invitrogen™
by life technologies™

Champion™ pET104 BioEase™ Gateway® Expression System

For cloning and expression of biotinylated fusion proteins in *E. coli*

Catalog no. K104-01

Rev. Date: 7 July 2010
Manual part no. 25-0472

MAN0000262

User Manual

Table of Contents

Kit Contents and Storage

Shipping/Storage

The Champion™ pET104 BioEase™ Gateway® Expression System is shipped on dry ice and contains the following items. Upon receipt, store as detailed below.

Item	Storage
Vectors	-20°C
BL21 Star™(DE3) One Shot® Chemically Competent *E. coli*	-80°C

Vectors

The following vectors are supplied in the Champion™ pET104 BioEase™ Gateway® Expression System. **Store at -20°C.**

Vector	Composition	Amount
pET104.1-DEST vector	Lyophilized in TE Buffer, pH 8.0	6 µg
pET104.1/GW/*lacZ* control vector	Lyophilized in TE Buffer, pH 8.0	10 µg

Important

If you have previously purchased the p Champion™ pET104 BioEase™ Gateway® Expression System, note that the pET104-DEST vector and the pET104/GW/*lac*Z control vector have been replaced with pET104.1-DEST and pET104.1/GW/*lac*Z, respectively. The new pET104.1 vectors include a T7 gene 10 leader sequence upstream of the BioEase™ tag for increased expression of the biotinylated recombinant protein.

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. Transformation efficiency is 1 x 10^8 cfu/µg DNA. **Store 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	10 µl

continued on next page

Kit Contents and Storage, continued

Genotype of BL21 Star™ (DE3)

Use this 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 4).

Additional Products

Some of the reagents supplied in the Champion™ pET104 BioEase™ Gateway® Expression System and other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 20).

Item	Amount	Catalog no.
Gateway® LR Clonase™ Enzyme Mix	20 reactions	11791-019
	100 reactions	11791-043
Library Efficiency® DB3.1™ Competent Cells	1 ml (5 x 0.2 ml)	11782-018
One Shot® TOP10 Chemically Competent *E. coli*	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
BL21-AI™ One Shot® Chemically Competent *E. coli*	20 x 50 µl	C6070-03
Ampicillin	5 g	Q100-16
Carbenicillin	5 g	10177-012
IPTG (isopropylthio-β-galactoside)	1 g	15529-019
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
EKMax™	250 units	E180-01
β-gal Antiserum	50 µl*	R901-25
Streptavidin-HRP Conjugate	50 µl*	SA100-01
Streptavidin-AP Conjugate	125 µl*	SA100-03
Streptavidin Agarose	5 ml packed bed	SA100-04

Introduction

Overview

Introduction

The Champion™ pET104 BioEase™ Gateway® Expression System is designed to allow high-level, inducible expression of biotinylated recombinant fusion proteins in *E. coli* using the pET system. Biotinylated recombinant protein may then be easily detected or immobilized to a solid support for other downstream applications. The main component of the System is the pET104.1-DEST vector into which the gene of interest is inserted through Gateway® cloning. For more information about the pET104.1-DEST vector, see below. For more information about the Gateway® Technology, see the next page.

The pET Expression System

The pET system was 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 T7-regulated expression, see page 3.

Features of the Vector

The pET104.1-DEST vector contains 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)
- Ribosome binding site and T7 gene 10 leader sequence for efficient translation of the gene of interest in *E. coli*
- BioEase™ tag to allow biotinylation of the recombinant protein of interest for easy detection or use in other applications (see the next page for more information)
- Two recombination sites, *att*R1 and *att*R2, downstream of the CMV promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- *ccd*B gene located between the *att*R sites for negative selection
- *lacI* gene encoding the lac repressor to reduce basal transcription from the T7*lac* promoter in the pET104.1-DEST vector and from the *lacUV5* promoter in the *E. coli* host chromosome
- Ampicillin resistance gene for selection in *E. coli*
- pBR322 origin for low-copy replication and maintenance of the plasmid in *E. coli*

The control plasmid, pET104.1/GW/*lacZ*, is included for use as a positive control for expression in *E. coli*.

continued on next page

Overview, continued

BioEase™ Tag

The BioEase™ tag is a 72 amino acid peptide derived from the C-terminus (amino acids 524-595) of the *Klebsiella pneumoniae* oxalacetate decarboxylase α subunit (Schwarz *et al.*, 1988). Biotin is covalently attached to the oxalacetate decarboxylase α subunit and peptide sequencing has identified a single biotin binding site at lysine 561 of the protein (Schwarz *et al.*, 1988). When fused to a heterologous protein, the BioEase™ tag is both necessary and sufficient to facilitate *in vivo* biotinylation of the recombinant protein of interest (see the diagram on page 6 for the location of the biotin binding site). The entire 72 amino acid domain is required for recognition by the cellular biotinylation enzymes. For more information about the cellular biotinylation enzymes and the mechanism of biotinylation, refer to the review by Chapman-Smith and Cronan, 1999.

The Gateway® Technology

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 the Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone.
2. Perform an LR recombination reaction between the entry clone and a Gateway® destination vector (*e.g.* pET104.1-DEST). Transform *E. coli* and select for an expression clone.
3. Purify plasmid and transform your expression construct into BL21 Star™(DE3) *E. coli*. Induce expression of your recombinant protein with IPTG.

For more detailed information about the Gateway® Technology, generating an entry clone, and performing the LR recombination reaction, refer to the Gateway® Technology manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 20).

T7-Regulated Expression

The Basis of T7-Regulated Expression

The pET expression system uses elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*. In the pET104.1-DEST vector, 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™ pET104 BioEase™ Gateway® Expression System, T7 RNA polymerase is supplied by the BL21 Star™(DE3) host *E. coli* strain in a regulated manner (see below).

Regulating Expression of T7 RNA Polymerase

The BL21 Star™(DE3) *E. coli* strain is specifically included in the kit for expression of T7-regulated genes. This strain carries the DE3 bacteriophage lambda lysogen, which 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-thiogalacto-side (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 the next page.

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 pET104.1-DEST vector contains 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.

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 the next page and page 13.

BL21 Star™ *E. coli* Strains

BL21 Star™ Strains

The BL21 Star™(DE3) *E. coli* strain is included in the kit for use as a host for expression. Other BL21 Star™ strains are also available from Invitrogen (see below). The BL21 Star™(DE3) strains contain the following features to facilitate high-level expression in *E. coli*:

- The λDE3 lysogen to allow high-level expression of T7-regulated genes
- The *rne131* mutation to enhance recombinant protein yields

Important: Once you have generated your expression clone, **do not** use BL21 Star™(DE3) *E. coli* to propagate or maintain your expression construct. Use an *end*A, *rec*A strain (*e.g.* TOP10) instead.

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 vi 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 Service (see page 20).

Note

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.

Methods

Using pET104.1-DEST

Important

The pET104.1-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual has previously recommended using a linearized destination vector for more efficient recombination, further testing at Invitrogen has found that linearization of pET104.1-DEST is **not** required to obtain optimal results for any downstream application.

Resuspending the Vector

pET104.1-DEST is supplied as 6 µg of plasmid, lyophilized in TE, pH 8.0. To use, simply resuspend the destination plasmid in 40 µl of sterile water to a final concentration of 150 ng/µl.

Propagating the Vector

If you wish to propagate and maintain the pET104.1-DEST vector, we recommend using Library Efficiency® DB3.1™ Competent Cells from Invitrogen (Catalog no. 11782-018) for transformation. The DB3.1™ *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 as these strains are sensitive to CcdB effects.

Entry Clone

To recombine your gene of interest into pET104.1-DEST, you should have an entry clone containing your gene of interest. Refer to the Gateway® Technology manual for details on choosing a Gateway ™ entry vector and constructing an entry clone. The Gateway® Technology manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 20).

Points to Consider Before Recombining into pET104.1-DEST

pET104.1-DEST is an N-terminal fusion vector and contains an ATG initiation codon. A Shine-Dalgarno ribosome binding site (RBS) is included upstream of the ATG in the N-terminal tag to ensure optimal spacing for proper translation initiation. Your gene of interest in the entry clone must:

- Be in frame with the N-terminal gene 10 leader sequence and BioEase™ tag after recombination
- Contain a stop codon

Refer to the diagram of the recombination region of pET104.1-DEST on the next page for more information.

continued on next page

Using pET104.1-DEST, continued

Recombining Your Gene of Interest

Each entry clone contains *att*L sites flanking the gene of interest. Genes in an entry clone are transferred to the destination vector backbone by mixing the DNAs with the Gateway® LR Clonase™ Enzyme Mix (see page vi for ordering information). The resulting LR recombination reaction is then transformed into *E. coli* (*e.g.* TOP10 or DH5α™-T1^R) and the expression clone selected using ampicillin. Recombination between the *att*R sites on the destination vector and the *att*L sites on the entry clone replaces the chloramphenicol (Cm^R) gene and the *ccd*B gene with the gene of interest and results in the formation of *att*B sites in the expression clone.

Follow the instructions in the Gateway® Technology manual to set up the LR recombination reaction, transform *E. coli*, and select for the expression clone.

Recombination Region of pET104.1-DEST

The recombination region of the expression clone resulting from pET104.1-DEST x entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pET104.1-DEST vector by recombination. Non-shaded regions are derived from the pET104.1-DEST vector.
- Bases 604 and 2266 of the pET104.1-DEST sequence are marked.
- The biotin binding site is labeled with a *.

continued on next page

Using pET104.1-DEST, continued

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 should not grow in the presence of chloramphenicol.

Sequencing

You may sequence your expression construct to confirm that your gene of interest is in frame with the BioEase™ tag, if desired. We recommend using the priming sites indicated in the diagram on the previous page (BioEase™ forward and T7 reverse) to help you sequence your insert. For your convenience, Invitrogen offers a custom primer synthesis service. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 20).

General Guidelines for Expression

Introduction

BL21 Star™(DE3) One Shot® *E. coli* are included with the Champion™ pET104 BioEase™ Gateway® Expression System for use as the host for expression. You will need purified plasmid DNA of your pET104.1-DEST 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. The pET104.1/GW/*lacZ* vector is included in the kit for use as a positive expression control (see below).

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). **Do not use this strain to propagate and maintain your plasmid. Use a general cloning strain (*e.g.* TOP10 or DH5α) instead.** 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.

Positive Control

The pET104.1/GW/*lacZ* vector is supplied with the kit for use as a positive expression control, and was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pET104.1-DEST. To use pET104.1/GW/*lacZ*, transform 10 ng of the plasmid into *E. coli* (*e.g.* TOP10 or DH5α) and select for transformants using 50-100 µg/ml ampicillin.

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.

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.

continued on next page

General Guidelines for Expression, continued

Choosing a Selection Agent

For most purposes, ampicillin works well for selection of transformants and expression experiments. However, if you find that your expression level is low, you may want to use carbenicillin instead. The resistance gene for ampicillin encodes a protein called β-lactamase. This protein 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 pET104.1-DEST expression 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 pET104.1-DEST 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.

Expressing Your Recombinant Protein

Materials to Have on Hand

Be sure to have the following solutions and equipment on hand before starting the experiment:

- SOB or LB containing 100 µg/ml ampicillin (plus 1% glucose, if desired)
- 37°C incubator (shaking and nonshaking)
- 42°C water bath
- IPTG (Invitrogen, Catalog no. 15529-019; prepare a 1 M stock solution in sterile water)
- Lysis Buffer (see page 19 for a recipe)
- Liquid nitrogen
- 1X and 2X SDS-PAGE sample buffer (see page 19 for a recipe)
- Reagents and apparatus for SDS-PAGE gel (see page 12)
- Boiling water bath
- Sterile water

Transforming BL21 Star™(DE3) One Shot® Cells

To transform your construct or the positive control (10 ng each) into BL21 Star™(DE3) One Shot® cells, follow the instructions below. You will need one vial of cells per transformation.

Note that 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 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 100 µg/ml ampicillin or 50 µg/ml carbenicillin (and 1% glucose, if desired).
9. Grow overnight at 37°C with shaking. Proceed to **Pilot Expression**, next page.

continued on next page

Expressing Your Recombinant Protein, continued

Pilot Expression

1. Inoculate 10 ml of LB containing 100 µg/ml ampicillin or 50 µg/ml carbenicillin with 500 µl of the overnight culture from Step 8, previous page.
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 the next section.

Preparing Samples

Before starting, prepare SDS-PAGE gels or use one of the pre-cast polyacrylamide gels available from Invitrogen (see the next page) to analyze all the samples you collected.

Note: If you wish to analyze your samples for soluble protein, see the next section.

1. When all the samples have been collected from Steps 5 and 7, above, resuspend each cell pellet in 80 µl of 1X SDS-PAGE sample buffer.
2. Boil 5 minutes and centrifuge briefly.
3. Load 5-10 µl of each sample on an SDS-PAGE gel and electrophorese. Save your samples by storing them at -20°C.

Preparing Samples for Soluble/Insoluble Protein

1. Thaw and resuspend each pellet in 500 µl of Lysis Buffer (see **Recipes**, page 19).
2. Freeze sample in dry ice or liquid nitrogen and then thaw at 42°C. Repeat 2 to 3 times.

   Note: To facilitate lysis, you may need to add lysozyme or sonicate the cells.
3. Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at +4°C to pellet insoluble proteins. Transfer supernatant to a fresh tube and store on ice.
4. Mix together equivalent amounts of supernatant and 2X SDS-PAGE sample buffer and boil for 5 minutes.
5. Add 500 µl of 1X SDS-PAGE sample buffer to the pellets from Step 3 and boil 5 minutes.
6. Load 10 µl of the supernatant sample and 5 µl of the pellet sample onto an SDS-PAGE gel and electrophorese.

continued on next page

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 20).

Analyzing Samples

To determine the success of your expression experiment, you may want to perform the following types of analyses:

1. Stain the polyacrylamide gel with Coomassie blue and look for a band of increasing intensity in the expected size range for the recombinant protein. Use the uninduced culture as a negative control.
2. Perform a western blot to confirm that the overexpressed band is your desired protein (see below).
3. Use the positive control to confirm that growth and induction were performed properly. The size of the β-galactosidase fusion protein expressed from pET104.1/GW/*lacZ* is 127 kDa.

   Note: β-galactosidase Antiserum is available from Invitrogen (Catalog no. R901-25) to detect β-galactosidase fusion proteins by western blot.

Detecting Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use:

- Streptavidin-HRP or Streptavidin-AP conjugates available from Invitrogen (see page vi for ordering information)
- An antibody to your protein of interest

Note: The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of streptavidin conjugates by colorimetric or chemiluminescent methods. For more information, refer to our Web site or contact Technical Service (see page 20).

Note

The N-terminal peptide containing the gene 10 leader sequence, BioEase™ tag, and EK recognition site will add approximately 11 kDa to the size of your protein. Be sure to account for any additional amino acids between the fusion tag and the start of your protein.

The Next Step

If you are satisfied with expression of your gene of interest, proceed to **Purifying the PCR Product**, page 15.

If you have trouble expressing your protein or wish to optimize expression, see the next page.

Troubleshooting Expression

Introduction

Use the information provided below to troubleshoot your expression experiment.

No Expression

- Sequence your construct and make sure it is in frame with the N-terminal peptide.
- If the positive control expressed, but you don't see any expression from your construct on a Coomassie-stained gel, re-run your samples on an SDS-PAGE gel and perform a western blot. For detection, use an antibody to your protein or one of the streptavidin conjugates available from Invitrogen (*i.e.* strept-avidin-HRP or streptavidin-AP).

Low Expression Due to Plasmid Instability

If you are using ampicillin for selection in your expression experiments and see low levels of expression, you may be experiencing plasmid instability due to the absence of selective conditions. This occurs as the ampicillin is destroyed by β-lactamase or hydrolyzed under the acidic media conditions generated by bacterial metabolism. You may substitute carbenicillin for ampicillin in your transformation and expression experiments (see page 9 for more information).

Low Expression Due to Toxicity

If your protein expresses, but the levels are low, it is possible that expression of your gene is toxic to *E. coli*. This is the most common reason for poor expression. Evidence of toxicity includes the following:

- Slow growth relative to the control
- Loss of plasmid

To reduce the toxicity of your gene, basal levels of T7 RNA polymerase must be reduced. There are a number of methods to reduce basal level expression of T7 RNA polymerase:

- Transform your expression construct into a pLysS-containing strain (*e.g.* BL21 Star™(DE3)pLysS). Substantial levels of T7 lysozyme are produced; however, growth rate may be reduced.
- Transform your expression construct into an *E. coli* strain in which expression of T7 RNA polymerase is tightly regulated (*e.g.* BL21-AI™). The BL21-AI™ strain contains a chromosomal insertion of the gene encoding T7 RNA polymerase into the *ara*B locus of the *araBAD* operon, allowing expression of T7 RNA polymerase to be tightly regulated by L-arabinose. For more information, refer to out Web site (www.invitrogen.com) or contact Technical Service (see page 20).
- Infect TOP10F′ (or any other suitable host strain) with M13 or lambda phage expressing T7 RNA polymerase. In this case, T7 RNA polymerase is not present in the cell until infection. This method requires growth and maintenance of phage stocks.

continued on next page

Troubleshooting Expression, continued

Tip

Many researchers use the leakiness of the T7 system to their advantage. In some cases, basal-level, constitutive expression produces sufficient protein for analysis and purification, particularly if the host strain containing the construct of interest is grown at room temperature. We recommend growing the strain for 24-48 hours at room temperature to produce sufficient protein. Using this method to express your construct can result in substantial production of soluble protein.

Note: To optimize production of soluble protein using the above method, use BL21 Star™(DE3) cells, which do not express T7 lysozyme.

Obtaining Other BL21 Strains

BL21 Star™(DE3)pLysS One Shot® chemically competent *E. coli* and BL21-AI™ One Shot® chemically competent *E. coli* are available from Invitrogen (see page vi for ordering information). Visit our Web site (www.invitrogen.com) or contact Technical Service (see page 20) for more information.

Infecting with Phage

In about 5% of all cases, there will be some genes that are so toxic that they require infection with phage expressing T7 RNA polymerase (Tabor, 1990). You will need to use an *E. coli* host strain that contains the F′ episome (*e.g.* TOP10F′ or DH5αF′). Remember that the BL21 Star™(DE3) and BL21 Star™(DE3)pLysS strains should **not** be used in this situation. A protocol for infecting with M13 phage expressing T7 polymerase can be found in *Current Protocols in Molecular Biology*, pp. 16.2.1 to 16.2.11 (Ausubel *et al.*, 1994). Information for infecting *E. coli* with lambda phage expressing T7 polymerase is also available (Studier *et al.*, 1990). Contact Technical Service for more information (see page 20).

Purifying the Recombinant Protein

Introduction

The presence of the N-terminal BioEase™ tag in pET104.1-DEST allows your recombinant fusion protein to be biotinylated. Once biotinylated, you may take advantage of the strong association between biotin and avidin (and its analogs including streptavidin) to purify your recombinant fusion protein using streptavidin agarose-conjugated beads available from Invitrogen (see page vi for ordering information). General guidelines are provided below. Other streptavidin conjugates are suitable.

Streptavidin-Agarose Beads

The streptavidin-agarose resin available from Invitrogen can be used for affinity purification of recombinant fusion proteins containing the BioEase™ tag, and is constructed by covalently linking streptavidin to cross-linked agarose beads via a 15-atom hydrophilic spacer arm specifically designed to reduce non-specific binding and ensure optimal binding of biotinylated molecules. Streptavidin is bound to a final concentration of 2-3 mg streptavidin per ml of packed resin.

General Guidelines for Purification

Recombinant fusion proteins may be purified with streptavidin-agarose under native or denaturing conditions. We generally scale up the expression to a 50 ml volume and use the soluble fraction of the cell lysate in a native buffer for initial experiments.

To purify your fusion protein using streptavidin-agarose, refer to the Streptavidin-Agarose manual, which is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 20).

Note: Since recombinant proteins expressed in *E. coli* are often insoluble, using mildly denaturing conditions can facilitate increased capture and recovery of expressed proteins.

Important

Because of the extremely strong interaction between streptavidin and biotin, the recombinant fusion protein must be eluted from the streptavidin-agarose resin by cleavage using an enterokinase. We generally use EKMax™ available from Invitrogen (see below) and incubate overnight. For more information, refer to the Streptavidin-Agarose manual.

Removing the N-terminal BioEase™ Tag with Enterokinase

pET104.1-DEST contains an enterokinase (EK) recognition site to allow removal of the BioEase™ tag from your recombinant fusion protein, if desired. Note that after digestion with enterokinase, 11 amino acids will remain at the N-terminus of your protein (see diagram on page 6).

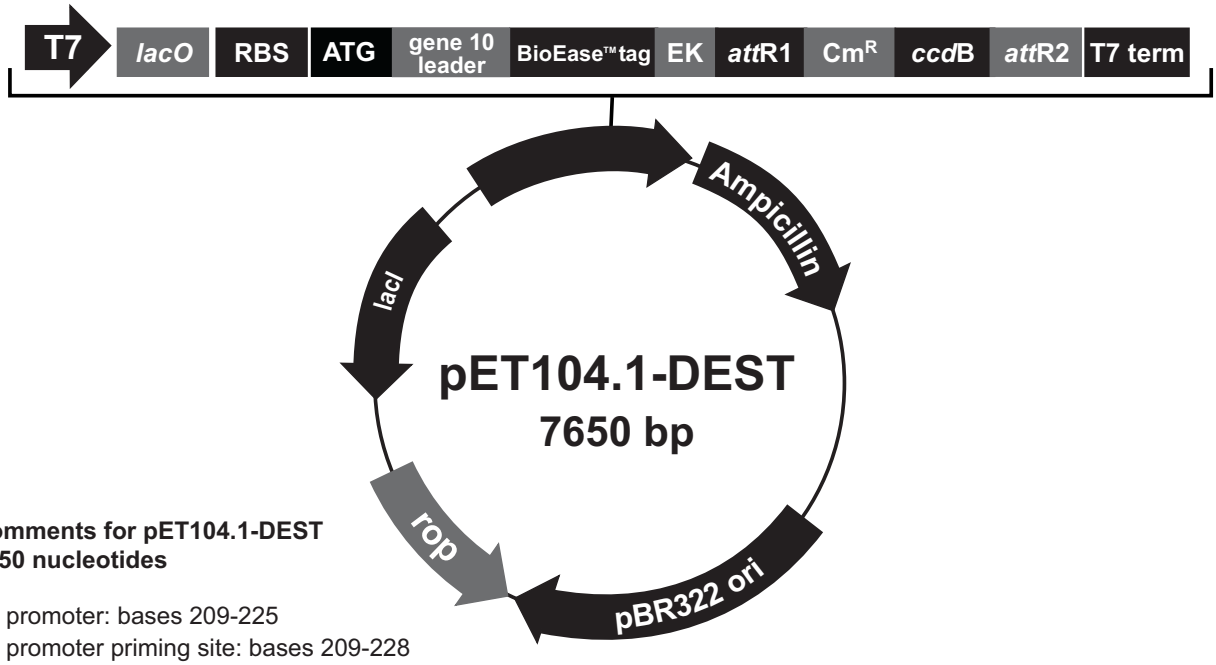
A recombinant preparation of the catalytic subunit of bovine enterokinase (EKMax™) available from Invitrogen (Catalog no. E180-01) to remove the BioEase™ tag from your recombinant fusion protein. Instructions for digestion are included with the product. For more information, refer to our Web site or contact Technical Service (see page 20).

Appendix

Map and Features of pET104.1-DEST

Map of pET104.1-DEST

The map below shows the elements of pET104.1-DEST. DNA from the entry clone replaces the region between bases 604 and 2266. The complete sequence for pET104.1-DEST is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 20).



Comments for pET104.1-DEST
7650 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
T7 gene 10 leader sequence: bases 297-329
BioEase™ tag: bases 336-551
BioEase™ forward priming site: bases 529-546
Enterokinase (EK) recognition site: bases 573-587
*att*R1: bases 597-721
Chloramphenicol resistance gene: bases 830-1489
*ccd*B gene: bases 1810-2115
*att*R2: bases 2156-2280
T7 reverse priming site: bases 2345-2364
T7 transcription termination region: bases 2306-2434
bla promoter: bases 2739-2837
Ampicillin (*bla*) resistance gene: bases 2838-3698
pBR322 origin: bases 3843-4516
ROP ORF: bases 4887-5078 (complementary strand)
lacI ORF: bases 6390-7481 (complementary strand)

continued on next page

Map and Features of pET104.1-DEST, continued

Features of pET104.1-DEST

The pET104.1-DEST vector (7650 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.
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 initiation ATG in the N-terminal tag for efficient translation of PCR product.
T7 gene 10 leader sequence	Sequence from bacteriophage T7 gene 10 that optimizes translation of the gene of interest
BioEase™ tag	Allows biotinylation of recombinant fusion protein (Schwarz *et al.*, 1988).
BioEase™ forward priming site	Allows sequencing in the sense orientation.
Enterokinase (EK) recognition site (Asp-Asp-Asp-Asp-Lys)	Allows removal of the BioEase™ tag from your recombinant fusion protein using an enterokinase such as EKMax™ (Catalog no. E180-01).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that allow recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	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.

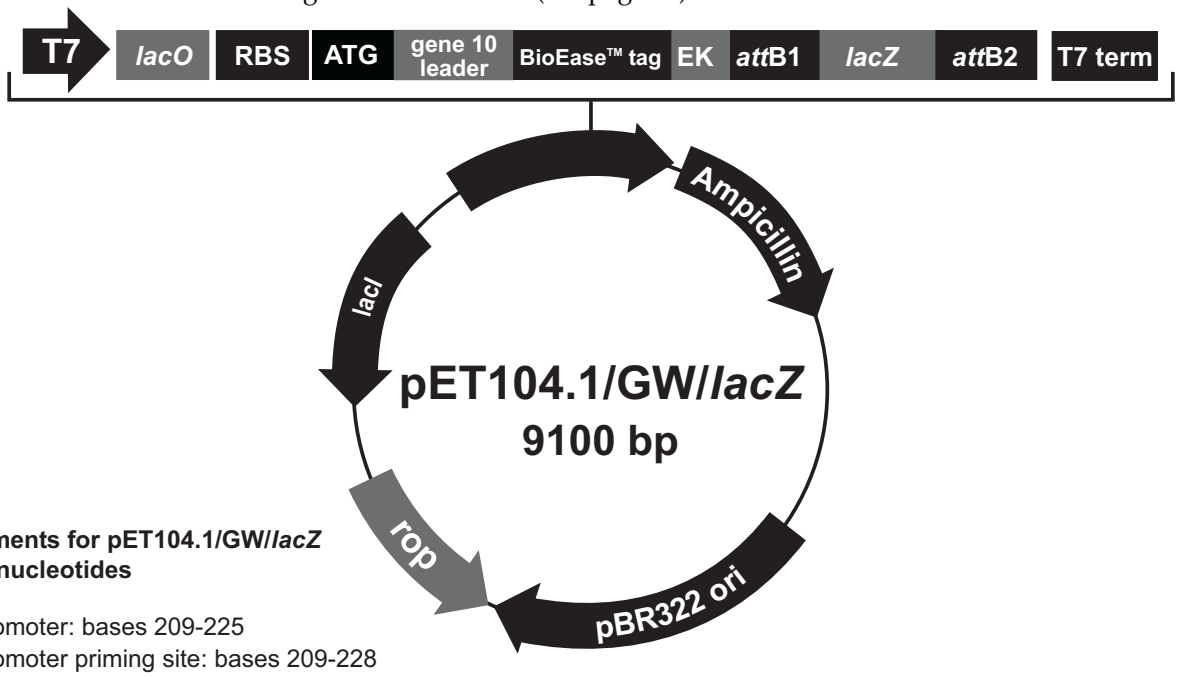
Map of pET104.1/GW/*lacZ*

Description

pET104.1/GW/*lacZ* is a 9100 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pET104.1-DEST. β-galactosidase is expressed as an N-terminal fusion protein with a molecular weight of approximately 127 kDa.

Map of pET104.1/GW/*lacZ*

The map below shows the elements of pET104.1/GW/*lacZ*. The complete sequence of the vector is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 20).



Comments for pET104.1/GW/*lacZ*
9100 nucleotides

T7 promoter: bases 209-225
T7 promoter priming site: bases 209-228
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
T7 gene 10 leader sequence: bases 297-329
BioEase™ tag: bases 336-551
BioEase™ forward priming site: bases 529-546
Enterokinase (EK) recognition site: bases 573-587
*att*B1: bases 597-621
lacZ gene: bases 627-3701
*att*B2: bases 3706-3730
T7 reverse priming site: bases 3795-3814
T7 transcription termination region: bases 3756-3884
bla promoter: bases 4189-4287
Ampicillin (*bla*) resistance gene: bases 4288-5148
pBR322 origin: bases 5293-5966
ROP ORF: bases 6337-6528 (complementary strand)
lacI ORF: bases 7840-8931 (complementary strand)

Recipes

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.

2X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2.0 ml
β-mercaptoethanol	0.4 ml
Bromophenol Blue	0.02 g
SDS	0.4 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

Technical Service

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 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.

continued on next page

Technical Service, continued

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

Introduction

Use of the Champion™ pET104 BioEase™ Gateway® Expression System is covered under a number of different licenses including those detailed below.

Information for European Customers

The BL21 Star™(DE3) *E. coli* strain is genetically modified and carries the bacteriophage λ DE3 lysogen. 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.

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.

continued on next page

Purchaser Notification, continued

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.

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.

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 26.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 50: *rne*131 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.

Limited Use Label License No. 142: Gateway® BioEase™ Technology

No license is conveyed to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose.

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

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Chapman-Smith, A., and J.E. Cronan, J. (1999). Molecular Biology of Biotin Attachment to Proteins. J. Nutr. *129*, 477S-484S.

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.

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.

Schwarz, E., Oesterhelt, D., Reinke, H., Beyreuther, K., and Dimroth, P. (1988). The Sodium Ion Translocating Oxalacetate Decarboxylase of *Klebsiella pneumoniae*. J. Biol. Chem. *263*, 9640-9645.

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.

Tabor, S. (1990) Expression Using the T7 RNA Polymerase/Promoter System. In *Current Protocols in Molecular Biology*, K. Struhl, ed. (New York: Greene Publishing Associates and Wiley-Interscience), pp. 16.2.1-16.2.11.

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**