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Instruction Manual

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

Cell-free protein synthesis system for expression of up to milligram quantities of recombinant protein

Catalog nos. K9900-96 and K9900-97

Version A
13 May 2010
25-0808

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Amount	Catalog no.
Expressway™ Milligram Cell-Free *E. coli* Expression System *with pEXP5-NT/TOPO® and pEXP5-CT/TOPO®*	5 reactions	K9900-96
Expressway™ Milligram Cell-Free *E. coli* Expression System	5 reactions	K9900-97

Kit Components

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

Component	Catalog no.	
	K9900-96	**K9900-97**
Expressway™ High Yield Expression Module	√	√
Expressway™ Milligram Amino Acids Module	√	√
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.

Shipping/Storage

The Expressway™ Milligram Cell-Free *E. coli* Expression System kits are shipped as described below. Upon receipt, store as detailed. For more detailed information about the reagents supplied in the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits, see the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual.

Box	Component	Shipping	Storage
1	Expressway™ High Yield Expression Module	Dry ice	-80ºC
2	Expressway™ Milligram Amino Acids Module	Dry ice	-20ºC
3-4	pEXP5-NT/TOPO® TA Expression Kit	Dry ice	pEXP5-NT/TOPO® Reagents: -20°C One Shot® TOP10 Chemically Competent *E. coli*: -80°C
5-6	pEXP5-CT/TOPO® TA Expression Kit	Dry ice	pEXP5-CT/TOPO® Reagents: -20°C One Shot® TOP10 Chemically Competent *E. coli*: -80°C

Kit Contents and Storage, continued

Expressway™ High Yield Expression Module

The following reagents are included in the Expressway™ High Yield Expression Module (Box 1). 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	Composition	Amount	Storage
Expressway™ IVPS High-Yield *E. coli* Extract	Proprietary	5 × 400 µl	-80°C
Expressway™ 2.5X IVPS *E. coli* Reaction Buffer (-A.A.)	Proprietary	5 × 400 µl	-80°C
Expressway™ 2X IVPS Feed Buffer	Proprietary	5 x 500 µl	-80ºC
DNase/RNase-Free Distilled Water	---	2 × 2 ml	-20°C or -80°C
T7 Enzyme Mix	Proprietary	200 µl	-80°C -20°C after initial use
pEXP5-NT/CALML3 Expression Control Plasmid	0.5 µg/µl in TE Buffer, pH 8.0	20 µl	-20°C

Expressway™ Milligram Amino Acids Module

The following reagents are included in the Expressway™ Milligram Amino Acids Module (Box 2). **Store at -20°C.**

Item	Composition	Amount
50 mM Amino Acids (-Met, -Cys)	Contains all amino acids (50 mM) except for Met and Cys in 50 mM HEPES, pH 11	250 µl
75 mM Methionine (Met)	75 mM Met in 50 mM HEPES, pH 7.5, 4 mM DTT	200 µl
75 mM Cysteine (Cys)	75 mM Cys in 50 mM HEPES, pH 7.5, 4 mM DTT	200 µl

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

The Expressway™ Milligram Cell-Free *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 3-6) to facilitate TOPO® Cloning-based generation of plasmid templates to express your gene of interest in the Expressway™ Milligram System. Each kit contains:

- pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA reagents (Boxes 3 and 5)
- One Shot® TOP10 Chemically Competent *E. coli* (Boxes 4 and 6)

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.

Accessory Products

Accessory Products

Some of the reagents supplied in the Expressway™ Milligram 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, see www.invitrogen.com or contact Technical Service (page 27).

Product	Quantity	Catalog no.
pEXP5-NT/TOPO® TA Expression Kit	10 reactions	V960-05
pEXP5-CT/TOPO® TA Expression Kit	10 reactions	V960-06
DNase/RNase-Free Distilled Water	500 ml	10977-015
Ampicillin	20 ml (10 mg/ml)	11593-019
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Coomassie Brilliant Blue R®-250 Protein Stain	10 g	15528-011
SimplyBlue™ SafeStain	1 L	LC6060
BenchMark™ Protein Ladder	2 x 250 µl	10747-012

Products to Detect Recombinant Fusion Protein

If you are expressing your recombinant protein from pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, 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.

Product	Epitope	Catalog no.
Anti-HisG Antibody	Detects the N-terminal polyhistidine (6xHis) tag followed by glycine: HHHHHHG	R940-25
Anti-HisG-HRP Antibody		R941-25
Anti-HisG-AP Antibody		R942-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997): HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25

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Accessory 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 from pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, respectively, 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

Introduction

Overview

Introduction

The Expressway™ Milligram Cell-Free *E. coli* Expression System is designed for *in vitro* transcription and translation of target DNA to protein in a single reaction, and allows synthesis of up to milligram quantities of a recombinant protein of interest from an expression construct in only 4-6 hours. This flexible system allows production of recombinant protein containing unnatural amino acids (*e.g.* selenomethionine or selenocysteine). Once purified, the resulting recombinant protein is suitable for use in downstream structural analyses including x-ray crystallography.

The System is available with the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors to allow rapid and easy generation (using TOPO® Cloning technology) of an expression construct containing an N- or C-terminal tag, respectively, to produce a recombinant fusion protein that may be easily detected and purified. For more information about the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vectors, see page 3. For more information about cell-free expression systems, see published references (Katzen *et al.*, in press).

Note: The Expressway™ Milligram protein synthesis reaction does not require the use of specialized equipment, and yields milligram amounts of recombinant protein in a relatively small volume, allowing use of significantly lower amounts of unnatural amino acids than cell-based expression systems.

Applications

The Expressway™ Milligram 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:

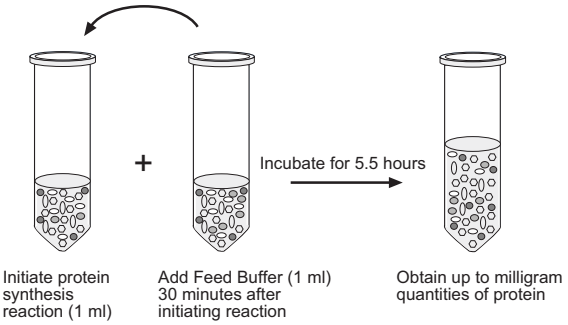
- Producing up to milligram quantities of recombinant protein (or radiolabeled protein) suitable for use in downstream applications including biochemical, physical, and structural characterization
- Producing up to milligram quantities of recombinant protein containing incorporated unnatural amino acids (*e.g.* selenomethionine or selenocysteine) for use in x-ray crystallography
- Analyzing mutants
- Verifying cloned gene products
- Producing proteins that are toxic to cells

continued on next page

Overview, continued

How the System Works

The Expressway™ Milligram 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 6 hours.



Components of the System

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

- An optimized S30 *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, -Cys) required for protein synthesis to occur
- Methionine and cysteine provided separately to allow production of radiolabeled recombinant protein or recombinant protein containing unnatural amino acids
- Proprietary T7 Enzyme Mix containing T7 RNA polymerase and other components optimized for T7-based expression from DNA templates (Studier *et al.*, 1990)
- Two optimized expression vectors, pEXP5-NT/TOPO® and pEXP5-CT/TOPO®, to allow rapid generation of N- or C-terminal fusion constructs, respectively (Catalog no. K9900-96 only; see the next page)
- 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 25 for more information)

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Overview, continued

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

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors are supplied with the 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™ Milligram 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 27).

Other Expressway™ Systems

If you have used other Invitrogen Expressway™ Cell-Free *E. coli* Expression Systems, note that some of the components from these other Systems are compatible with the Expressway™ Milligram Cell-Free *E. coli* Expression System (*e.g.* T7 Enzyme Mix, 75 mM Methionine). Other components including the Expressway™ IVPS *E. coli* Extract and the Expressway™ 2.5X IVPS *E. coli* Reaction Buffer supplied with other Expressway™ kits contain different formulations and may not be compatible with the Expressway™ Milligram System.

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

Experimental Outline

The table below describes the major steps required to synthesize your recombinant protein of interest using the Expressway™ Milligram 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.	4-6
2	Purify your DNA template.	7
3	Perform the protein synthesis reaction.	8–12
4	Analyze recombinant protein by polyacrylamide gel electrophoresis or other method of choice.	13-15
5	Purify your recombinant protein, if desired.	18

Methods

General Guidelines to Generate the DNA Template

Introduction

Successful use of the Expressway™ Milligram 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.

The pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors supplied with the kit (Catalog no. K9900-96 only) allow you to generate a plasmid DNA template containing your gene of interest and transcription and translation regulatory elements in the optimal context for use in the Expressway™ Milligram Cell-Free *E. coli* Expression System to produce high yields of recombinant protein. For more information about the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® vectors, see page 6.

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)
- 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.

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General Guidelines to Generate the DNA Template, continued

DNA Templates

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

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

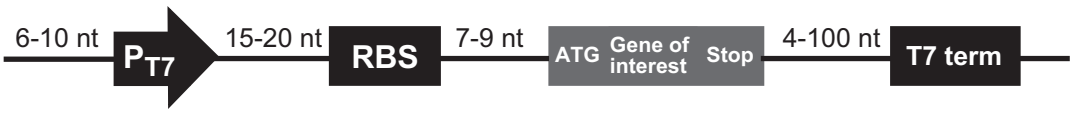
For proper expression, all templates must contain the T7 promoter, an initiation codon, and a prokaryotic Shine-Dalgarno ribosome binding site (RBS) upstream of the gene of interest. See below for a discussion of template optimization.

Optimal Configuration of DNA Template

Optimized expression vectors (*i.e.* pEXP5-NT/TOPO® and pEXP5-CT/TOPO®; see the next page) are supplied with the Expressway™ Milligram Cell-Free *E. coli* Expression System (Catalog no. K9900-96); however, other expression vectors or DNA templates may be used. 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).

Note: For examples, refer to the diagrams of the TOPO® Cloning site for pEXP5-NT/TOPO® or pEXP5-CT/TOPO® (see the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual).

- 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™ Milligram 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.



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General Guidelines to Generate the DNA Template, continued

Expressway™ Milligram Compatible Vectors

Many T7-based expression vectors are suitable for use as templates for the Expressway™ Milligram System. At a minimum, these vectors must contain the following:

- T7 promoter, RBS, and T7 terminator with the suitable spacing and sequence configuration for optimal expression of protein (see previous page)
- Bacteriophage ϕ10-s10 segment (*i.e.* ϕ10 promoter and the translation initiation region for the gene 10 protein) contains a region that forms a hypothetical stem-and-loop structure as described by Studier *et al.*, 1990

For recommended T7-based vectors to use with the Expressway™ Milligram System, see **pEXP5-NT/TOPO® and pEXP5-CT/TOPO® Vectors,** 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™ Milligram System. Both vectors contain the bacteriophage ϕ10-s10 segment described above. In addition, the vectors contain the following features:

- T7 promoter, RBS, and T7 terminator with spacing and sequence configuration optimized to allow the highest levels of protein expression in the Expressway™ Milligram System (see page 20 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™ Milligram System. Protein yields can vary significantly depending on whether the recombinant protein of interest is expressed as an N- or C-terminal fusion. For an example, see page 19.

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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® or pEXP5-CT/TOPO®, 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:

- 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.
- **Do not gel-purify** your DNA template. Purified DNA solution obtained from agarose gels significantly inhibits the protein synthesis reaction.
- 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™ Milligram Cell-Free *E. coli* Expression System. 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 (*i.e.* 50 ml conical tubes and pipette tips) when performing the protein synthesis reaction.

General Guidelines to Perform the Protein Synthesis Reaction

Follow the general guidelines below to perform the protein synthesis reaction:

- **Reaction volume:** The standard volume of the protein synthesis reaction is 2 ml (*i.e.* 1 ml initial reaction + 1 ml Feed Buffer) to generate up to milligram quantities of recombinant protein. Note that protein yields may vary depending on the nature of the protein expressed and the template used.

  Note: The protein synthesis reaction is scalable. Simply adjust the reagent volumes used proportionately according to the reaction volume.
- **Amount of DNA template required:** For a standard 2 ml protein synthesis reaction, use 10-15 µg of template DNA (plasmid or linear DNA). For optimal results, purify DNA template before use (see page 7).
- **Reaction vessel:** Use a reaction vessel that contains a large enough surface area to allow moderate mixing to occur. We recommend performing the 2 ml protein synthesis reaction in a sterile, RNase-free 50 ml conical tube. Other reaction vessels including 6-well or 12-well untreated culture plates are suitable. For other reaction sizes, use an appropriate reaction vessel.
- **Incubation conditions:** To obtain optimal protein yield, **it is critical to mix the reaction thoroughly throughout the incubation period.** We recommend using a floor shaking incubator set to shake at 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%.
- **Incubation temperature:** 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).
- **Amino acid concentration:** Use an amino acid concentration ranging from 1 mM to 4 mM (for each amino acid) 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 **Note** and **Using Unnatural Amino Acids**, next page).
- **Feed Buffer:** Add 1 volume (*i.e.* 1 ml for a 2 ml reaction) of Feed Buffer (containing Expressway™ 2X IVPS Feed Buffer and amino acids) to the protein synthesis reaction after an initial 30-minute incubation (see the protocol on page 12). Higher protein yields may be obtained by adding one half-volume of Feed Buffer (*i.e.* 0.5 ml for a 2 ml reaction) at 30 minutes and again at 2 hours after initiating the protein synthesis reaction.

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Performing the Protein Synthesis Reaction, continued

Note

The amount of amino acid solutions supplied in the kit (*i.e.* 50 mM Amino Acids (-Met, -Cys), 75 mM Methionine, and 75 mM Cysteine) is sufficient to synthesize 5 recombinant proteins using an amino acid concentration of 1.25 mM each. If you wish to use a higher concentration of amino acids in your synthesis reaction, note that you may need to obtain your own supply of amino acids.

Using Unnatural Amino Acids

Methionine and cysteine are 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 of choice, you may use the following types of unnatural amino acids:

- Heavy metal-labeled methionine or cysteine: Use selenomethionine (Budisa *et al.*, 1995; Doublie, 1997; Hendrickson *et al.*, 1990) or selenocysteine (Strub *et al.*, 2003) to produce labeled protein for use in X-ray crystallographic studies.
- Radiolabeled methionine: Use ^{35}S-Methionine to produce radiolabeled protein for use in expression and purification studies.

To incorporate one of these unnatural amino acids into your recombinant protein, reduce the amount of unlabeled 75 mM Methionine or Cysteine used in the reaction, as desired, and substitute with the same amount of the unnatural methionine or cysteine.

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®. The 19.5 kDa fusion protein may be detected by Western blot using an Anti-HisG Antibody, and purified using metal-chelating resin. For details about the vector, refer to page 25. To propagate and maintain the 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.

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Performing the Protein Synthesis Reaction, continued

Materials Needed

You should have the following materials on hand before beginning:

Supplied by the user:

- pEXP5-NT/TOPO® or pEXP5-CT/TOPO® expression construct or other suitable DNA template (purified; resuspended in TE or water at a concentration greater than 500 ng/µl)
- Labeled methionine (*e.g.* selenomethionine) or cysteine (optional; if producing labeled recombinant protein)
- Sterile, RNase-free, 50 ml conical tubes (one for each reaction)
- RNase-free pipette tips and microcentrifuge tubes
- Standard floor shaking incubator (set to 30°C or 37°C and 300 rpm)

Supplied with the kit:

- Expressway™ IVPS High Yield *E. coli* Extract (thaw on ice one vial per sample; see **Important Note** on the next page)
- Expressway™ 2.5X IVPS *E. coli* Reaction Buffer (-A.A.) (thaw on ice one vial per sample; see **Important Note** on the next page)
- Expressway™ 2X IVPS Feed Buffer (thaw on ice one vial per sample; see **Important Note** on the next page)
- T7 Enzyme Mix (keep on ice; store at -20°C after initial use)
- 50 mM Amino Acids (-Met, -Cys)
- 75 mM Methionine
- 75 mM Cysteine
- DNase/RNase-free distilled water (supplied with the kit)
- pEXP5-NT/CALML3 control plasmid (optional; 1 µg/µl in TE Buffer, pH 8.0)

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Performing the Protein Synthesis Reaction, continued

Important

The kit supplies five tubes each of Expressway™ IVPS High Yield *E. coli* 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). If you perform a standard 2 ml protein synthesis reaction to obtain up to milligram quantities of recombinant protein, you will need **one tube each** of the High Yield *E. coli* Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer.

Depending on your needs, it is possible to synthesize recombinant protein in a smaller reaction volume (*e.g.* for pilot experiments or to compare different expression constructs). Under these circumstances, you will not obtain up to milligram quantities of protein, and you will not use the entire contents of a tube of High Yield *E. coli* Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer in a single experiment. If you wish to synthesize protein in a smaller reaction size, we recommend the following.

1. **Thaw on ice** the High Yield *E. coli* Extract, *E. coli* Reaction Buffer (-A.A.), and 2X Feed Buffer.
2. Remove the amount of High Yield *E. coli* 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.

   Note: To prevent contamination, use RNase-free, sterile pipette tips and wear gloves when removing reagents from the tubes.

Do not store the High Yield *E. coli* 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.

Note: Freezing and thawing the High Yield *E. coli* 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.

Note

When thawing the 50 mM Amino Acids (-Met, -Cys), the solution may have a brown or yellowish tint. This is normal and does not affect the activity of the amino acids.

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 in a standard 2 ml reaction. If you are scaling up or down the reaction, adjust the volume of reagents used accordingly.

1. **For each sample**, add the following reagents to a sterile, RNase-free 50 ml conical tube.

Reagent	Amount
IVPS High Yield *E. coli* Extract	400 µl
2.5X IVPS *E. coli* Reaction Buffer (-A.A.)	400 µl
50 mM Amino Acids (-Met, -Cys)	25 µl
75 mM Methionine*	20 µl
75 mM Cysteine*	20 µl
T7 Enzyme Mix	20 µl
DNA Template	10-15 µg
DNase/RNase-free Distilled Water	to a final volume of 1 ml

***Note:** To generate labeled protein using selenomethionine, selenocysteine, or ^{35}S-Methionine, use 1-20 µl of the labeling reagent, as desired and bring up the remaining volume to 20 µl with unlabeled 75 mM Methionine or Cysteine, as appropriate.

2. Tighten the screw cap on the conical 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	500 µl
50 mM Amino Acids (-Met, -Cys)	25 µl
75 mM Methionine*	20 µl
75 mM Cysteine*	20 µl
DNase/RNase-free Distilled Water	to a final volume of 1 ml

***Note:** If you are generating labeled protein using selenomethionine, selenocysteine, or ^{35}S-Methionine, use 1-20 µl of the labeling reagent, as desired and bring up the remaining volume to 20 µl with unlabeled 75 mM Methionine or Cysteine, as appropriate.

4. After 30 minutes of incubation (from Step 2 above), add 1 ml of the Feed Buffer to the sample (total volume = 2 ml).
5. Tighten the screw cap on the conical tube and return the sample to the shaking incubator (300 rpm). Incubate for 4-6 hours at 30°C (or 37°C as appropriate).
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™ Milligram 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 (see page 19 for an example). 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 16).

Materials Needed

You should have the following materials on hand before proceeding:

- Acetone (room temperature)
- 1X SDS-PAGE sample buffer (see page 26 for a recipe)
- Appropriate polyacrylamide gel to resolve your protein of interest (see the next page)
- Coomassie Brilliant Blue R®-250 Stain (Catalog no. 15528-011) or other appropriate protein stain (*e.g.* SimplyBlue™ SafeStain; Invitrogen, 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 27).

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 appropriate antibody to the epitope tag (see table) or an antibody to your protein.

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

For more information about the Anti-HisG Antibodies or the Anti-His(C-term) Antibodies, see www.invitrogen.com or call Technical Service (page 27). For ordering information, see page vii.

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. To detect the CALML3 fusion protein by Western blot analysis, use one of the Anti-HisG Antibodies available from Invitrogen (see page vii 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 the 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 18.

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 12 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 12 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/1% sodium pyrophosphate 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 12 into separate glass tubes. Add 3 ml of 10% TCA to each glass tube and incubate tubes at +4ºC for 20 minutes.
2. Wet individual glass fiber (GF/C) filters with 10% TCA and place onto the vacuum filtration device.
3. Turn the vacuum on and pour the TCA solution from each glass tube into a sample well.
4. Wash filters twice with 5% TCA.
5. Wash filters once with 100% ethanol. Leave the vacuum on for 1 minute to allow the filters to dry.
6. Turn the vacuum off and remove the filters. Place the filters in scintillation vials, and add scintillation fluid. Count samples in a scintillation counter.
7. 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:	$\text{total cpm per 5 µl spotted} \times \frac{\text{total reaction volume}}{5}$
Specific activity:	$\frac{\text{total counts}}{\text{pmoles of methionine}}$
pmoles methionine incorporated:	$\frac{\left[(\text{TCA precipitable counts - background}) \times \frac{50}{5}\right]}{\text{specific activity}}$
pmoles of protein:	$\frac{\text{pmoles of methionine incorporated into protein}}{\text{number of methionines in protein}}$
Yield of protein:	$\text{pmoles of protein} \times \text{molecular weight of protein}$

Purifying the Recombinant Fusion Protein

Introduction

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

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™ Milligram kit):** Dilute the Expressway™ Milligram reaction (from Step 6, page 12) 1:1 with Binding Buffer (50 mM $NaPO_4$, pH 8.0, 500 mM NaCl). **Example:** For a 2 ml reaction, add 2 ml of Binding Buffer.
3. Centrifuge the Expressway™ Milligram reaction at 15,000 x g for 10 minutes at room temperature to remove aggregates and 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 containing your purified protein and dialyze, if necessary.

Sample Protein Synthesis Experiments

Introduction

This section provides examples of typical protein synthesis experiments performed using the Expressway™ Milligram Cell-Free *E. coli* Expression System and results obtained.

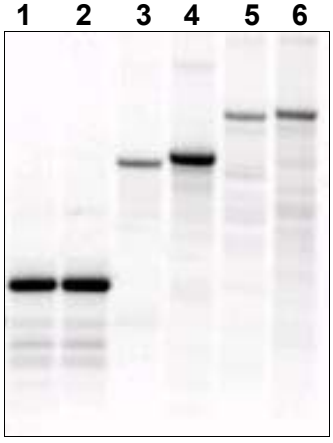
Example 1: Expressing Human ORFs from pEXP5-NT/TOPO® or pEXP5-CT/TOPO®

In this experiment, 3 human open reading frames (ORFs) encoding calmodulin-like 3 (CALML3; GenBank accession no. NM_005185), brain creatine kinase B-chain (CKB; GenBank accession no. NM_001823), and receptor-interacting serine/threonine kinase 2 (RIPK2; GenBank accession no. NM_003821) were amplified with *Taq* polymerase and TOPO® Cloned into pEXP5-NT/TOPO® or pEXP5-CT/TOPO® using the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA Expression Kit. Plasmid DNA for each expression construct was purified and used in a 100 µl protein synthesis reaction with the components supplied in the Expressway™ Milligram kit and according to the protocol on page 12. ^{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 exposed to x-ray film.

Results:

- All 3 human ORFs were expressed as N- or C-terminal fusions from pEXP5-NT/TOPO® or pEXP5-CT/TOPO®, respectively.
- The amount of CALML3 fusion protein obtained was similar when expressed as an N- or C-terminal fusion. However, the amount of CKB and RIPK2 fusion protein obtained varied significantly when expressed as an N- or C-terminal fusion. CKB and RIPK2 were expressed at higher levels as N-terminal fusions. These results illustrate the benefit of testing different template configurations when optimizing protein expression.

  Note: The pEXP5-NT/CALML3 expression construct used in this experiment (Lane 2) is identical to the pEXP5-NT/CALML3 control plasmid supplied in this kit.



Lane 1: pEXP5-CT/CALML3
Lane 2: pEXP5-NT/CALML3
Lane 3: pEXP5-CT/CKB
Lane 4: pEXP5-NT/CKB
Lane 5: pEXP5-CT/RIPK2
Lane 6: pEXP5-NT/RIPK2

continued on next page

Sample Protein Synthesis Experiments, continued

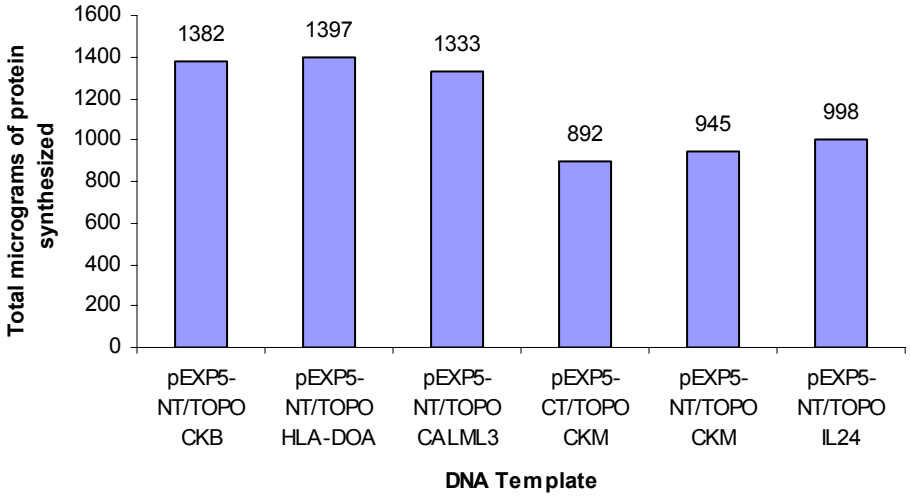
Example 2: Expressing Human ORFs Using the Expressway™ Milligram System

In this experiment, 5 human open reading frames (ORFs) encoding brain creatine kinase B-chain (CKB; GenBank accession no. NM_001823), HLA Class II alpha chain (HLA-DOA; GenBank accession no. NM_002119), calmodulin-like 3 (CALML3; GenBank accession no. NM_005185), muscle creatine kinase (CKM; GenBank accession no. NM_001823), and interleukin 24 (IL24; GenBank accession no. BC009681) were amplified with *Taq* polymerase and TOPO® Cloned into pEXP5-NT/TOPO® or pEXP5-CT/TOPO® using the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® TA Expression Kit. Plasmid DNA for each expression construct was purified and used in a 2 ml protein synthesis reaction with the components supplied in the Expressway™ Milligram kit and according to the protocol on page 12. ^{35}S-Methionine was included in each reaction for trace labeling. Radiolabeled protein was TCA precipitated, counted in a scintillation counter, and the data used to calculate total protein yield. The figure below shows the total amount of protein synthesized for each expression construct, with the total amount of protein obtained listed above each construct.

Results:

- When expressing 5 different human ORFs using the Expressway™ Milligram System, a high yield of recombinant protein was obtained in every case. Total protein yields ranged from nearly one milligram for 2 human ORFs to over 1.3 milligrams for 3 human ORFs.
- The amount of recombinant protein synthesized varies depending on the nature of the protein and on the DNA template. Note that levels of CKM fusion protein obtained varied when expressed from pEXP5-NT/TOPO® or pEXP5-CT/TOPO®.

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



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 5 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 (*e.g.* in pEXP5-NT/TOPO® or pEXP5-CT/TOPO®	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 7.
	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	Protein synthesis reaction performed in an inappropriately sized vessel	Perform the 2 ml protein synthesis reaction in a sterile, RNase-free, 50 ml conical tube, 6-well, or 12-well plate.
	Sample incubated in a non-shaking incubator or water bath	Incubate sample in a floor incubator with shaking (275-325 rpm).
	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.* 0.5 ml Feed Buffer to 1 ml sample) 30 minutes and 2 hours after initiating protein synthesis. **Note:** It is possible to add smaller volumes of Feed Buffer to the sample more frequently (*e.g.* 0.25 ml Feed Buffer to 1 ml sample every 45 minutes over 3 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.
Recombinant protein is pelleted after centrifugation	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™ High Yield *E. coli* Extract, Expressway™ 2.5X IVPS *E. coli* Reaction Buffer, and Expressway™ 2X IVPS Feed Buffer. Follow guidelines on page 11. 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.
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™ IVPS High Yield *E. coli* 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 26 to prepare 1X SDS-PAGE sample buffer.

continued on next page

Troubleshooting, continued

Analyzing Proteins, continued

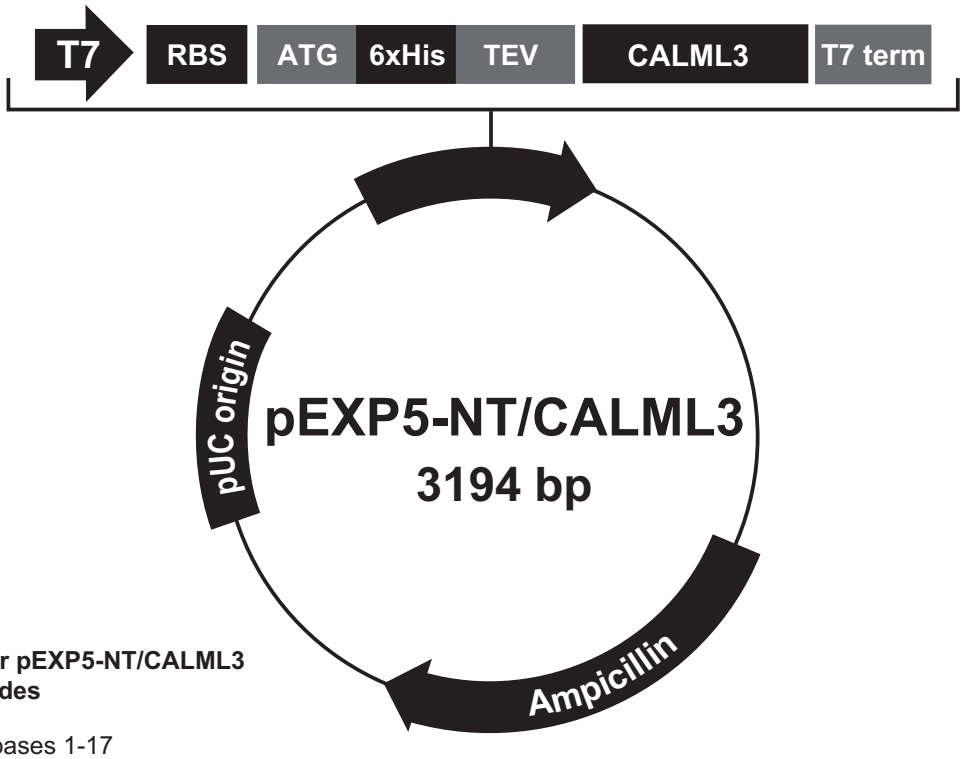
Problem	Reason	Solution
Multiple bands observed on the polyacrylamide gel, continued	Internal ATG codons in the context of RBS-like sequences	• Check the sequence of your gene and search for potential RBSs with the proper spacing from internal methionines. • Replace the methionine or change RBS sequence(s) 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 13.
	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 27).**



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 Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

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

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MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

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Limited Warranty

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Purchaser Notification

Introduction

Use of the Expressway™ Milligram 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.

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continued on next page

Purchaser Notification, continued

Limited Use Label License No. 30: T7 Expression System

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Limited Use Label License No. 266: *In Vitro* Protein Synthesis Technologies

This product is the subject of U.S. Patent Number 6,548,276, filed September 5, 2001, U.S. Patent Number 6,337,191 filed July 21, 2000, and U.S. Patent Number 6,168,931 filed March 17, 1999. For Research Use Only - Not for any clinical, therapeutic or diagnostic use in Humans or Animals.

Product Qualification

Introduction	This section describes the criteria used to qualify the components of the Expressway™ Milligram Cell-Free *E. coli* Expression System.
Protein Synthesis Reagents	Each lot of the Expressway™ Milligram Cell-Free *E. coli* Expression System is functionally tested for protein generation by expressing the CALML3 fusion protein from the pEXP5-NT/CALML3 Control Plasmid using the protocol on page 12. The yield and molecular weight of the CALML3 fusion protein is confirmed by SDS-PAGE analysis with the BenchMark™ Protein Ladder and known amounts of a purified protein. A 2 ml Expressway™ Milligram protein synthesis reaction must yield greater than 1.5 mg of CALML3 fusion protein.
Expressway™ Milligram Amino Acids Module	The pH of each lot of amino acids supplied in the kit (*i.e.* 50 mM Amino Acids (-Met, -Cys), 75 mM Methionine, and 75 mM Cysteine) is confirmed. In addition, the amino acid solutions are functionally tested in a cell-free protein synthesis reaction with the other components supplied in the kit (see above).
pEXP5-NT/ CALML3 Vector	The structure of the pEXP5-NT/CALML3 vector is verified by restriction enzyme digestion. In addition, the vector is used as the template in a cell-free protein synthesis reaction to qualify the other components of the Expressway™ Milligram kit (see above).
pEXP5-TOPO® Kits	Refer to the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits manual for a detailed description of the criteria used to qualify the components of the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kits.

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