/CAT
6518 nucleotides

T7 promoter/priming site: bases 21-40
lac operator (lacO): bases 40-64
Ribosome binding site (RBS): bases 94-101
Initiation ATG: bases 109-111
*att*B1 site: bases 154-178
CAT gene: bases 199-855
*att*B2 site: bases 872-896
Lumio™ tag: bases 925-942
Polyhistidine (6xHis) region: bases 958-975
T7 transcription termination region: bases 990-1118
T7 reverse priming site: bases 1029-1048
bla promoter: bases 1419-1517
Ampicillin (*bla*) resistance gene: bases 1518-2378
pBR322 origin: bases 2523-3196
ROP ORF (c): bases 3567-3758
*lac*I ORF (c): bases 5070-6161

(c) = complementary strand

Technical Support

Web Resources

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

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

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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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SDS

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

Product Qualification

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

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Technical Support, continued

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Information for European Customers

The BL21 Star™(DE3) strain is genetically modified and carries the bacteriophage λ DE3 lysogen containing the T7 RNA polymerase gene. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 60.

Limited Use Label License No. 22: Vectors and Clones Encoding Histidine Hexamer

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continued on next page

Purchaser Notification, continued

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continued on next page

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Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

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