invitrogen™
by life technologies™

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

Cell-free protein synthesis system for expression of recombinant protein

Catalog nos. K9901-00, K9900-96, K9900-97

Rev. Date: 27 September 2011
Manual part no. 25-0890

MAN0000554

User Manual

Table of Contents

Kit Contents and Storage

Important

Kits K9900-96 and K9900-97 have been reconfigured. New reagents are supplied and quantities of reagents are different. If you have used these products in the past, discard old versions of this manual and use the instructions provided in this manual.

Types of Kits

This manual is supplied with the following products.

Product	Amount*	Catalog no.
Expressway™ Maxi Cell-Free *E. coli* Expression System with pEXP5-NT/TOPO® and pEXP5-CT/TOPO®	100 rxns	K9900-96
Expressway™ Maxi Cell-Free *E. coli* Expression System	100 rxns	K9900-97
Expressway™ Mini Cell-Free *E. coli* Expression System	20 rxns	K9901-00

* Amount based on 100 µl reaction size.

Kit Components

The Expressway™ Cell-Free *E. coli* Expression System kits include the following components.

Component	Catalog no.		
	K9900-96	**K9900-97**	**K9901-00**
Expressway™ Maxi Expression Module	√	√	
Expressway™ Mini Expression Module			√
Expressway™ Maxi Amino Acid Module	√	√	
Expressway™ Mini Amino Acid Module			√
Expressway™ Control Vector	√	√	√
pEXP5-NT/TOPO® TA Expression Kit	√		
pEXP5-CT/TOPO® TA Expression Kit	√		

For a detailed description of the contents of the Expressway™ Modules, see the next page. For a detailed description of the contents of the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits and how to use the reagents supplied, see the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual.

Kit Contents and Storage, continued

Shipping/Storage

The Expressway™ Cell-Free *E. coli* Expression System kits are shipped as described below. Upon receipt, store as described below.

Box	Component	Shipping	Storage
1	Expressway™ Mini or Maxi Expression Module	Dry ice	-80ºC
2	Expressway™ Mini or Maxi Amino Acids Module	Dry ice	-20ºC
3-5	pEXP5-TOPO® TA Expression Kit: **(Catalog no. K9900-96 only)** pEXP5-NT/TOPO® Reagents pEXP5-CT/TOPO® Reagents: One Shot® TOP10 Chemically Competent *E. coli*	 Dry ice Dry ice Dry ice	 -20°C -20°C -80°C

Expressway™ Mini Expression Module

The following reagents are included in the Expressway™ Mini Expression Module. Note that the Expressway™ 2.5X IVPS *E. coli* Reaction Buffer (-A.A.) does not contain amino acids.

Store the entire box at -80°C or store individual components as listed below.

Item	Amount	Storage
E. coli slyD$^-$ Extract	400 µl	-80°C
2.5X IVPS *E. coli* Reaction Buffer (-A.A.)	400 µl	-80°C
2X IVPS Feed Buffer	500 µl	-80ºC
T7 Enzyme Mix	20 µl	-80°C -20°C after initial use
DNase/RNase-Free Distilled Water	1.75 ml	-20°C or -80°C

Expressway™ Maxi Expression Module

The following reagents are included in the Expressway™ Maxi Expression Module. Note that the Expressway™ 2.5X IVPS *E. coli* Reaction Buffer (-A.A.) does not contain amino acids.

Store the entire box at -80°C or store individual components as listed below.

Item	Amount	Storage
E. coli slyD$^-$ Extract	5 x 400 µl	-80°C
2.5X IVPS *E. coli* Reaction Buffer (-A.A.)	5 x 400 µl	-80°C
2X IVPS Feed Buffer	5 x 500 µl	-80ºC
T7 Enzyme Mix	100 µl	-80°C -20°C after initial use
DNase/RNase-Free Distilled Water	5 x 1.75 ml	-20°C or -80°C

Continued on next page

Kit Contents and Storage, continued

Expressway™ Mini Amino Acids Module

The following reagents are included in the Expressway™ Mini Amino Acids Module. **Store at -20°C.**

Item	Composition	Amount
Amino Acid Mix (-Methionine)	Contains all amino acids (50 mM) except for methionine in 50 mM HEPES, pH 11	160 µl
75 mM Methionine	75 mM in 50 mM HEPES, pH 7.5, 4 mM DTT	120 µl

Expressway™ Maxi Amino Acids Module

The following reagents are included in the Expressway™ Maxi Amino Acids Module. **Store at -20°C.**

Item	Composition	Amount
Amino Acid Mix (-Methionine)	Contains all amino acids (50 mM) except for methionine in 50 mM HEPES, pH 11	800 µl
75 mM Methionine	75 mM in 50 mM HEPES, pH 7.5, 4 mM DTT	600 µl

Expressway™ Control Vector

The pEXP5-NT/CALML3 control vector (10 µg) is included in the kit for use as a positive control and allows expression of an N-terminally-tagged human calmodulin-like 3 (CALML3) protein from pEXP5-NT/TOPO®. The vector is supplied as 20 µl at 0.5 µg/µl in TE buffer, pH 8.0. **Store at -20°C.**

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

The Expressway™ Maxi *E. coli* Expression System with pEXP5-NT/TOPO® and pEXP5-CT/TOPO® (Catalog no. K9900-96 only) includes the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits (Boxes 3-5) to facilitate TOPO® Cloning-based generation of plasmid templates to express your gene of interest with the Expressway™ Maxi Expression System. Each kit contains:

- pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA reagents
- One Shot® TOP10 Chemically Competent *E. coli*

Refer to the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual for a detailed description of the reagents provided with the kit and instructions to produce an expression construct.

Additional Products

Accessory Products

Some of the reagents supplied in the Expressway™ Cell-Free *E. coli* Expression System as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below. For more information, go to www.invitrogen.com or contact Technical Service (page 35).

Product	Quantity	Catalog no.
pEXP5-NT/TOPO® TA Expression Kit	10 reactions	V960-05
pEXP5-CT/TOPO® TA Expression Kit	10 reactions	V960-06
pEXP1-DEST Vector	1 kit	V960-01
pEXP2-DEST Vector	1 kit	V960-02
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
DNase/RNase-Free Distilled Water	500 ml	10977-015
RNase AWAY™	250 ml	10328-011
Ampicillin	20 ml (10 mg/ml)	11593-019
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
PureLink™ PCR Purification Kit	50 reactions	K3100-01
SimplyBlue™ SafeStain	1 L	LC6060
BenchMark™ Protein Ladder	2 x 250 µl	10747-012

Continued on next page

Additional Products, continued

Products to Detect Recombinant Fusion Protein

If you are expressing your recombinant protein from pEXP5-NT/TOPO®, pEXP5-CT/TOPO®, pEXP1-DEST or pEXP2-DEST, you may detect expression of your recombinant fusion protein using an antibody to the appropriate epitope. The table below describes the products available from Invitrogen for detection of fusion proteins expressed from these vectors. The amount of antibody supplied is sufficient for 25 western blots.

Note: To detect the CALML3 fusion protein from the pEXP5-NT/CALML3 control plasmid, use one of the Anti-HisG antibodies.

For use with	Product	Epitope	Catalog no.
pEXP5-NT/TOPO®, pEXP5-CT/TOPO®, pEXP1-DEST pEXP2-DEST	Anti-HisG Antibody Anti-HisG-HRP Antibody Anti-HisG-AP Antibody	Detects the N-terminal polyhistidine (6xHis) tag followed by glycine: HHHHHHG	R940-25 R941-25 R942-25
	Anti-His (C-term) Antibody Anti-His (C-term)-HRP Antibody Anti-His (C-term)-AP Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25 R931-25 R932-25
	Penta-His™ mouse IgG1 monoclonal Antibody	Detects both N and C-terminal polyhistidine (6xHis) tag	P21315
pEXP1-DEST	Anti-Xpress™ Antibody Anti-Xpress™-HRP Antibody	Detects the 8 amino acid Xpress™ epitope: DLYDDDDK	R910-25 R911-25
pEXP2-DEST	Anti-V5 Antibody Anti-V5-HRP Antibody Anti-V5-AP Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus SV5 (Southern *et al.*, 1991): GKPIPNPLLGLDST	R960-25 R961-25 R962-25

Continued on next page

Additional Products, continued

Products to Purify Recombinant Fusion Protein

If you have expressed your protein of interest in frame with the N- or C-terminal polyhistidine (6xHis) tag, you may use a nickel-charged agarose resin such as ProBond™ or Ni-NTA to purify your recombinant fusion protein. See the table below for ordering information.

Product	Quantity	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-chelating Resin	50 ml	R801-01
	150 ml	R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
Purification Columns (10 ml polypropylene columns)	50	R640-50
AcTEV™ Protease (pEXP5-NT/TOPO® only)	1,000 units	12575-015

Expressway™ Kits

Other Expressway™ Cell-Free *E. coli* Expression System Modules suitable for Lumio™ detection of protein and production of proteins for NMR analysis are available separately from Invitrogen. Ordering information is provided below. For more information, go to www.invitrogen.com or contact Technical Service (page 35).

Product	Quantity	Catalog no.
Expressway™ N-terminal Lumio™ Expression Kit	1 kit	K9900-70
Expressway™ C-terminal Lumio™ Expression Kit	1 kit	K9900-90
pEXP3-DEST Vector Kit	1 kit	V960-03
pEXP4-DEST Vector Kit	1 kit	V960-04

Introduction

Overview

Introduction

The Expressway™ Cell-Free *E. coli* Expression System is designed for *in vitro* transcription and translation of target DNA to protein in a single reaction. This flexible system allows production of recombinant protein of interest from an expression construct in as little as 3 hours. Once purified, the resulting recombinant protein is suitable for use in other downstream applications including biochemical, physical, and structural characterization.

The System is available in two formats: Expressway™ Maxi (scalable from 20-100 reactions) and Expressway™ Mini (20 reactions).

Applications

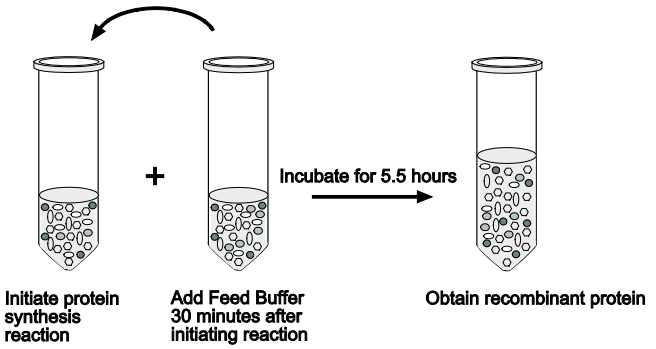
The Expressway™ Cell-Free *E. coli* Expression System provides a means to produce high levels of recombinant protein that may be easily detected and purified. Once protein expression is verified, the recombinant protein may be used for the following applications:

- Producing recombinant protein (or radiolabeled protein) suitable for use in downstream applications
- Analyzing mutants
- Verifying cloned gene products
- Producing proteins that are toxic to cells

For more information on the downstream applications of cell free protein expression technologies, refer to published reviews (Katzen *et al.*, 2005).

How the System Works

The Expressway™ Cell-Free *E. coli* Expression System uses an optimized *E. coli* extract, a reaction buffer containing an ATP regenerating system, and amino acids to allow high-level synthesis of your recombinant protein of interest. At one or several time points after initiating the protein synthesis reaction, the reaction is supplemented with an optimized Feed Buffer containing a proprietary mixture of salts, amino acids, and other substrates that are depleted or degraded over time during protein synthesis (see Figure below). Addition of this Feed Buffer to the reaction replenishes these components and allows continuous cell-free protein synthesis to occur, resulting in the achievement of significantly enhanced recombinant protein yields in up to 3-6 hours.



Continued on next page

Overview, continued

Components of the System

The major components of the Expressway™ Cell-Free *E. coli* Expression System include:

- An optimized *E. coli* extract (Zubay, 1973) for increased stability of DNA constructs during transcription and translation and increased production of soluble protein
- An optimized reaction buffer composed of an ATP regenerating system (Kim *et al.*, 1996; Lesley *et al.*, 1991; Pratt, 1984) to provide an energy source for protein synthesis
- An optimized feed buffer containing salts and other substrates (Kim and Swartz, 1999) to replenish components depleted or degraded during protein synthesis, thus enhancing recombinant protein yield
- Amino acids (-Met) required for protein synthesis to occur
- Methionine provided separately to allow production of radiolabeled recombinant protein, if desired
- Proprietary T7 Enzyme Mix containing T7 RNA polymerase and other components optimized for T7-based expression from DNA templates (Studier *et al.*, 1990)
- The pEXP5-NT/CALML3 control plasmid (expressing the human calmodulin like 3 fusion protein) for use as a positive control for protein synthesis (see page 33 for more information)
- Two optimized expression vectors, pEXP5-NT/TOPO® and pEXP5-CT/TOPO®, to allow rapid generation of N- or C-terminal fusion constructs, respectively (supplied with Catalog no. K9900-96 only; also available separately from Invitrogen)

E. coli slyD- Extract

All Invitrogen Expressway™ System Kits contain an optimized *E. coli slyD-* extract. The *slyD-* extract promotes the high yield expression of full-length, active protein from DNA constructs under the reaction conditions specified in this manual.

Other Expressway™ Systems

If you have used other Invitrogen Expressway™ Cell-Free *E. coli* Expression Systems, note that some of the components including the Expressway™ IVPS *E. coli* Extract and the Expressway™ 2.5X IVPS *E. coli* Reaction Buffer supplied with older Expressway™ kits contain different formulations and **may not** be compatible with this system.

For optimal results, use the components supplied in this kit to perform the protein synthesis reaction.

Continued on next page

Overview, continued

pEXP5-NT/TOPO® and pEXP5-CT/TOPO® Vectors

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors are supplied with the Expressway™ Maxi kit (Catalog no. K9900-96) or available separately from Invitrogen to facilitate rapid, TOPO® Cloning-mediated generation of expression constructs containing your gene of interest. The vectors contain all of the necessary regulatory elements in a configuration optimal for high-level production of your recombinant protein in the Expressway™ System. In addition, the vectors allow fusion of your gene of interest with an N- or C-terminal peptide, as appropriate, containing a polyhistidine (6xHis) tag for production of protein that can be readily detected with commercially available antibodies, and purified with metal-chelating resin.

For more information about the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vectors, TOPO® Cloning technology, and how to generate expression constructs, refer to the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual. This manual is supplied with Catalog no. K9900-96, but is also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).

Experimental Outline

The table below describes the major steps required to synthesize your recombinant protein of interest using the Expressway™ Cell-Free *E. coli* Expression System. Refer to the specified pages for details to perform each step.

Step	Action	Pages
1	Generate the DNA template.	13-15
2	Purify your DNA template.	17
3	Perform the protein synthesis reaction.	18–21
4	Analyze recombinant protein by polyacrylamide gel electrophoresis or other method of choice.	22-24
5	Purify your recombinant protein, if desired.	27

Methods

General Guidelines to Generate the DNA Template

Introduction

Successful use of the Expressway™ Cell-Free *E. coli* Expression System to synthesize recombinant protein requires the addition of a DNA template containing the gene of interest placed within the proper context of transcription and translation regulatory elements including a bacteriophage T7 RNA polymerase promoter ("T7 promoter"), prokaryotic Shine-Dalgarno ribosome binding site (RBS), ATG initiation codon, stop codon, and T7 terminator. However, protein yield can be significantly enhanced if the DNA template is optimally configured.

If you wish to design your own expression construct, general guidelines are provided in this section.

Factors Affecting Protein Yield

The yield of protein produced in cell-free systems is generally dependent on many factors, including:

- Size of the protein
- Sequence of the gene of interest
- Positioning of the gene of interest relative to the T7 promoter and the Shine-Dalgarno ribosome binding site in the DNA template
- Expression of protein as a fusion with an N- or C-terminal tag (typically added to facilitate detection and purification of recombinant protein)
- Optimized codon usage
- Quality of the DNA template
- Stability of mRNA

Recommendations and guidelines to generate a DNA template with the optimal configuration and to purify the DNA template are provided in this section. The size of the protein and its sequence will vary depending on your gene of interest. Any variability in protein yield due to these two factors will require empiric experimentation to optimize expression conditions.

Continued on next page

General Guidelines to Generate the DNA Template, continued

DNA Templates

The following DNA templates may be used in the Expressway™ Cell-Free *E. coli* Expression System:

- Supercoiled plasmid DNA (recommended to obtain the highest yields)
- Linear DNA
- PCR product

Many expression vectors or DNA templates may be used. For proper expression, all templates must contain the T7 promoter, an initiation codon, and a prokaryotic Shine-Dalgarno ribosome binding site (RBS) upstream of the gene of interest. See below for a discussion of template optimization.

Designing Your Own Template

If you are designing your own expression construct, we recommend generating a DNA template that contains the following elements (see the figure below for reference).

- Gene of interest placed downstream of a T7 promoter and a ribosome binding site (RBS). The gene of interest **must** contain an ATG initiation codon and a stop codon.
- Sequence upstream of the T7 promoter containing a minimum of 6-10 nucleotides (nt) for efficient promoter binding (**required** for linear PCR products). This sequence need not be specific.
- Sequence following the T7 promoter containing a minimum of 15-20 nt which forms a potential stem-and-loop structure as described by Studier *et al*., 1990 (see **Expressway™ Compatible Vectors**, next page, for more information).
- Sequence of 7-9 nt between the RBS and the ATG initiation codon for optimal translation efficiency of the protein of interest. This sequence need not be specific.
- A T7 terminator located 4-100 nt downstream of the gene of interest for efficient transcription termination and message stability.

Continued on next page

General Guidelines to Generate the DNA Template, continued

Expressway™ Compatible Vectors

Many T7-based expression vectors are suitable for use as templates for the Expressway™ Cell-Free *E. coli* System. At a minimum, these vectors must contain the T7 promoter, RBS, and T7 terminator with the suitable spacing and sequence configuration for optimal expression of protein (see previous page).

For recommended T7-based Gateway® and TOPO® Cloning vectors to use with the Expressway™ System, see below.

pEXP5-NT/TOPO® and pEXP5-CT/TOPO® Vectors

The pEXP5-NT/TOPO® (Catalog no. V960-05) and pEXP5-CT/TOPO® (Catalog no. V960-06) vectors available from Invitrogen are ideal for use with the Expressway™ Mini or Maxi Kit. Both vectors contain the following features:

- T7 promoter, RBS, and T7 terminator with spacing and sequence configuration optimized to allow the high levels of protein expression in the Expressway™ System (see previous page for an example)
- Adapted with topoisomerase I to allow highly efficient, 5-minute, TOPO® Cloning of *Taq* polymerase-amplified PCR products for rapid generation of expression constructs
- An N-terminal peptide containing the 6xHis tag and a TEV recognition site to allow production of a recombinant fusion protein that may be easily detected and purified (pEXP5-NT/TOPO® only). The TEV recognition site allows TEV protease-mediated removal of the N-terminal tag to generate nearly native (only 2 amino acids added to N-terminus) recombinant protein
- A C-terminal tag containing the 6xHis tag to allow production of a recombinant fusion protein that may be easily detected and purified (pEXP5-CT/TOPO® only)

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits are supplied with Catalog no. K9900-96, but are also available separately from Invitrogen. For more information about the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors, and instructions to generate the expression construct, refer to the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual.

Note

If you are expressing your recombinant protein for the first time and wish to maximize the yield obtained, we recommend generating expression constructs in pEXP5-NT/TOPO® and pEXP5-CT/TOPO®, and testing both constructs in the Expressway™ System. Protein yields can vary significantly depending on whether the recombinant protein of interest is expressed as an N- or C-terminal fusion.

Continued on next page

pEXP1-DEST and pEXP2-DEST Vectors

The pEXP1-DEST and pEXP2-DEST vectors (available separately from Invitrogen) contain the following elements:

- Bacteriophage T7 promoter for high-level, inducible expression of the recombinant protein of interest in the Expressway™ System
- N-terminal or C-terminal fusion tags for detection and purification of recombinant fusion proteins
- Two recombination sites, *att*R1 and *att*R2 downstream of the T7 promoter for Gateway® 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 attR sites for negative selection

For more information, refer to the pEXP1/2-DEST Gateway Vector Kit manual available at www.invitrogen.com or by contacting Technical Service (page 35).

Continued on next page

General Guidelines to Generate the DNA Template, continued

Sequencing

Once you have generated your DNA template, we recommend that you sequence the expression construct to confirm the presence and orientation of the gene of interest. If you have generated a fusion construct in pEXP5-NT/TOPO®, pEXP5-CT/TOPO®, pEXP1-DEST, or pEXP2-DEST, verify that your gene of interest is in frame with the appropriate N- or C-terminal tag.

Purifying the DNA Template

After you have generated the DNA template, you must purify the DNA before proceeding to the protein synthesis reaction. You may use a variety of methods to purify your DNA template including commercial DNA purification kits (see below) or CsCl gradient centrifugation. For protocols to purify DNA, refer to published reference sources (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989). When purifying your DNA template, keep the following in mind:

- **Do not gel-purify** your DNA template. Purified DNA solution obtained from agarose gels significantly inhibits the protein synthesis reaction.
- For rapid isolation of high quality purified plasmid DNA, we recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) available from Invitrogen. Other commercial DNA purification kits are suitable.
- For rapid isolation of high quality DNA from PCR reactions, we recommend using the PureLink™ PCR Purification Kit (Catalog no. K3100-01) available from Invitrogen.
- Ammonium acetate is not recommended for use in DNA precipitation as any residual contamination may inhibit translation. Use sodium acetate.
- Make sure that the purified DNA is free of RNase contamination. Wear gloves and use RNase-free reagents when preparing DNA.
- Make sure that purified DNA is free of excess ethanol or salt as both can inhibit translation.

  Note: Carefully wash ethanol precipitated DNA with 70% ethanol to remove excess salt and dry.
- Resuspend purified DNA in 1X TE Buffer or water to a final concentration of at least 500 ng/µl.

Performing the Protein Synthesis Reaction

Introduction

After you have obtained purified template DNA, you are ready to synthesize recombinant protein using the Expressway™ Maxi Kit or Expressway™ Mini Kit. This section provides guidelines and a protocol to synthesize your protein.

Important

RNase contamination may affect protein yield. To reduce the chances of RNase contamination, wear gloves and use RNase-free reagents when performing the protein synthesis reaction. To eliminate RNase from surfaces, use RNase AWAY™ available separately from Invitrogen (see page 7).

Reaction Volumes

The volume of the protein synthesis reaction may be scaled, based on your needs. For screening reactions, the standard volume is 100 µl (50 µl initial reaction + 50 µl Feed Buffer), but this can be decreased to 25 µl reaction volume and increased up to 2 ml reaction volume. Note that protein yields may vary depending on the nature of the protein expressed and the template used.

Amount of DNA Template

For a 100 µl protein synthesis reaction, use 1 µg of template DNA (plasmid or linear DNA). For a 2 ml reaction, use 10-15 µg of template DNA. For optimal results, purify DNA template before use (see page 17).

Reaction Vessel

Use a reaction vessel that contains a large enough surface area to allow moderate mixing to occur. We recommend performing the 100 µl protein synthesis reaction in a sterile, RNase-free 1.5 ml tube. If you are performing larger reaction volumes, you may use sterile, RNase-free 50 ml conical tubes. Other reaction vessels including 96-well, 6-well or 12-well untreated culture plates are suitable.

Incubation Conditions

- To obtain optimal protein yield, **it is critical to mix the reaction thoroughly throughout the incubation period.** We recommend using a thermomixer set to 1,200 rpm or a shaking incubator set to 300 rpm. **Do not** use stationary incubators such as incubator ovens or water baths as protein yields may be reduced by up to 30-50%.
- Incubate the protein synthesis reaction at a temperature ranging from 30°C to 37°C. The optimal temperature to use depends on the solubility of your recombinant protein, and should be determined empirically. Higher protein yields are generally obtained with incubation at higher temperatures (*i.e.* 37°C); however, protein solubility generally improves with incubation at lower temperatures (*i.e.* 30°C).
- You may obtain your protein of interest in as little as 2.5 hours of incubation after feeding (3 hours total). Many reactions yield 80-90% of total protein within 3 hours. However, for maximum yield, we recommend incubating the reaction for the full 6 hours.

Continued on next page

Performing the Protein Synthesis Reaction, continued

Amino Acid Concentration

Use an amino acid concentration ranging from 1 mM to 4 mM in the protein synthesis reaction. The recommended amino acid concentration is 1.25 mM each, but may be adjusted according to the protein being synthesized and your application (see **Using Unnatural Amino Acids**, below).

Feed Buffer

Add 1 volume of Feed Buffer (containing Expressway™ 2X IVPS Feed Buffer and amino acids) to the protein synthesis reaction after the initial 30-minute incubation. Higher protein yields may be obtained by adding one half-volume of Feed Buffer at 30 minutes and one half-volume of Feed Buffer again at 2 hours after initiating the protein synthesis reaction.

Using Unnatural Amino Acids

Methionine is supplied separately in the kit to allow you to incorporate unnatural amino acids into your recombinant protein and adjust the amino acid concentration in the protein synthesis reaction. Depending on your application, you may use the following unnatural amino acids:

- **Radiolabeled methionine:** Use ^{35}S-Methionine to produce radiolabeled protein for use in expression and purification studies. See Performing the Protein Synthesis Reaction, page 21 for recommended amounts of labeled and unlabeled methionine.
- **Heavy metal-labeled methionine:** Use selenomethionine (Budisa *et al.*, 1995; Doublie, 1997; Hendrickson *et al.*, 1990) to produce labeled protein for use in X-ray crystallographic studies. See **Performing the Protein Synthesis Reaction**, page 21 for recommended amounts of labeled methionine.

  Note: When using selenomethionine, do not use any unlabeled methioinine in the protein synthesis reaction.

Handling Reagents

- **Do not** store the *E. coli slyD*- Extract, *E. coli* Reaction Buffer (-A.A.), or 2X Feed Buffer at -20°C or room temperature as this may result in loss of activity.
- Freezing and thawing the *E. coli slyD*- Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer once or twice is acceptable. However, avoid multiple freeze/thaw cycles as this may result in loss of activity.

Positive Control

The pEXP5-NT/CALML3 control vector is provided in the kit for use as a positive control for protein expression and allows expression of an N-terminally-tagged human calmodulin-like 3 (CALML3) protein from pEXP5-NT/TOPO®. A 100 µl protein synthesis reaction should yield at least 75 µg of the 19.5 kDa fusion protein. For details about the vector, refer to page 33. To propagate and maintain the control plasmid:

1. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α™-T1^R, or equivalent. Use 10 ng of plasmid for transformation.
2. Select transformants on LB agar plates containing 100 µg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Continued on next page

Performing the Protein Synthesis Reaction, continued

Materials Needed

Supplied by the user:

- Expression construct or other suitable DNA template (purified; resuspended in TE or water at a concentration greater than 500 ng/µl)
- For each reaction, sterile, RNase-free 1.5 ml Eppendorf tubes or other suitable reaction vessels (see previous page)
- RNase-free pipette tips and microcentrifuge tubes
- Standard shaking incubator (set to 30°C or 37°C and 300 rpm)
- Thermomixer (optional; set to 1,200 rpm instead of shaking incubator)
- Labeled methionine (optional; if producing labeled recombinant protein, see previous page)

Supplied with the kit:

- Expressway™ *E. coli slyD*- Extract (thaw on ice)
- Expressway™ 2.5X IVPS *E. coli* Reaction Buffer (-A.A.) (thaw on ice)
- Expressway™ 2X IVPS Feed Buffer (thaw on ice)
- T7 Enzyme Mix (keep on ice; store at -20°C after initial use)
- 50 mM Amino Acids (-Met)

  Note: When thawing the 50 mM Amino Acids (-Met), the solution may have a brown or yellowish tint. This is normal and does not affect the activity of the amino acids.
- 75 mM Methionine
- DNase/RNase-free distilled water (supplied with the kit)
- pEXP5-NT/CALML3 control plasmid (optional; 1 µg/µl in TE Buffer, pH 8.0)

Scaling Expressway™ Maxi

The Expressway Maxi™ kit supplies five tubes each of Expressway™ *E. coli slyD*- Extract (400 µl per tube), Expressway™ 2.5X IVPS *E. coli* Reaction Buffer (-A.A.) (400 µl per tube), and Expressway™ 2X IVPS Feed Buffer (500 µl per tube).

It is possible to synthesize recombinant protein in various reaction volumes, such as for screening experiments (*i.e.* 100 µl reaction) or to obtain larger amounts of protein (*i.e.* up to 2 ml reaction). Depending on your experimental design, you may not use the entire contents of a tube of *E. coli slyD*- Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer in a single experiment. If you wish to synthesize protein in small reaction volumes, we recommend the following:

1. **Thaw on ice** the *E. coli slyD*- Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer.
2. Remove the amount of *E. coli slyD*- Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer needed for the protein synthesis reaction and return tubes to a -80°C freezer.

Continued on next page

Performing the Protein Synthesis Reaction, continued

Performing the Protein Synthesis Reaction

Use the protocol below to synthesize your recombinant protein from the DNA template. The amounts given are for a standard 100 µl reaction; if you are scaling up the reaction, adjust the volume of reagents accordingly.

1. **For each sample**, add the following reagents to the appropriate reaction vessel (see page 18):

Reagent	Amount
E. coli slyD- Extract	20 µl
2.5X IVPS *E. coli* Reaction Buffer (-A.A.)	20 µl
50 mM Amino Acids (-Met)	1.25 µl
75 mM Methionine*	1 µl
T7 Enzyme Mix	1 µl
DNA Template	1 µg
DNase/RNase-free Distilled Water	To a final volume of 50 µl

***Note:** To generate radiolabeled protein using ^{35}S-methionine, use 2 µl of ^{35}S-methionine, and 1µl unlabeled 75 mM methionine. To generate labeled protein using selenomethionine, use 2 µl of selenomethionine only, do not add unlabeled methionine.

2. Close the tube and incubate sample in a standard shaking incubator (300 rpm) at 30°C for 30 minutes. If the protein you are synthesizing is known to be soluble, you may incubate the sample at 37°C.
3. During the 30 minute incubation, prepare the Feed Buffer. **For each sample**, add the following reagents to a sterile, RNase-free microcentrifuge tube. For multiple samples, you may scale up the volume of reagents used accordingly and prepare one master mix.

Reagent	Amount
2X IVPS Feed Buffer	25 µl
50 mM Amino Acids (-Met)	1.25 µl
75 mM Methionine*	1 µl
DNase/RNase-free Distilled Water	To final volume of 50 µl

***Note:** To generate radiolabeled protein using ^{35}S-methionine, use 2 µl of ^{35}S-methionine, and 1 µl unlabeled 75 mM methionine. To generate labeled protein using selenomethionine, use 2 µl of selenomethionine only, do not add unlabeled methionine.

4. After 30 minutes of incubation (from Step 2 above), add 50 µl of the Feed Buffer to the sample (total volume = 100 µl).
5. Cap the tube and return the sample to the shaking incubator (300 rpm). Incubate for 3-6 hours at 30-37°C as appropriate (see page 18).
6. Place the reaction on ice and proceed to **Analyzing Samples**, next page or store the sample at -20°C for future processing or analysis.

Analyzing Samples

Introduction

Once you have performed the protein synthesis reaction, you may use any method of choice to analyze your sample. Generally, the amount of protein produced in an Expressway™ Mini or Maxi reaction is sufficient to allow detection on a Coomassie-stained protein gel, by western blot analysis, by enzymatic activity assay, or by affinity purification (if affinity tag is present). Note that expression levels may vary from protein to protein and depends on the nature of the protein and the configuration of the DNA template. If you plan to analyze your sample using polyacrylamide gel electrophoresis, you should first precipitate the proteins with acetone to remove background smearing. A protocol to perform acetone precipitation and other general guidelines for gel electrophoresis are provided in this section.

If you have performed trace labeling using ^{35}S-Methionine, you may use TCA precipitation to determine the amount of radiolabeled methionine incorporated and to calculate the yield of protein (see **Determining Protein Yield**, page 25).

Materials Needed

- Acetone (room temperature)
- 1X SDS-PAGE sample buffer (see page 34 for a recipe)
- Appropriate polyacrylamide gel to resolve your protein of interest (see the next page)
- Appropriate protein stain (*e.g.* SimplyBlue™ SafeStain; Catalog no. LC6060)

Acetone Precipitation

Before starting, prepare an SDS-PAGE gel or use one of the pre-cast polyacrylamide gels available from Invitrogen (see the next page) to analyze your samples. Use the following protocol to precipitate your proteins prior to loading on the polyacrylamide gel.

1. Add 5 µl of the protein reaction product from Step 6, previous page, to 20 µl of acetone. Mix well.
2. Centrifuge for 5 minutes at room temperature in a microcentrifuge at 12,000 rpm.
3. Carefully remove the supernatant, taking care not to disturb the protein pellet.
4. Resuspend pellet in 20 µl of 1X SDS-PAGE sample buffer.
5. Heat at 70-80°C for 10-15 minutes and centrifuge briefly. Proceed to **Polyacrylamide Gel Electrophoresis**, next page.

   Note: Alternatively, samples may be stored at -20°C until needed.

Continued on next page

Analyzing Samples, continued

Recommended Gels and Protein Standard

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE®, Novex® Tris-Glycine, and E-PAGE™ polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. In addition, the BenchMark™ Protein Ladder allows easy visualization of molecular weight ranges of your recombinant protein. The BenchMark™ Protein Ladder consists of 15 distinct protein bands in the range of ~10-220 kDa that are easily detected using Coomassie blue, SimplyBlue™ SafeStain, or other protein stains.

For more information about pre-cast gels available from Invitrogen as well as the BenchMark™ Protein Ladder, visit www.invitrogen.com or contact Technical Service (page 35).

Polyacrylamide Gel Electrophoresis

1. Load 5-10 µl of the sample from Step 6, previous page on an SDS-PAGE gel and electrophorese at 120V. You may save your sample by storing at -20°C, if desired.
2. Depending on your assay of choice, perform the following:

If you are…	Then…
Visualizing your protein using Coomassie blue stain (or other protein stain)	Stain gel with Coomassie blue stain or other stain. Refer to manufacturer's instructions. **Note:** For radiolabeled proteins, the signal may be enhanced by placing the gel in a commercially available reagent that enhances the signal. Dry the gel and expose to x-ray film for 1-4 hours.
Analyzing your protein by Western blot	Transfer proteins electrophoretically to a suitable membrane and use an appropriate antibody to detect the protein of choice (see below).

Detecting Fusion Proteins

If you have expressed your recombinant protein from pEXP5-NT/TOPO® or pEXP5-CT/TOPO® as a fusion to the N- or C-terminal tag, respectively, you may perform Western blot analysis and detect expression of your recombinant fusion protein using an antibody to your protein an appropriate antibody to the epitope tag. For more information about the Anti-HisG Antibodies or the Anti-His(C-term) Antibodies, see www.invitrogen.com or call Technical Service (page 35). For ordering information, see page 8.

Continued on next page

Analyzing Samples, continued

Detecting CALML3 Control Protein

If you use pEXP5-NT/CALML3 as a positive control for protein expression, you should be able to detect the CALML3 fusion protein on a Coomassie blue-stained gel using 5 µl of the reaction. To detect the CALML3 fusion protein by Western blot analysis, use one of the Anti-HisG Antibodies available from Invitrogen (see page 8 for ordering information).

What to Do Next

Once you have verified expression, you may use the recombinant protein in any downstream application of your choice. If you plan to use the recombinant protein for structural analyses including x-ray crystallography, note that you must purify the recombinant protein before use. Use any method of choice to purify your recombinant protein.

If you have expressed your recombinant protein from pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, and have cloned your gene in frame with the N-terminal or C-terminal 6xHis tag in each vector, you may purify your recombinant protein using a metal-chelating resin such as ProBond™ or Ni-NTA. For guidelines to purify recombinant protein using ProBond™ or Ni-NTA, see page 27.

Note: Other metal-chelating resins are suitable.

Determining Protein Yield

Introduction

If you have included radiolabeled methionine in the protein synthesis reaction, you may use TCA precipitation to determine the amount of radiolabeled methionine incorporated and to calculate the yield of protein.

Determining Total Counts

1. Mix and spot 5 µl of each radiolabeled reaction from Step 6, page 21 on a glass microfiber filter (Type GF/C; Whatman, Catalog no. 1822-021).
2. Set aside and let dry. **Do not** wash or TCA precipitate these filters.

Performing TCA Precipitation

Two protocols are provided below for performing TCA precipitation; one to perform standard TCA precipitation and one to perform TCA precipitation using a vacuum filtration device (*e.g.* Millipore 1225 Sampling Manifold). Choose the protocol that best fits your needs.

Performing Standard TCA Precipitation

1. Mix and spot 5 µl of each radiolabeled reaction from Step 6, page 21 on a separate set of individual glass fiber (GF/C) filters and allow to air dry for approximately 5-10 seconds.
2. Place filter in a beaker and wash once with cold 10% TCA for 10 minutes at room temperature while shaking gently (use approximately 10-20 ml per filter).
3. Wash with 5% TCA for 5 minutes at room temperature while shaking gently. Repeat wash.
4. Rinse filters with methanol to facilitate drying.
5. Allow filters to dry, place in scintillation vials, and add scintillation fluid. Count samples in a scintillation counter.
6. Proceed to **Calculating Protein Yield**, next page.

Performing TCA Precipitation Using a Vacuum Filtration Device

1. Aliquot 5 µl of each radiolabeled reaction from Step 6, page 21 into separate glass tubes.
2. Add 100 µl of 1 N NaOH to each reaction and incubate for 5 minutes at room temperature.
3. Add 3 ml of 10% TCA to each glass tube and incubate tubes at +4ºC for 20 minutes.
4. Wet individual glass fiber (GF/C) filters with 10% TCA and place onto the vacuum filtration device.
5. Turn the vacuum on and pour the TCA solution from each glass tube into a sample well.
6. Wash filters twice with 5% TCA.
7. Wash filters once with 100% ethanol. Leave the vacuum on for 1 minute to allow the filters to dry.
8. Turn the vacuum off and remove the filters. Place the filters in scintillation vials, and add scintillation fluid. Count samples in a scintillation counter.
9. Proceed to **Calculating Protein Yield**, next page.

Continued on next page

Determining Protein Yield, continued

Calculating Protein Yield

Use the equations below to calculate the yield of protein obtained. You will need to determine the pmoles of methionine present in your specific reaction. Remember to account for both radiolabeled and unlabeled methionine. You will also need to determine the total counts incorporated using TCA precipitation (see previous page).

Total counts:

$$\text{total cpm per 5 } \mu\text{l spotted} \times \frac{\text{total reaction volume}}{5}$$

Specific activity:

$$\frac{\text{total counts}}{\text{pmoles of methionine}}$$

pmoles methionine incorporated:

$$\frac{\left[(\text{TCA precipitable counts} - \text{background}) \times \frac{\text{total reaction volume}}{5}\right]}{\text{specific activity}}$$

pmoles of protein:

$$\frac{\text{pmoles of methionine incorporated into protein}}{\text{number of methionines in protein}}$$

Yield of protein (in μg):

$$\frac{\text{moles of protein} \times \text{molecular weight of protein}}{10^6}$$

Purifying the Recombinant Soluble Fusion Protein

Introduction

The presence of the N-terminal or C-terminal 6xHis tag in the pEXP5-NT/TOPO®, pEXP5-CT/TOPO®, pEXP1-DEST, and pEXP2-DEST vectors allows purification of your recombinant protein with a metal-chelating resin such as ProBond™ or Ni-NTA (see page 9 for ordering information). This section provides guidelines for purification.

Note

The pEXP5-NT/TOPO® vector contains a Tobacco Etch Virus (TEV) recognition site to allow removal of the N-terminal tag from your recombinant fusion protein using TEV protease. See page 9 for additional products.

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 purify your fusion protein using ProBond™ or Ni-NTA, follow the guidelines below and detailed instructions included with each product. You may download the appropriate manuals from www.invitrogen.com.
- To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.

Guidelines for Purification

Follow these guidelines when purifying your recombinant fusion protein using ProBond™ or Ni-NTA. Remember to use criteria appropriate for purification under native conditions. For details, refer to the ProBond™ or Ni-NTA manual, as appropriate.

1. Prepare the purification column containing ProBond™ or Ni-NTA agarose resin. After applying the resin to the purification column, wash with 4 volumes of water followed by 8 volumes of Binding Buffer (supplied with the kit; 50 mM $NaPO_4$, pH 8.0, 500 mM NaCl) to equilibrate the column.
2. **Optional (applies only to protein synthesis reactions containing extra components (*e.g.* detergents, chaperones) other than those supplied with the Expressway™ kit):** Dilute the Expressway™ reaction (from Step 6, page 21) 1:1 with Binding Buffer (50 mM $NaPO_4$, pH 8.0, 500 mM NaCl).
3. Centrifuge the Expressway™ Mini or Maxi reaction at 15,000 x g for 10 minutes at room temperature to remove insoluble material.
4. Load the supernatant containing soluble protein onto the equilibrated resin and incubate (*i.e.* batch binding) for 30 minutes at the desired temperature.
5. Wash the column twice with 2 volumes of Binding Buffer each time.
6. Wash the column twice with 2 volumes of Binding Buffer containing 20 mM imidazole.
7. Elute the protein using an Elution buffer containing an appropriate amount of imidazole (*e.g.* 250 mM imidazole).
8. Analyze the fractions using SDS-PAGE.
9. Pool the desired fractions and dialyze, if necessary.

Sample Protein Synthesis Experiment

Introduction

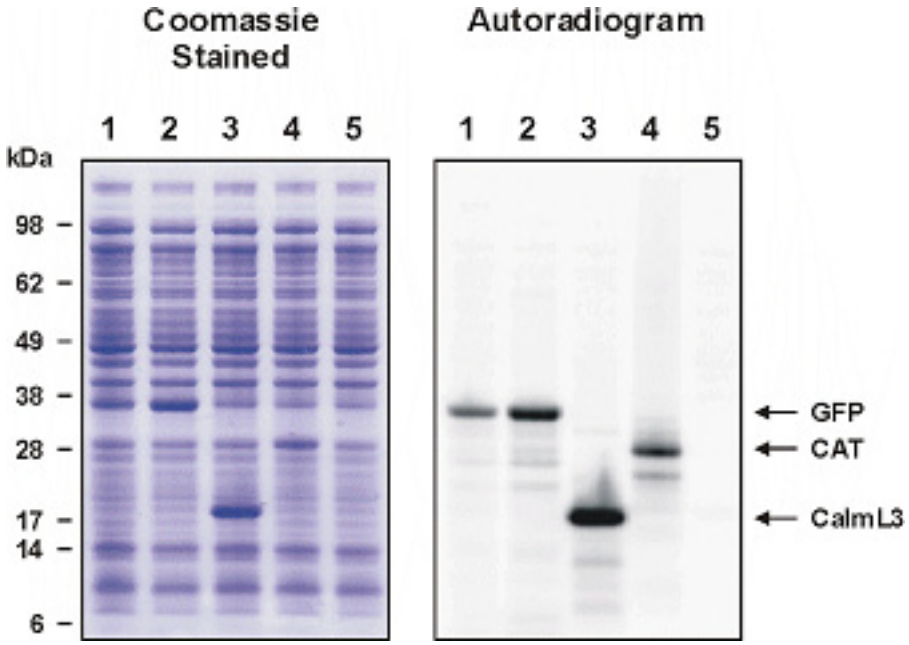
This section provides an example of a typical protein synthesis experiment performed using the Expressway™ Cell-Free *E. coli* Expression System and results obtained.

Experimental Conditions

In this experiment, 3 open reading frames (ORFs) encoding green fluorescent protein (GFP), calmodulin-like 3 (CALML3; GenBank accession no. NM_005185), and chloramphenicol acetyl transferase (CAT) were amplified with *Taq* polymerase. The ORFS were cloned into pCR2.1, pEXP5-NT TOPO® or pEXP3-DEST to generate pCR2.1-GFP, pEXP5-CALML3, and pEXP3-CAT, respectively. Linear template was generated by PCR amplification of pCR2.1-GFP.

Plasmid or linear DNA was purified and 1 µl used in a 100 µl protein synthesis reaction with the components supplied in the Expressway™ Mini kit and according to the protocol on page 21. ^{35}S Methionine was included in each reaction for trace labeling. One microliter of each reaction was electrophoresed on a NuPAGE® 4-12% Bis-Tris Gel and stained (Coomassie Stained, below) or exposed to x-ray film (Autoradiogram, below).

Note: The pEXP5-NT/CALML3 expression construct used in this experiment (Lane 3) is identical to the pEXP5-NT/CALML3 control plasmid supplied in this kit.



Lane 1: GFP (linear template)
Lane 2: GFP (plasmid template)
Lane 3: pEXP5-NT/CALML3
Lane 4: pEXP3-CAT
Lane 5: No DNA

Troubleshooting

Introduction

Review the information in this section to troubleshoot your cell-free expression experiment.

Synthesizing Proteins

The table below lists some potential problems and possible solutions that may help you troubleshoot your protein synthesis experiments.

Problem	Reason	Solution
Low or no yield of protein (but control reaction produces protein)	DNA template not optimally configured	• Use the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector or follow the guidelines on page 14 to generate a DNA template with the optimal configuration. • Make sure that the ATG initiation codon is in the proper context for expression (*i.e.* check spacing and placement after the RBS). • Fusion of your protein to an N- or C-terminal tag may affect RNA structure and lower translation levels. Try moving the fusion tag to the other terminus.
	Gene of interest not cloned in frame with the N- or C-terminal tag	Generate a new expression construct, making sure that your gene of interest is cloned in frame with the N- or C-terminal tag; confirm by sequencing.
	DNA template not pure • Contaminated with ethanol, sodium salt, or ammonium acetate • Contaminated with RNases	• Prepare new DNA template taking care to remove excess ethanol and/or salt after precipitation. • Do not use ammonium acetate to precipitate DNA. Use sodium acetate. • Wear gloves and use RNase-free reagents when preparing DNA.
	DNA template purified from agarose gel	Do **not** purify your DNA from a gel. See the purification guidelines on page 17.
	Insufficient amount of DNA template used	• Use 10-15 µg of template DNA in a 2 ml protein synthesis reaction. • If you are expressing a large protein, increase the amount of DNA template used in the protein synthesis reaction to 20 µg.

Continued on next page

Troubleshooting, continued

Synthesizing Proteins, continued

Problem	Reason	Solution
Low or no yield of protein (but control reaction produces protein), continued	Sample incubated in a non-shaking incubator or water bath	Incubate sample in a thermomixer or incubator with shaking (see page 18).
	Insufficient feeding	• Add one volume of Feed Buffer to the sample (*i.e.* 1 ml Feed Buffer to 1 ml sample) 30 minutes after initiating protein synthesis. • Add one-half volume of Feed Buffer to the sample (*i.e.* 25 µl Feed Buffer to 50 µl sample) 30 minutes and 2 hours after initiating protein synthesis.
	Large protein being expressed	• Protein yield may decrease as the size of the protein increases; optimize expression conditions. • Reduce incubation temperature to 25°C-30°C during protein synthesis.
	Sample not mixed before spotting on filter for TCA precipitation (radiolabeled samples only)	Mix sample before spotting on filter for TCA precipitation.
	Protein forms aggregates	• Reduce the incubation temperature to 25°C-30°C during protein synthesis. • Add mild detergents (*e.g.* up to 0.05% Triton-X-100, 0.025% sodium dodecyl maltoside, 0.1% CHAPS, or 0.05% Brij-58) to the reaction and Feed Buffer. • Add molecular chaperones to the reaction.

Continued on next page

Troubleshooting, continued

Synthesizing Proteins, continued

Problem	Reason	Solution
Control reaction produces no protein	Reagents have lost activity	Store reagents at -80°C. Store the T7 Enzyme Mix at -20°C after initial use. • Use care when freezing and thawing the Expressway™ *E. coli slyD*-Extract, Expressway™ 2.5X IVPS *E. coli* Reaction Buffer, and Expressway™ 2X IVPS Feed Buffer. Follow handling guidelines on page 19. One or two freeze/thaw cycles are acceptable. Avoid multiple freeze/thaw cycles.
	Reagent(s) contaminated with RNases	Wear gloves and use RNase-free supplies when handling the reagents supplied in the kit. Use RNase AWAY™ available from Invitrogen (Catalog no. 10328-011) to eliminate RNase from surfaces.
Protein has low biological activity	Improper protein folding	Reduce incubation temperature to as low as 25°C during protein synthesis.
	Post-translational modifications required	The Expressway™ *E. coli slyD*- Extract will not introduce post-translational modifications such as phosphorylation or glycosylation to the recombinant protein.
	Synthesized protein requires co-factors for complete activity	Add required co-factors to the protein synthesis reaction.

Analyzing Proteins

The table below lists some potential problems and possible solutions that may help you troubleshoot your electrophoresis experiments.

Problem	Reason	Solution
Multiple bands observed on the polyacrylamide gel	Proteins denatured for too long	Add 1X SDS-PAGE sample buffer to the sample and incubate at 70°C-80°C for 10-15 minutes before loading on the gel.
	Old ^{35}S-Methionine used (radiolabeled samples only)	Use fresh ^{35}S Methionine.
	Not enough SDS in the 1X SDS-PAGE sample buffer	Follow the recipe on page 34 to prepare 1X SDS-PAGE sample buffer.

Continued on next page

Troubleshooting, continued

Analyzing Proteins, continued

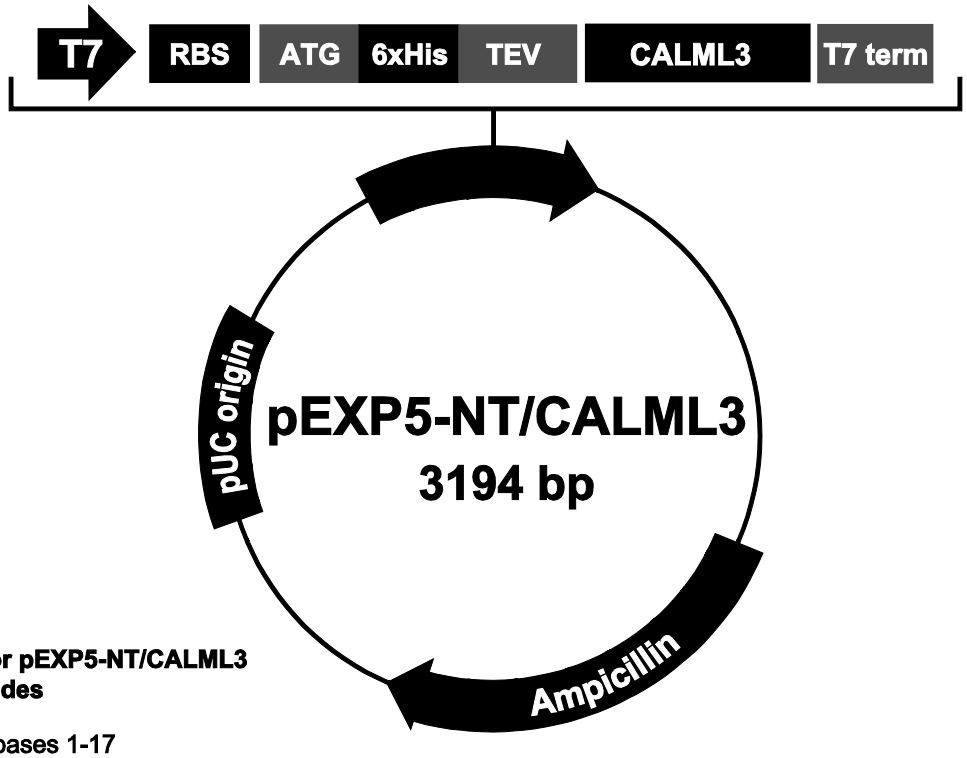
Problem	Reason	Solution
Multiple bands observed on the polyacrylamide gel, continued	Internal ATG codons in the context of RBS-like sequences	• Check the sequence of your gene and search for potential RBSs with the proper spacing from internal methionines. • Replace the methionine or change RBS sequence(s) using point mutation(s). • Clone your gene of interest into pEXP5-NT/TOPO® or pEXP5-CT/TOPO®.
Smearing on the gel	Samples not precipitated with acetone	Precipitate the proteins with acetone to remove background smearing. Follow the protocol provided on page 22.
	Too much protein loaded	Reduce the amount of protein loaded on the gel.
	Gel not clean	Rinse the gel briefly before exposing to film. If you have stained the gel with Coomassie blue, destain the gel in water or 50% methanol, 7.5% glacial acetic acid for 15-30 minutes before drying. If you have already destained the gel, repeat destaining procedure.
	Ethanol present in the protein synthesis reaction	Make sure that any residual ethanol is removed during DNA purification.
	Old pre-cast gels	Do not use pre-cast gels after the expiration date.

Appendix

Map and Features of pEXP5-NT/CALML3

pEXP5-NT/CALML3 Map

The pEXP5-NT/CALML3 vector (3194 bp) contains a human calmodulin-like 3 gene (CALML3; GenBank accession number NM_005185) that has been TOPO® Cloned into pEXP5-NT/TOPO® in frame with the N-terminal tag. The size of the CALML3 fusion protein is approximately 19.5 kDa. **The complete sequence of pEXP5-NT/CALML3 is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 35).**



Comments for pEXP5-NT/CALML3
3194 nucleotides

T7 promoter: bases 1-17
T7 forward priming site: bases 1-20
Ribosome binding site (RBS): bases 68-73
Initiation ATG: bases 80-82
Polyhistidine (6xHis) region: bases 92-109
HisG epitope: bases 92-112
TEV recognition site: bases 122-142
CALML3: bases 146-595
T7 reverse priming site: bases 647-666
T7 transcription terminator: bases 608-736
bla promoter: bases 848-946
Ampicillin resistance gene: bases 947-1807
pUC origin: 1952-2625

Recipes

1X SDS-PAGE Sample Buffer

1. Combine the following reagents:

0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol (100%)	2 ml
β-mercaptoethanol	0.4 ml
Bromophenol blue	0.02 g
SDS	0.4 g

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

Technical Support

Obtaining support

For the latest services and support information for all locations, go to www.lifetechnologies.com.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

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.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited warranty

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 a Life Technologies product or service, contact our Technical Support Representatives.

All Life Technologies 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.

Life Technologies 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 Expressway™ Cell-Free *E. coli* Expression System is covered under the licenses detailed below. For additional licenses related to use of the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA Expression Kits, refer to the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual.

Limited Use Label License: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License

This product is licensed from Hoffmann-LaRoche, Inc., Nutley, NJ and 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: ULB *ccd*B Selection Technology

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: outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

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).

Budisa, N., Steipe, B., Demange, P., Eckerskorn, C., Kellermann, J., and Huber, R. (1995). High-Level Biosynthetic Substitution of Methionine in Proteins by its Analogs 2-Aminohexanoic Acid, Selenomethionine, Telluromethionine and Ethionine in *Escherichia coli*. Eur. J. Biochem. *230*, 788-796.

Doublie, S. (1997). Preparation of Selenomethionyl Proteins for Phase Determination. Meth. Enzymol. *276*, 523-530.

Hendrickson, W. A., Horton, J. R., and LeMaster, D. M. (1990). Selenomethionyl Proteins Produced for Analysis by Multiwavelength Anomalous Diffraction (MAD): A Vehicle for Direct Determination of Three-Dimensional Structure. EMBO J. *9*, 1665-1672.

Katzen, F., Chang, G., and Kudlicki, W. (2005). The past, present and future of cell-free protein synthesis systems. Trends Biotechnol. *23*, 150-156.

Kim, D. M., Kigawa, T., Choi, C. Y., and Yokoyama, S. (1996). A Highly Efficient Cell-free Protein Synthesis System from *E. coli*. Eur. J. Biochem. *239*, 881-886.

Kim, D. M., and Swartz, J. R. (1999). Prolonging Cell-Free Protein Synthesis with a Novel ATP Regeneration System. Biotechnol. Bioeng. *66*, 180-188.

Lesley, S. A., Brow, M. A., and Burgess, R. R. (1991). Use of *in vitro* Protein Synthesis from Polymerase Chain Reaction-generated Templates to Study Interaction of *Escherichia coli* Transcription Factors with Core RNA Polymerase and for Epitope Mapping of Monoclonal Antibodies. J. Biol. Chem. *266*, 2632-2638.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Pluckthun, A. (1997). Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions. BioTechniques *22*, 140-149.

Pratt, J. M. (1984). Transcription and Translation (Oxford: S.J. IRL Press).

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

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.

Zubay, G. (1973). *In vitro* Synthesis of Protein in Microbial Systems. Annu. Rev. Genet. *7*, 267-287.

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**