invitrogen™
by life technologies™

Champion™ pET Gateway® Expression Kits with Lumio™ Technology

For inducible expression and specific detection of Lumio™ fusion proteins in *E. coli*

Catalog nos. 12583-035 and 12583-043

Rev. Date: 4 August 2010
Manual part no. 25-0698

MAN0000429

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Quantity	Catalog no.
Champion™ pET160 Gateway® Expression Kit with Lumio™ Technology	20 reactions	12583-035
Champion™ pET161 Gateway® Expression Kit with Lumio™ Technology	20 reactions	12583-043

Shipping/Storage

The Champion™ pET Gateway® 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-DEST Gateway® Vector	-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

pET-DEST Gateway® Vector

The vectors provided with the Champion™ pET Gateway® Expression Kits with Lumio™ Technology (Box 1) are listed below.

Store Box 1 at -20°C.

Item	Concentration	Amount
Gateway® Destination Vector (pET160-DEST or pET161-DEST)	lyophilized in TE Buffer, pH 8.0	6 µg
Control Plasmid (pET160-GW/CAT or pET161-GW/CAT)	lyophilized in TE Buffer, pH 8.0	100 ng

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 is ≥ 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 ≥ 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 strain for propagation and general maintenance of your pET expression construct.

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 5).

Lumio™ Green Detection Kit and BenchMark™ Fluorescent Protein Standard

The Champion™ pET Gateway® 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 Gateway® 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.
Gateway® LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
One Shot® TOP10 Chemically Competent *E. coli*	10 x 50 µl 20 x 50 µl	C4040-10 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
Lumio™ Green Detection Kit	100 reactions	LC6090
BenchMark™ Fluorescent Protein Standard	250 µl	LC5928
PureLink™ HQ Plasmid Purification Kit	100 reactions	K2100-01
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

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 150 ml	R801-01 R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 ml 25 ml	R901-01 R901-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The Champion™ pET Gateway® Expression Kits with Lumio™ Technology contain Gateway®-adapted destination vectors that allow high-level expression of recombinant proteins in *E. coli*, and are designed for use with the Lumio™ Technology. Using the kit 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 Gateway® Destination Vectors

The pET Gateway® destination vectors contain the following elements:

- 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)
- N-terminal or C-terminal Lumio™ tag for specific detection of recombinant proteins using the Lumio™ Green Detection Kit
- Two recombination sites, *att*R1 and *att*R2, downstream of the T7*lac* promoter for recombinational cloning of the gene of interest from an entry clone
- 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-DEST only)
- *lacI* gene encoding the lac repressor to reduce basal transcription from the T7*lac* promoter in the pET Gateway® vector and from the *lacUV5* promoter in the *E. coli* host chromosome (see page 3 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 3.

continued on next page

Overview, continued

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 in *E. coli* using Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector to create an entry clone.
2. Generate an expression clone by performing a LR recombination reaction between the entry clone and a Gateway® destination vector (*e.g.* pET160-DEST or pET161-DEST).
3. Transform your expression clone into BL21 Star™ *E. coli* and assay for expression.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support Technical Support (page 34).

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 6.

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-DEST and pET161-DEST, 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 Gateway® 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 Gateway® 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 5.

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-DEST and pET161-DEST 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.

continued on next page

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 5.

Using TOP10 Cells

One Shot® TOP10 competent *E. coli*, which do not contain T7 RNA polymerase, are included in each Champion™ pET Gateway® 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 Gateway® LR recombination 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 Gateway® 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 that 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 viii 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 34).

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 Gateway® 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 avoid 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 of 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.

Methods

Generating an Entry Clone

Introduction

To recombine your gene of interest into pET160-DEST or pET161-DEST, you will need a Gateway® entry clone containing the gene of interest. Many entry vectors including pENTR/D-TOPO® (Catalog no. K2400-20) are available from Invitrogen to facilitate generation of entry clones. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Support (page 34). Refer to the manual for the specific entry vector you are using for detailed instructions to construct an entry clone.

Points to Consider for pET160-DEST

pET160-DEST 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.

If you wish to...	Then your insert...
include the 6xHis and Lumio™ tags	• should **not** contain a ribosome binding site and ATG initiation codon • should be in frame with the N-terminal 6xHis and Lumio™ tags after recombination (see page 11 for a diagram)
express your protein with a native N-terminus, *i.e.* without the N-terminal peptide	• should contain a stop codon at the 5′ end to terminate the N-terminal peptide • should contain a second ribosome binding site 9-10 base pairs 5′ of the ATG initiation codon of your protein

continued on next page

Generating an Entry Clone, continued

Points to Consider for pET161-DEST

pET161-DEST 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 24 additional amino acids will be present at the N-terminus of your protein (see diagram on page 12).

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).

If you wish to...	Then your insert...
express your protein with a native N-terminus, *i.e.* without the additional N-terminal amino acids	• should contain a stop codon at the 5′ end to terminate the N-terminal peptide • should contain a second ribosome binding site 9-10 base pairs 5′ of the ATG initiation codon of your protein
include the C-terminal Lumio™ and 6xHis tags	• should **not** contain a stop codon at the 3′ end • should be in frame with the Lumio™ tag after recombination (see page 12 for a diagram)
not include the C-terminal Lumio™ and 6xHis tags	• should contain a stop codon at the 3′ end

Creating an Expression Clone

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into pET160-DEST or pET161-DEST to create your expression clone. To ensure that you obtain the best results, we recommend that you read this section and the next section entitled **Performing the LR Recombination Reaction** (page 13) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing pET160-DEST or pET161-DEST vector.
2. Transform the reaction mixture into One Shot® TOP10 Chemically Competent *E. coli* (supplied with the kit).
3. Select for expression clones (refer to pages 11-12 for diagrams of the recombination region of the resulting expression clones).

Resuspending the Vectors

pET160-DEST and pET161-DEST are supplied as 6 µg of plasmid, lyophilized in TE, pH 8.0. To use, simply resuspend the destination vector in 40 µl of sterile water to a final concentration of 150 ng/µl.

Propagating the Vectors

If you wish to propagate and maintain pET160-DEST or pET161-DEST, we recommend using One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli* (Catalog no. C7510-03) from Invitrogen for transformation. The *ccd*B Survival T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50-100 µg/ml ampicillin and 15-30 µg/ml chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5α for propagation and maintenance of pET160-DEST or pET161-DEST as these strains are sensitive to CcdB effects.

LR Clonase™ II Enzyme Mix

LR Clonase™ II enzyme mix is available separately from Invitrogen (Catalog no. 11791-020) to catalyze the LR recombination reaction. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase™ Reaction Buffer previously supplied as separate components in LR Clonase™ enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 13 to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix provided in this manual as reaction conditions differ.

continued on next page

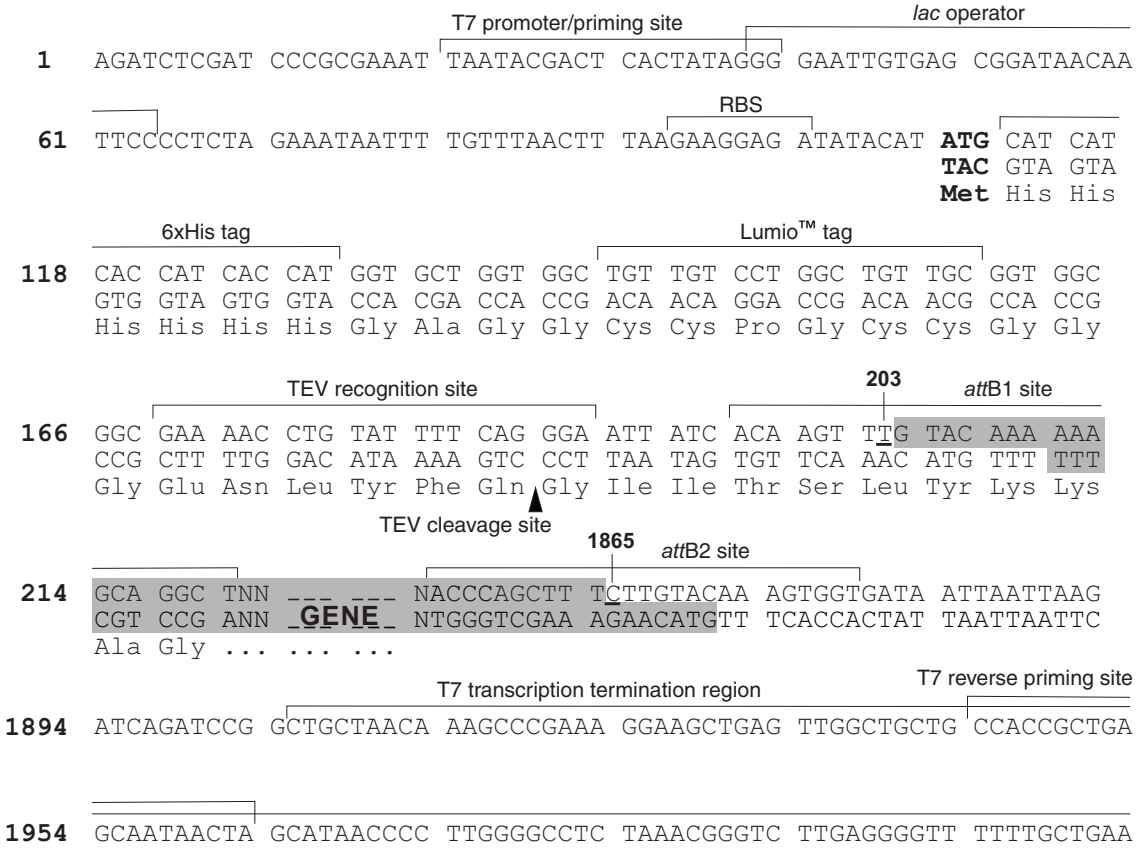
Creating an Expression Clone, continued

Recombination Region for pET160-DEST

The recombination region of the expression clone resulting from pET160-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the entry clone into pET160-DEST by recombination. Non-shaded regions are derived from the pET160-DEST vector.
- Bases 203 and 1865 of the pET160-DEST vector sequence are marked.



continued on next page

Creating an Expression Clone, continued

Recombination Region for pET161-DEST

The recombination region of the expression clone resulting from pET161-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the entry clone into pET161-DEST by recombination. Non-shaded regions are derived from the pET161-DEST vector.
- Bases 161 and 1844 of the pET161-DEST vector sequence are marked.

Performing the LR Recombination Reaction

Introduction

Once you have an entry clone containing your gene of interest, you may perform an LR recombination reaction between the entry clone and pET160-DEST or pET161-DEST to generate your expression clone. We recommend including the pENTR™-gus positive control supplied with the LR Clonase™ II enzyme mix in your experiments to help you evaluate your results.

Materials Needed

You should have the following materials on hand before beginning:

- Purified plasmid DNA of your entry clone (50-150 ng/µl in TE, pH 8.0)
- pET160-DEST or pET161-DEST vector (150 ng/µl in TE, pH 8.0)
- LR Clonase™ II enzyme mix (Invitrogen, Catalog no. 11791-020; keep at -20°C until immediately before use)
- pENTR™-gus positive control, optional (50 ng/µl in TE, pH 8.0; supplied with the LR Clonase™ II 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 LR Clonase™ II enzyme mix; thaw and keep on ice until use)

Setting Up the LR Recombination Reaction

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix. To include a negative control, set up a second sample reaction and substitute TE Buffer for the LR Clonase™ II enzyme mix (Step 4).

Component	Sample	Positive Control
Entry clone (50-150 ng/reaction)	1-7 µl	--
Destination vector (150 ng/µl)	1 µl	1 µl
pENTR™-gus (50 ng/µl)	--	2 µl
TE Buffer, pH 8.0	to 8 µl	5 µl

2. Remove the LR Clonase™ II enzyme mix from -20°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 2 µl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return LR Clonase™ II enzyme mix to -20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µl of Proteinase K to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

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

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the LR recombination reaction, you will transform your pET expression clone into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are supplied with the kit to facilitate transformation.

To maintain the stability of your construct, we recommend that you transform your LR recombination 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:

- LR recombination reaction from **Performing the LR Recombination Reaction**, Step 6 (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. You may sequence your construct to confirm the orientation and reading frame, if desired (see next page).

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.

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Add 1 µl of the LR recombination reaction from Step 6, page 13 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 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.

What You Should See

If you use One Shot® TOP10 Chemically Competent *E. coli* supplied with the kit, the LR reaction should give > 5000 colonies if the entire LR reaction is transformed and plated.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/ml chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

Sequencing

You may sequence your construct to confirm that your gene is in frame with the appropriate N-terminal or C-terminal fusion tag, if desired. We suggest using the T7 Promoter and T7 Reverse primer sequences (see below). Refer to the diagram on page 11 or page 12 for the location of the primer binding sites.

Primer	Sequence
T7 Promoter Primer	5´-TAATACGACTCACTATAGGG-3´
T7 Reverse Primer	5´-TAGTTATTGCTCAGCGGTGG-3´

Expressing Recombinant Proteins

Introduction

BL21 Star™(DE3) One Shot® *E. coli* (Box 3) are supplied with each Champion™ pET Gateway® Expression Kit with Lumio™ Technology for use as the host for expression. You will need pure plasmid DNA of your expression clone 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 5.

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.

continued on next page

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-DEST or pET161-DEST expression clone. 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-DEST expression clone (> 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

continued on next page

Expressing Recombinant Proteins, continued

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 30 minutes 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 34).

continued on next page

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 18
- Lysis Buffer (see page 27 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 18) and resuspend each pellet in 50 µl of Lysis Buffer (see page 27 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 Gateway® vector. Note that the expected sizes take into account any additional amino acids between the gene of interest and the fusion peptide (see page 11 or page 12 for a diagram).

Vector	Fusion	Expected Size Increase
pET160-DEST	N-terminal	4 kDa
pET161-DEST	N-terminal (if using the vector-encoded ATG initiation codon)	2.5 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-DEST, 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-DEST, 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).

continued on next page

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 viii 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-DEST, you may use recombinant TEV protease available from Invitrogen (see page viii 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 34).

Note: After digestion with TEV protease, twelve 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-DEST and pET161-DEST 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).

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

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

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

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

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

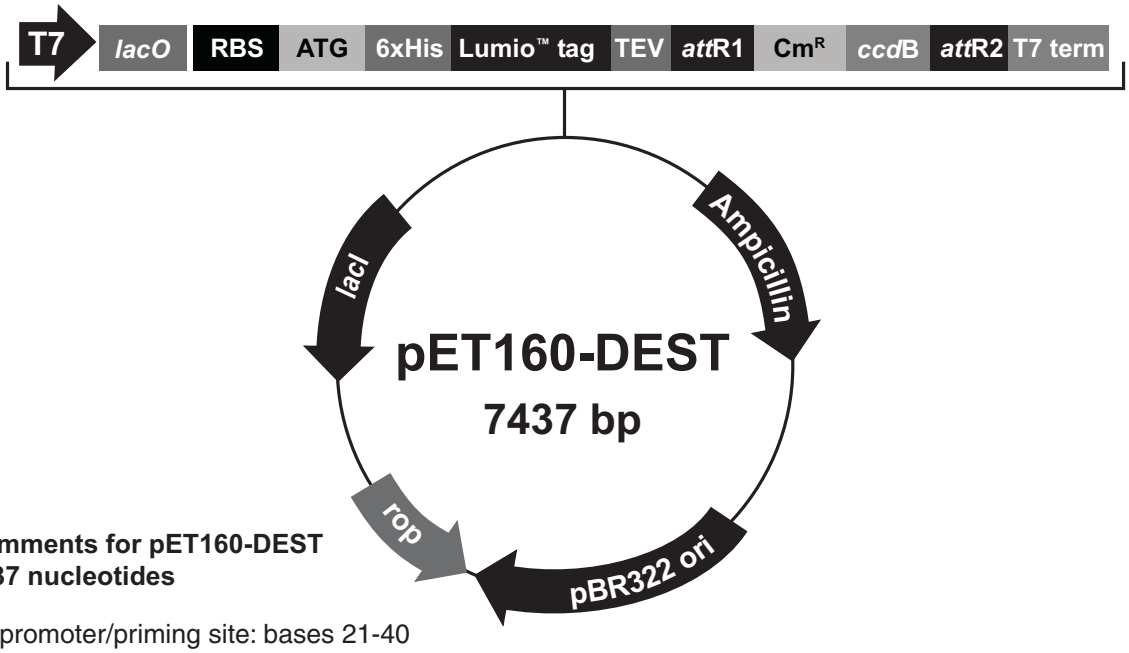
1. Prepare 1 M stock solutions of KH_2PO_4 and K_2HPO_4.
2. For 100 ml, dissolve the following reagents in 90 ml of deionized water:

   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-DEST

Map of pET160-DEST

The figure below shows the elements of pET160-DEST (7437 bp). **The complete sequence of each vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support (page 34).**



Comments for pET160-DEST
7437 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*R1 site: bases 196-320
Chloramphenicol resistance gene: bases 429-1088
*ccd*B gene: bases 1409-1714
*att*R2 site: bases 1755-1879
T7 transcription termination region: bases 1905-2033
T7 reverse priming site: bases 1944-1963
bla promoter: bases 2338-2436
Ampicillin (*bla*) resistance gene: bases 2437-3297
pBR322 origin: bases 3442-4115
ROP ORF (c): bases 4486-4677
*lac*I ORF (c): bases 5989-7080

(c) = complementary strand

continued on next page

Map and Features of pET160-DEST, continued

Features of pET160-DEST

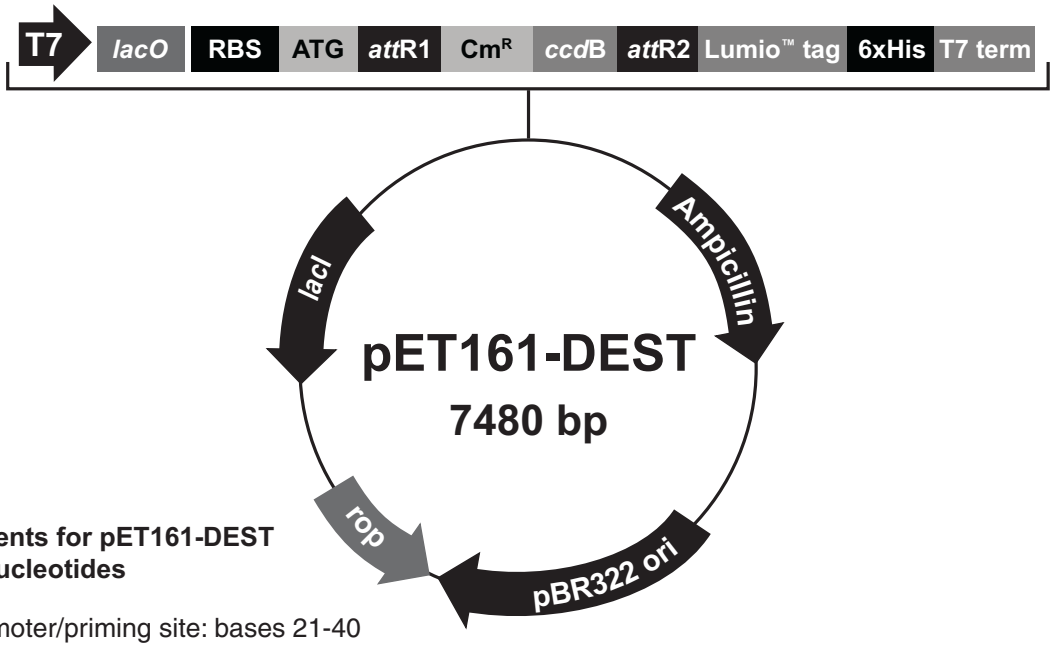
pET160-DEST (7437 bp) contains the following elements. All features have been functionally tested.

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*R1 and *attR*2 sites	Allows recombinational cloning of the gene of interest from an entry clone.
Chloramphenicol resistance gene	Allows counterselection of the plasmid.
*ccd*B gene	Allows negative selection of the plasmid.
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-DEST

Map of pET161-DEST

The figure below shows the elements of pET161-DEST (7480 bp). **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support (page 34).**



Comments for pET161-DEST
7480 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*R1 site: bases 154-278
Chloramphenicol resistance gene: bases 387-1046
*ccd*B gene: bases 1388-1693
*att*R2 site: bases 1734-1858
Lumio™ tag: bases 1887-1904
Polyhistidine (6xHis) region: bases 1920-1937
T7 transcription termination region: bases 1952-2080
T7 reverse priming site: bases 1991-2010
bla promoter: bases 2381-2479
Ampicillin (*bla*) resistance gene: bases 2480-3340
pBR322 origin: bases 3485-4158
ROP ORF (c): bases 4529-4720
*lac*I ORF (c): bases 6032-7123

(c) = complementary strand

continued on next page

Map and Features of pET161-DEST, continued

Features of pET161-DEST

pET161-DEST (7480 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*R1 and *att*R2 sites	Allows recombinational cloning of the gene of interest from an entry clone.
Chloramphenicol resistance gene	Allows counterselection of the plasmid.
*ccd*B gene	Allows negative selection of the plasmid.
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.

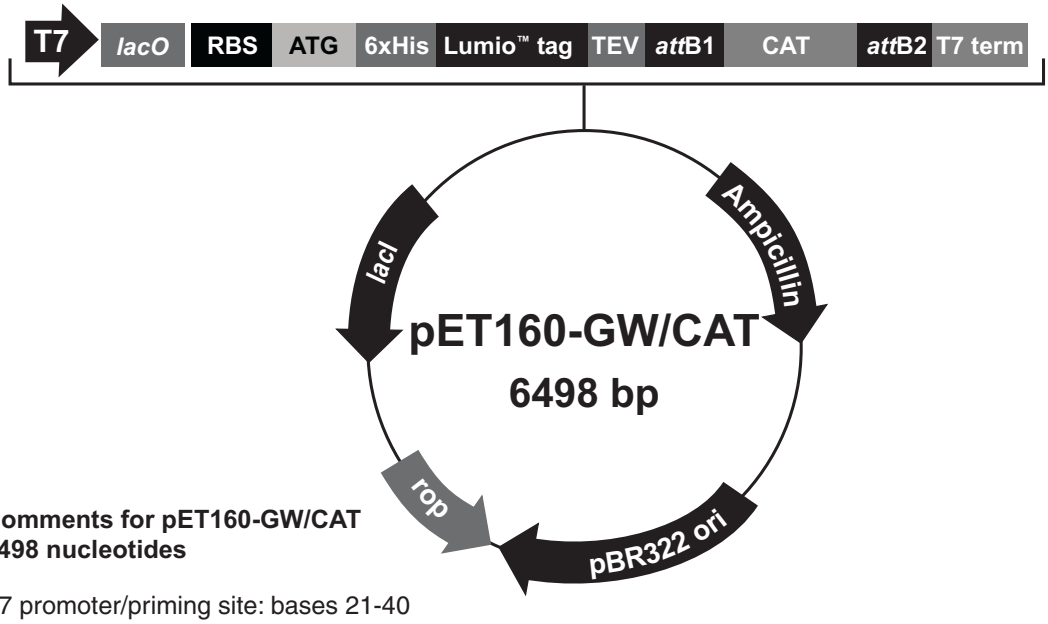
Map of pET160-GW/CAT

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.

Map

The complete sequence of pET160-GW/CAT is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Support (page 34).



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

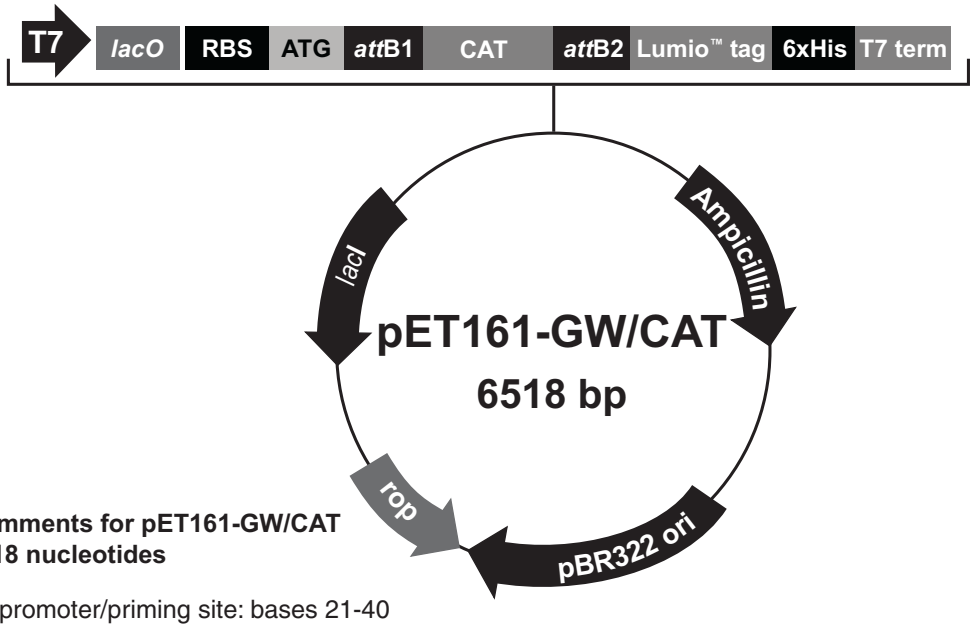
Map of pET161-GW/CAT

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.

Map

The complete sequence of this vector is available for downloading from Web site (www.invitrogen.com) or by contacting Technical Support (page 34).



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

Contact Us

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

Corporate Headquarters:
5791 Van Allen Way
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_support@invitrogen.com

Japanese Headquarters:
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

SDS

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

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited Warranty

Invitrogen (a part of Life Technologies Corporation) is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Support Representatives.

All Invitrogen products are warranted to perform according to specifications stated on the certificate of analysis. The Company will replace, free of charge, any product that does not meet those specifications. This warranty limits the Company's liability to only the price of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. The Company reserves the right to select the method(s) used to analyze a product unless the Company agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore the Company makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, report it to our Technical Support Representatives.

Life Technologies Corporation shall have no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

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, use of 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.

Limited Use Label License No. 5: Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For products that are subject to multiple limited use label licenses, the terms of the most restrictive limited use label license shall control. Life Technologies Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Life Technologies Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information about purchasing a license to use this product or the technology embedded in it for any use other than for research use please contact Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008; Phone (760) 603-7200 or e-mail: outlicensing@lifetech.com.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 19: Gateway® Cloning Products

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of Clonase™ purchased from Life Technologies Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Notwithstanding the preceding, any buyer who is employed in an academic or government institution may transfer materials made with this product to a third party who has a license from Life Technologies under the patents identified above to distribute such materials. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (i) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200.

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 39.

continued on next page

Purchaser Notification, continued

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

This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

Limited Use Label License No. 30: T7 Expression System

The composition and/or use of this product may be claimed in U.S. Patent No. 5,693,489 licensed to Life Technologies Corporation by Brookhaven Science Associates, LLC. The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy, and is the subject of patents and patent applications assigned to Brookhaven Science Associates, LLC (BSA,). By provisions of the Distribution License Agreement granted to Life Technologies covering said patents and patent applications, Life Technologies grants you a non-exclusive sub-license under patents assigned to BSA for the use of this technology, including the enclosed materials, based upon the following conditions: 1 – these materials are to be used for non-commercial research purposes only. A separate license under patents owned by BSA is required for any commercial use, including the use of these materials for research purposes or production purposes by any commercial entity. Information about commercial license may be obtained from The Office of Technology Transfer, Brookhaven National Laboratory, Bldg. 475D, P.O. Box 5000, Upton, New York 11973-5000. Phone (516) 344-7134. 2 - No materials that contain the cloned copy of the T7 gene 1, the gene for T7 RNA polymerase, may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this sub-license and agrees to be bound by its terms. This limitation applies to strains BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE, CE6, BL21-SI Competent Cells and any derivatives that are made of them. You may refuse this sub-license by returning this product unused in which case Life Technologies accept return of the product with a full refund. By keeping or using this product, you agree to be bound by the terms of this license.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 50: *rne131* Cells

Life Technologies Corporation ("Life Technologies") has an exclusive license to sell the *rne131* genotype to scientists for research or commercial evaluation purposes only, under the terms described below. Use of the *rne131* genotype by commercial entities for Commercial Purposes, as defined below, beyond evaluation requires the user to obtain a commercial license as detailed below. Before using the BL21 Star™ strain, please read the following license agreement. If you do not agree to be bound by its terms, contact Life Technologies within 10 days for authorization to return the unused BL21 Star™ product and to receive a full credit.

The *rne131* genotype is covered by one or more French patents or patent applications and corresponding foreign patents or patent applications owned by CNRS. Information about commercial licences may be obtained from: Centre National de la Recherche Scientifique (CNRS) , 3, rue Michel Ange, 75794 PARIS Cedex 16, France, REF: L00084, Contact: Dale.Roche@fist.fr.

Life Technologies grants you a non-exclusive license to use the enclosed BL21 Star™ strain for research or for commercial evaluation purposes only. The strain is being transferred to you in furtherance of, and reliance on, such license. You may not use the strain, or the materials contained therein, for any Commercial Purpose, as defined below, beyond a one-year evaluation without a license for such purpose from CNRS.

If you are a commercial entity, each of your laboratories is allowed a one-year evaluation (not free use) period, after which time this right automatically terminates. To use any portion of BL21 Star™ strain for a Commercial Purpose as defined below, commercial entities must obtain a commercial license from CNRS for each of their laboratories. Contact information for commercial entities purchasing a BL21 Star™ strain will be provided to CNRS, who may contact them during the evaluation period regarding their desire for a commercial license.

Commercial Purposes include: Any use of *rne131* in a Commercial Product; Any use of *rne131* in the manufacture of a Commercial Product; Any sale of *rne131* or products having used in a commercial process the *rne131* genotype.

Access to the BL21 Star™ strain must be limited solely to those officers, employees and students of your institution who need access to perform the above-described research or evaluation. You must inform each such officer, employee and student of the provisions of this license agreement and require them to agree to be bound by the provisions of this license agreement. You may not distribute the BL21 Star™ strain to others, even to those within your own institution. You may only transfer modified, altered, or original material from the cell line to a third party following written notification of, and written approval from, Life Technologies so that the recipient can be licensed. You may not assign, sub-license, rent, lease or otherwise transfer this license agreement or any of the rights or obligation there under, except as expressly permitted by Life Technologies and CNRS.

This license agreement is effective until terminated. You may terminate it at any time by destroying all BL21 Star™ products in your control. It will also terminate automatically if you fail to comply with the terms and conditions of the license agreement. You shall, upon termination of the license agreement, destroy all BL21 Star™ strains in your control, and so notify Life Technologies in writing.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 54: ULB ccdB Selection Technology

This product is the subject of one or more of U.S. Patent Numbers 5,910,438, 6,180,407, and 7,176,029 and corresponding foreign patents and is sold under license from the Université Libre de Bruxelles for research purposes only. ccdB selection technology is described in Bernard et al., "Positive Selection Vectors Using the F Plasmid ccdB Killer Gene" Gene 148 (1994) 71-74. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). For licensing information for use in other than research, please contact: Out Licensing, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008; Phone (760) 603-7200 or e-mail.

Limited Use Label License No. 167: Target Sequences for Synthetic Molecules

This product and/or its use is the subject of one or more of U.S. Patent Nos. 5,932,474, 6,008,378, 6,054,271, and 6,451,569 and foreign equivalents owned by and/or licensed to Life Technologies Corporation.

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.

References

Adams, S. R., Campbell, R. E., Gross, L. A., Martin, B. R., Walkup, G. K., Yao, Y., Llopis, J., and Tsien, R. Y. (2002). New Biarsenical Ligands and Tetracysteine Motifs for Protein Labeling in Vitro and in Vivo: Synthesis and Biological Applications. J. Am. Chem. Soc. *124*, 6063-6076.

Carrington, J. C., and Dougherty, W. G. (1988). A Viral Cleavage Site Cassette: Identification of Amino Acid Sequences Required for Tobacco Etch Virus Polyprotein Processing. Proc. Natl. Acad. Sci. USA *85*, 3391-3395.

Deutscher, M. P. (1990) Guide to Protein Purification. In *Methods in Enzymology*, Vol. 182. (J. N. Abelson and M. I. Simon, eds.) Academic Press, San Diego, CA.

Dougherty, W. G., Carrington, J. C., Cary, S. M., and Parks, T. D. (1988). Biochemical and Mutational Analysis of a Plant Virus Polyprotein Cleavage Site. EMBO J. *7*, 1281-1287.

Dubendorff, J. W., and Studier, F. W. (1991). Controlling Basal Expression in an Inducible T7 Expression System by Blocking the Target T7 Promoter with lac Repressor. J. Mol. Biol. *219*, 45-59.

Griffin, B. A., Adams, S. R., and Tsien, R. Y. (1998). Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells. Science *281*, 269-272.

Grunberg-Manago, M. (1999). Messenger RNA Stability and its Role in Control of Gene Expression in Bacteria and Phages. Annu. Rev. Genet. *33*, 193-227.

Kido, M., Yamanaka, K., Mitani, T., Niki, H., Ogura, T., and Hiraga, S. (1996). RNase E Polypeptides Lacking a Carboxyl-terminal Half Suppress a *mukB* mutation in *Escherichia coli*. J. Bacteriol. *178*, 3917-3925.

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949.

Lopez, P. J., Marchand, I., Joyce, S. A., and Dreyfus, M. (1999). The C-terminal Half of RNase E, Which Organizes the *Escherichia coli* Degradosome, Participates in mRNA Degradation but not rRNA Processing *in vivo*. Mol. Microbiol. *33*, 188-199.

Rosenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J., and Studier, F. W. (1987). Vectors for Selective Expression of Cloned DNAs by T7 RNA Polymerase. Gene *56*, 125-135.

Studier, F. W., and Moffatt, B. A. (1986). Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-Level Expression of Cloned Genes. J. Mol. Biol. *189*, 113-130.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990). Use of T7 RNA Polymerase to Direct Expression of Cloned Genes. Meth. Enzymol. *185*, 60-89.

Notes

invitrogen™
by life technologies™

Corporate Headquarters
5791 Van Allen Way
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech_support@invitrogen.com

For country-specific contact information, visit our web site at **www.invitrogen.com**