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Instruction Manual

Expressway™ HTP Cell-Free *E. coli* Expression Kit

Pre-mixed components for cell-free protein synthesis in a high-throughput format

Catalog no. K9900-80

Version C
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Table of Contents

Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the Expressway™ HTP Cell-Free *E. coli* Expression Kit. If you are a first time user, we recommend following the detailed protocols in the manual.

Step	Action
Generate the DNA Construct	For optimal expression using the Expressway™ HTP Cell-Free *E. coli* Expression Kit, clone your gene of interest into a T7-based expression vector or use PCR to generate a DNA template containing the elements discussed on page 4.
Purify the DNA Construct	Use your method of choice or a commercially available kit to purify your plasmid or linear DNA template. **Do not gel-purify your linear DNA**. Resuspend purified DNA in 1X TE Buffer, pH 8.0 or water to a concentration of 200-500 ng/µl. Take care to avoid contamination of your DNA construct with ethanol, salt, or RNase.
Perform the Protein Synthesis Reaction	1. For each reaction, aliquot 22.5 µl of the Expressway™ HTP Cell-Free *E. coli* Expression Mix into a well. 2. Add 0.5 µg of purified DNA to each reaction. Do not exceed a total reaction volume of 25 µl per well. 3. Bring the final reaction volume to 25 µl with DNase/RNase-free water. Mix gently by pipetting up and down. 4. Securely place the lid or sealing tape onto the plate. You may briefly centrifuge the plate, if desired. 5. Shake the samples in a thermomixer (1,400 rpm) or a floor shaker (300 rpm) at 37°C for 2 hours. **Alternative:** Incubate plate at 37ºC for 2 hours in a standard incubator. **Note:** It is possible to incubate reactions for up to 4 hours to obtain greater protein yield. You may also incubate reactions at 30°C to decrease the rate of protein synthesis and to promote proper folding. 6. After the 2 hour incubation, condensation may appear on the lid or sealing tape. This is expected and will not affect your synthesis reaction. If you notice significant amounts of the reaction mix on the lid or sealing tape, we recommend briefly centrifuging the samples. Proceed to analyze samples.
Analyze Sample	Use any method of choice including Lumio™ detection, Coomassie® blue staining, Western blot, or activity assay to analyze your sample. If you plan to use Coomassie® blue staining or Western blot to analyze your sample, we recommend you precipitate the proteins with acetone prior to performing polyacrylamide gel electrophoresis (see page 10).

Important Information

Shipping/Storage

The Expressway™ HTP Cell-Free *E. coli* Expression Kit is shipped on dry ice. Upon receipt, store at -80ºC.

Item	Storage
Expressway™ HTP Cell-Free *E. coli* Expression Mix	-80ºC
pT7/CAT Control Plasmid	-20ºC or -80ºC

Kit Contents

The following components are supplied in the Expressway™ HTP Cell-Free *E. coli* Expression Kit. Store components at -80ºC or as detailed below.

Item	Composition	Amount
Expressway™ HTP Cell-Free *E. coli* Expression Mix	Expressway™ *E. coli* Extract Expressway™ 2.5X *E. coli* Reaction Buffer (includes methionine) T7 Enzyme Mix	5 x 2.5 ml
pT7/CAT Control Plasmid	Lyophilized in TE Buffer, pH 8.0	10 µg

Product Qualification

Each lot of the Expressway™ HTP Cell-Free *E. coli* Expression Kit is functionally tested for protein generation by incorporation of ^{35}S-methionine. Expressway™ HTP protein synthesis reactions are performed in 1.5 ml microcentrifuge tubes using the protocol on page 8 and must yield greater than 8 µg of CAT protein from the pT7/CAT control plasmid. The pT7/CAT control plasmid is verified by restriction enzyme digestion.

Accessory Products

Additional Products

Invitrogen products suitable for use with the Expressway™ HTP Cell-Free *E. coli* Expression Kit are available separately. Ordering information is provided below.

Item	Quantity	Catalog no.
DNase/RNase-Free Distilled Water	500 ml	10977-015
Ampicillin	200 mg	11593-019
E-PAGE™ *96* 6% High-Throughput (HTP) Protein Electrophoresis System	1 Starter Kit	EPST96-06
SimplyBlue™ SafeStain	1 L	LC6060
Lumio™ Green Detection Kit	1 kit	LC6090
CAT Antiserum*	50 µl	R902-25
PureLink™ HQ 96 Plasmid DNA Purification Kit	384 reactions	K2100-96

*The amount of antibody supplied is sufficient for 25 Western blots.

Expression Vectors

Invitrogen offers many T7-based expression vectors that contain an optimal configuration of transcription/translation elements for protein expression using the Expressway™ HTP Cell-Free *E. coli* Expression Kit. Refer to the table below for features of these expression vectors and for ordering information.

Vector	Feature	Catalog no.
pEXP1-DEST	Gateway® destination vector with N-terminal 6xHis tag, Xpress™ epitope, and EK cleavage site	V960-01
pEXP2-DEST	Gateway® destination vector with C-terminal V5 epitope and 6xHis tag	V960-02
pEXP3-DEST	Gateway® destination vector with N-terminal 6xHis tag, Lumio™ tag, and TEV cleavage site	V960-03
pEXP4-DEST	Gateway® destination vector with C-terminal Lumio™ tag and 6xHis tag	V960-04
pCR®T7/NT-TOPO®	TOPO®-adapted vector with N-terminal 6xHis tag, Xpress™ epitope, and EK cleavage site	K4200-01
pCR®T7/CT-TOPO®	TOPO®-adapted vector with C-terminal V5 epitope and 6xHis tag	K4210-01
pRSET	N-terminal 6xHis tag, Xpress™ epitope, and EK cleavage site	V351-20

Introduction

Overview

Introduction

The Expressway™ HTP Cell-Free *E. coli* Expression Kit is specifically designed for T7-based, *in vitro* transcription and translation of target DNA to protein in a high-throughput format. The Expressway™ Expression Mix provides all the necessary reagents required for performing *in vitro* expression reactions, except for the DNA template, pre-mixed and ready-to-use. The *E. coli* extract and T7 Enzyme Mix provided in the Expressway™ Expression Mix have been optimized for expressing full-length, active protein from circular or linear DNA templates in approximately 2 hours.

Components of the System

The major components of the Expressway™ HTP Cell-Free *E. coli* Expression Mix are provided pre-mixed and include:

- Optimized S30 *E. coli* extract (Zubay, 1973) for increased stability of circular and linear DNA constructs during transcription and translation
- Proprietary T7 Enzyme Mix containing T7 RNA polymerase and other components optimized for T7-based expression (Studier *et al.*, 1990)
- Optimized reaction buffer composed of an ATP regenerating system and all the required amino acids including methionine (Kim *et al.*, 1996; Lesley *et al.*, 1991; Pratt, 1984)

Applications

The Expressway™ HTP Cell-Free *E. coli* Expression Kit is suitable for use in the following applications:

- Characterizing proteins
- Analyzing mutants
- Verifying cloned gene products
- Producing proteins which are toxic to cells

Expressway™ HTP Scripts for Automation

The Expressway™ HTP Cell-Free *E. coli* Expression Mix is provided pre-mixed in 5 single tubes for easy set up with robotic systems. If you wish to automate your Expressway™ HTP protein synthesis reactions, you may obtain script files that have been tested and qualified for use on the Biomek® FX Automation Workstation and the Tecan Freedom EVO™ Workstation. Downloadable script files are available on our Web site at www.invitrogen.com/expressway.

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Overview, continued

Experimental Outline

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

Step	Action	Pages
1	Generate and purify the DNA template.	3-5
2	Perform the protein synthesis reaction.	6-8
3	Analyze your sample using polyacrylamide gel electrophoresis, Western blot analysis, or activity assay.	9-10

Methods

Generating the DNA Template

Introduction

Successful use of the Expressway™ HTP Cell-Free *E. coli* Expression Kit requires only the addition of a DNA template containing the gene of interest placed within the proper context of transcription/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. Guidelines are provided to produce your DNA template in an optimal configuration for protein expression using the Expressway™ HTP Cell-Free *E. coli* Expression Kit.

Factors Affecting Protein Yield

The yield of protein produced in *in vitro* transcription and translation systems is generally dependent on many factors, including:

- Size of the protein
- Sequence of the gene of interest
- Spacing of the T7 promoter and the gene of interest in the DNA template
- 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.

DNA Templates

The following DNA templates may be used in the Expressway™ HTP System.

- Supercoiled plasmid DNA
- Linear DNA
- PCR product

For proper expression using the Expressway™ HTP Expression Kit, 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 the next page for a discussion of template optimization.

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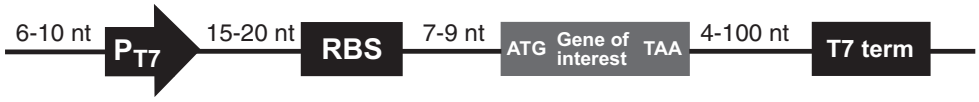
Generating the DNA Template, continued

Optimal Configuration of DNA Template

Optimized expression vectors for use with the Expressway™ HTP Expression Kit are available separately from Invitrogen (see page viii for ordering information). 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). See the next page for an example.

- 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 **T7 Expression Vectors**, below, 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. For the sequence of the T7 terminator, see the next page.



T7 Expression Vectors

Many T7-based expression vectors contain a T7 promoter, RBS, and T7 terminator with the suitable spacing and sequence configuration for optimal expression of protein. In general, T7-based expression vectors that contain the bacteriophage ϕ10-s10 segment (*i.e.* ϕ10 promoter and the translation initiation region for the gene 10 protein) are recommended for use (see Studier *et. al.*, 1990 for a list). The ϕ10-s10 segment contains a region that forms a hypothetical stem-and-loop structure as described by Studier *et al.*, 1990. Refer to page viii for a list of T7-based expression vectors available from Invitrogen that contain this configuration.

Note: T7 expression vectors that contain the T7*lac* promoter (*e.g.* pET101/D-TOPO® available from Invitrogen) are suitable for use with the Expressway™ HTP Cell-Free *E. coli* Expression Kit although protein yield may be reduced. In these vectors, the *lac* operator replaces the region containing the hypothetical stem-and-loop structure.

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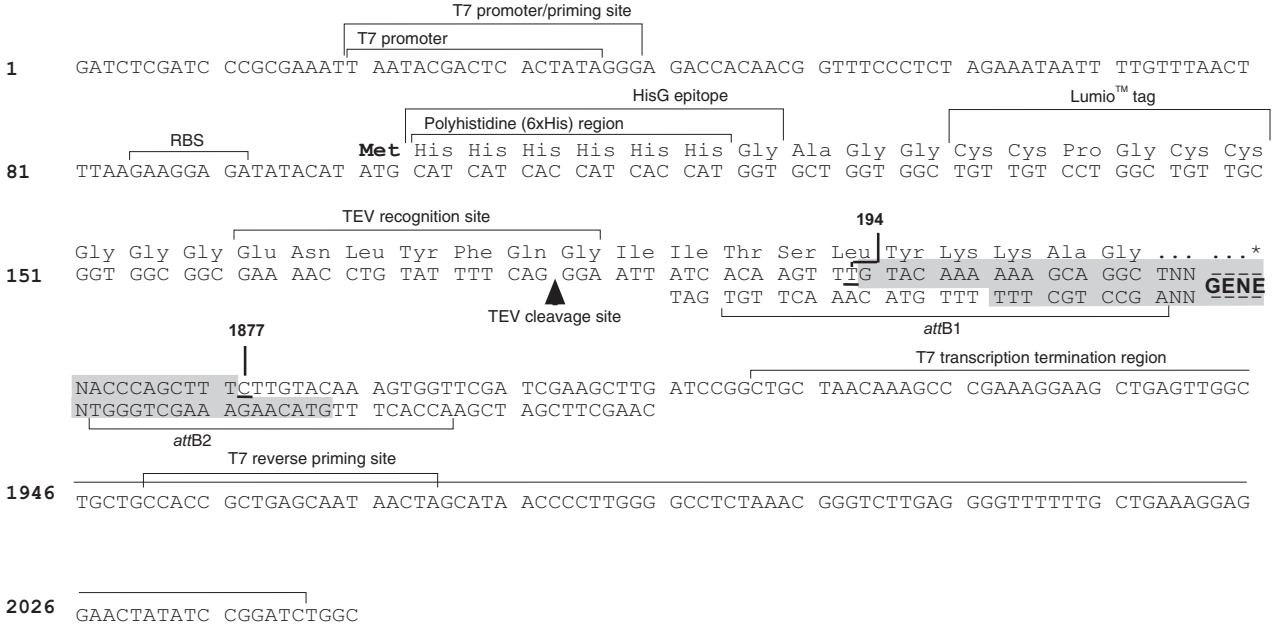
Generating the DNA Template, continued

Example

The T7-based Gateway® destination vector pEXP3-DEST (Invitrogen Catalog no. V960-03) allows you to generate an expression clone containing your gene of interest in an optimal configuration for expression using the Expressway™ HTP Cell-Free *E. coli* Expression Kit. To illustrate the points discussed on the previous page, the region surrounding the gene of interest after generating an expression clone with pEXP3-DEST is diagrammed below.



Purifying the DNA Template

We recommend purifying the DNA before proceeding to the protein synthesis reaction as purified DNA results in higher yield of active, full-length protein. You may use a variety of methods to purify your DNA template including commercial DNA purification kits or CsCl gradient centrifugation. For protocols to purify DNA, refer to published reference sources (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989). 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 PCR products or linear DNA, use a suitable purification kit.
- Do not use ammonium acetate for DNA precipitation as any residual contamination may inhibit translation. Use sodium acetate.
- Purified DNA **must** be free of RNases (wear gloves and use RNase-free reagents when preparing DNA).
- Make sure purified DNA is free of excess ethanol or salt as both can inhibit translation.

  Note: Wash ethanol precipitated DNA with 70% ethanol to remove excess salt.
- Resuspend purified DNA in 1X TE Buffer or water to a final concentration of 250-500 ng/µl.

Performing the Protein Synthesis Reaction

Introduction

Once you have obtained purified DNA, you are ready to synthesize your recombinant protein using the Expressway™ HTP Cell-Free *E. coli* Expression Mix. General guidelines and instructions to produce your recombinant protein are provided in this section.

Important

Five tubes containing 2.5 ml each of Expressway™ HTP Cell-Free *E. coli* Expression Mix are supplied with the kit. Each tube is sufficient for 96 protein synthesis reactions using a 25 µl reaction volume. If you will not be using the entire contents of a tube in a single experiment, you may wish to aliquot any remaining Expression Mix into multiple tubes before returning to a -80ºC freezer.

Note: The Expressway™ HTP Expression Mix may be thawed **on ice** up to two times without loss of activity.

If you will be synthesizing proteins in a 96-well format, we recommend using plates with round or U-shaped wells (*e.g.* Fisherbrand® Clear Polystyrene 96-well Plate, Fisher Scientific Catalog no. 12-565-500) for optimal yields. For a 384-well format, you may use plates with wells of any shape. Make sure that plates (96-well or 384-well) **do not** have high-binding capacity.

Additional Equipment

To obtain the optimal yield of protein, we recommend using a floor shaker or a thermomixer to shake your samples at 37ºC during the protein synthesis reaction (see Step 5 in the protocol on page 8). If you are using 96- or 384-well plates, you will need to equip the floor shaker or thermomixer with the appropriate adaptors.

If a floor shaker or thermomixer is unavailable, you may also incubate your samples in a 37ºC incubator, without shaking, although protein yields may not be optimal.

Amount of DNA to Use

You will need 0.5 µg of purified DNA for each 25 µl Expressway™ HTP protein synthesis reaction. Because you may add up to 2.5 µl of your DNA depending on the amount of radiolabeled methionine (if any) used in the reaction, we recommend that you resuspend your DNA to a final concentration of 200-500 ng/µl in TE Buffer, pH 8.0 or water.

Note: You may scale up or scale down the 25 µl Expressway™ HTP reaction volume depending on your particular experiment or application. However, we recommend that you keep the DNA:total reaction volume ratio constant (*i.e.* 0.5 µg template DNA per 25 µl reaction).

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Performing the Protein Synthesis Reaction, continued

Positive Control

The pT7/CAT vector is provided in the kit for use as positive control for protein expression. pT7/CAT allows expression of the chloramphenicol acetyltransferase (CAT) protein and may be detected by Western blot or functional assay. For details about the vector, refer to page 17. To propagate and maintain the plasmid:

1. Resuspend the vector in 20 µl of sterile water to prepare a 0.5 µg/µl stock solution.
2. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, OmniMAX™ 2, or equivalent. Use 10 ng of plasmid for transformation.
3. Select transformants on LB agar plates containing 50-100 µg/ml ampicillin.
4. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Important

RNase contamination may affect protein yield. To reduce the chances of RNase contamination, wear gloves and use RNase-free reagents (*i.e.* microcentrifuge tubes and pipette tips) when performing the protein synthesis reaction.

Generating Radiolabeled Proteins

If you are generating radiolabeled proteins, note that unlabeled methionine is present in the Expressway™ HTP Cell-Free *E. coli* Expression Mix. The final concentration of unlabeled methionine in a standard 25 µl reaction will be 1.5 mM.

Materials Needed

You should have the following materials on hand before beginning:

- Expressway™ HTP Cell-Free *E. coli* Expression Mix (supplied with the kit; thaw **on ice**)
- DNA template (purified; resuspended in TE Buffer, pH 8.0 or water at a recommended concentration of 200-500 ng/µl)
- pT7/CAT control plasmid, optional (supplied with the kit; resuspended to 0.5 µg/µl in sterile water)
- DNase/RNase-free water, optional (Invitrogen Catalog no. 10977-15)
- 96-well plate with round-shaped wells or equivalent (see Recommendation on previous page)
- 96-well plate lid, sealing tape, or equivalent
- Floor shaker or thermomixer adapted for use with plates (recommended; set to 37ºC) or standard incubator (set to 37°C)
- Centrifuge with centrifuge rotor adapted for use with plates

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Performing the Protein Synthesis Reaction, continued

Protein Synthesis Protocol

Use the protocol below to synthesize protein from your DNA template . You may perform this protocol using a multichannel pipettor or an automated robotic system (see page 1 for more information).

1. For each reaction, aliquot 22.5 µl of the Expressway™ HTP Cell-Free *E. coli* Expression Mix into a well
2. Add 0.5 µg of purified DNA to each reaction. Do not exceed a total reaction volume of 25 µl per well
3. Bring the final reaction volume to 25 µl with DNase/RNase-free water. Mix gently by pipetting up and down.
4. Securely place the lid or sealing tape onto the plate. You may briefly centrifuge the plate, if desired.
5. Shake samples in a thermomixer (1,400 rpm) or a floor shaker (300 rpm) at 37°C for 2 hours.

   Alternative: Incubate plate at 37ºC for 2 hours in a standard incubator.

   Note: It is possible to incubate reactions for up to 4 hours to obtain greater protein yield. You may also incubate reactions at 30°C to decrease the rate of protein synthesis and to promote proper folding.
6. After the 2 hour incubation, condensation may appear on the lid or sealing tape. This is expected and will not affect your synthesis reaction. If you notice significant amounts of the reaction mix on the lid or sealing tape, we recommend briefly centrifuging the samples. Proceed to **Analyzing Samples**, next page.

   Note: Alternatively, you may store your samples at -20ºC until needed. Make sure to properly cover the plate using foil sealing tape.

Analyzing Samples

Introduction

Once you have performed the protein synthesis reaction, you may use any method of choice to analyze your samples. Generally, sufficient protein is produced for analysis on a Coomassie®-stained protein gel, by Western blot analysis, by enzymatic activity, or by affinity purification. However, expression levels may vary depending on the nature of your protein and the configuration of the DNA construct (see page 4 for more details).

Lumio™ Technology

If you expressed your protein from pEXP3-DEST or pEXP4-DEST, you may directly detect your Lumio™-tagged fusion protein in the polyacrylamide gel without staining or performing Western blot analysis. For detailed instructions to perform in-gel Lumio™ detection, refer to the manual supplied with your particular Expressway™ vector. A sample Expressway™ HTP experiment using Lumio™ Technology is provided on page 11.

If you plan to analyze your samples using polyacrylamide gel electrophoresis, we recommend that you first precipitate the proteins with acetone to remove background smearing. A protocol for acetone precipitation is provided below.

If you experience difficulty precipitating your proteins due to the large number of samples, you may omit this step and directly add sample buffer to your unprecipitated proteins. Note, however, that this may result in smearing and lower resolution of the bands on your gel.

E-PAGE™ *96* System

E-PAGE™ High-Throughput (HTP) Protein Electrophoresis System is available from Invitrogen for fast, high-throughput protein electrophoresis in a horizontal format. The E-PAGE™ System consists of E-PAGE™ *96* Pre-cast Gels, E-Base™ Electrophoresis Device, E-PAGE™ Loading Buffers, and E-Editor™ 2.0 Software. Each E-PAGE™ *96* Gel contains 96 sample lanes and 8 marker lanes in a patented staggered well-format that is compatible with the standard 96-well plate format for automated robotic loading. For more information about the E-PAGE™ *96* System and other gels, standards, and stains available from Invitrogen, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 18).

Materials Needed

You should have the following materials on hand before proceeding:

- Acetone at -20ºC
- 1X SDS-PAGE sample buffer (see next page for a recipe) or equivalent
- Prepared SDS-PAGE gels or pre-cast polyacrylamide gels to resolve your protein of interest
- Coomassie® blue stain, optional

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Analyzing Samples, continued

1X SDS-PAGE Sample Buffer

Use this recipe to make the 1X SDS-PAGE sample buffer.

Note: If you are a commercially available electrophoresis or detection system (*e.g.* Invitrogen's E-PAGE™ Protein Electrophoresis System or Lumio™ Green Detection Kit), use the buffers provided with the kit.

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.

Acetone Precipitation

Use the following protocol to precipitate your proteins prior to loading on the polyacrylamide gel.

1. Add 5 µl of each protein synthesis reaction to 20 µl of cold acetone. Mix well by pipetting up and down.
2. Incubate samples at +4ºC for 20 minutes.
3. Centrifuge the samples for 5 minutes at room temperature at 12,000 rpm.
4. Carefully remove the supernatant without disturbing the protein pellet. Allow the residual acetone to evaporate.
5. Resuspend pellet in 20 µl of 1X SDS-PAGE sample buffer.
6. Heat at 70-80°C for 10-15 minutes and centrifuge briefly. Proceed to **Polyacrylamide Gel Electrophoresis**, next page.

   Note: Alternatively, you may store your samples at -20°C until needed.

Electrophoresis and Detection

1. Load 5-10 µl of the sample from Step 6, previous page, on an SDS-PAGE gel and electrophorese.
2. Detect protein by staining the gel with a Coomassie® blue stain or by Western blot analysis using an appropriate antibody.

Detecting CAT Protein

If you use pT7/CAT as a positive control for protein expression, you may assay for CAT protein using CAT Antiserum available from Invitrogen (see page viii for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT protein is approximately 25 kDa.

Sample Expressway™ HTP Cell-Free Experiment

Introduction

The following experiment is provided to illustrate the use of the Expressway™ HTP Cell-Free *E. coli* Expression Kit to synthesize proteins in a high-throughput format. This experiment begins with a Gateway® LR recombination reaction to transfer clones from Invitrogen's Ultimate™ ORF Collection to an Expressway™ destination vector and continues through detection of Lumio™-tagged proteins using the E-PAGE™ *96* System. Where appropriate, helpful tips are provided for high-throughput users. For detailed protocols, refer to the manuals provided with the respective products.

Performing the Gateway® LR Recombination Reaction

Ninety-six human kinases from the Ultimate™ ORF Collection were selected for expression. The ORFs of interest, provided in entry clones, were transferred to pEXP3-DEST by performing an LR recombination reaction using Gateway® LR Clonase™ II enzyme mix. Component volumes were scaled for 96 samples and the reaction was performed following the protocol provided with LR Clonase™ II enzyme mix.

Tip: To streamline this process, LR reactions were incubated at room temperature for 30 minutes instead of the recommended 1 hour. Also, samples were not incubated with Proteinase K solution. Although addition of Proteinase K reduces background levels and increases transformation efficiency, it is not essential for the LR reaction.

Transforming Competent *E. coli*

LR reactions were transformed into MultiShot™ TOP10 Chemically Competent *E. coli* which are provided pre-packaged in 96-well plates. Competent cells were transformed using 2 µl of each LR reaction and following the protocol provided with the TOP10 cells.

Selecting Transformants

Transformants containing the desired pEXP3-DEST expression clone were selected using the following protocol.

Tip: Transformants were selected in the wells of a 96-well assay block and not on selective plates. If you perform this protocol, transformants **will not** be clonal isolates.

1. Add 1.5 ml of LB media containing the appropriate antibiotic to select for your plasmid (*i.e.* ampicillin to select for pEXP3-DEST) to each well of a 96-well assay block.
2. Remove 20 µl of each transformation reaction and add to each well containing LB media (from Step 1, above).
3. Cover the assay block with breathable sealing tape and incubate 12-20 hours at 37ºC with shaking.
4. Using a centrifuge equipped with the appropriate adaptor, centrifuge the assay block at 1500 x *g* for 15 minutes.
5. Remove the sealing tape and carefully decant the supernatant. Tap the assay block on paper towels to remove any excess media.

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Sample Expressway™ HTP Cell-Free Experiment, continued

Synthesizing Proteins

pEXP3-DEST expression constructs were purified using a commercial DNA purification kit. Standard 25 µl Expressway™ HTP reactions were performed using 0.5 µg of purified DNA and the protocol provided on page 8.

Preparing Lumio™-Tagged Proteins

The following protocol was used to prepare protein samples for electrophoresis using the E-PAGE™ *96* System and the Lumio™ Green Detection Kit.

Tip: To streamline the process, samples were not precipitated with acetone before performing the detection step.

1. Preheat thermocycler to 70ºC.
2. Prepare the Lumio™ reaction mix as detailed below. Note that the components have been scaled 125X for 96 samples to ensure sufficient amounts.

Component	1 Sample	96 Samples
Lumio™ Gel Sample Buffer (4X)	2.5 µl	312.5 µl
Lumio™ Green Detection Reagent	0.1 µl	12.5 µl
Deionized water	6.4µl	800 µl
Final Volume	**9 µl**	**1125 µl**

3. Aliquot 9 µl of the Lumio™ reaction mix to each well of a 96-well V-bottom plate.
4. To the Lumio™ reaction mix, add 1 µl of each Expressway™ HTP reaction and mix by pipetting up and down.
5. Cover the 96-well plate with sealing tape and incubate at 70ºC for 10 minutes.
6. Remove the sealing tape. Add 1 µl of Lumio™ In-Gel Detection Enhancer to each well and mix by pipetting up and down. Incubate samples at room temperature for 5 minutes.

Performing Electrophoresis

Each well of an E-PAGE™ *96* 6% gel was loaded with 10 µl of deionized water followed by 10 µl of prepared sample from Step 6, above. For the marker lanes, 17 µl of deionized water was loaded to the wells followed by 3 µl of BenchMark™ Fluorescent Protein Standard.

Samples were electrophoresed following the guidelines provided in the E-PAGE™ *96* Protein Electrophoresis System manual.

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Sample Expressway™ HTP Cell-Free Experiment, continued

Detecting Lumio™-Tagged Proteins

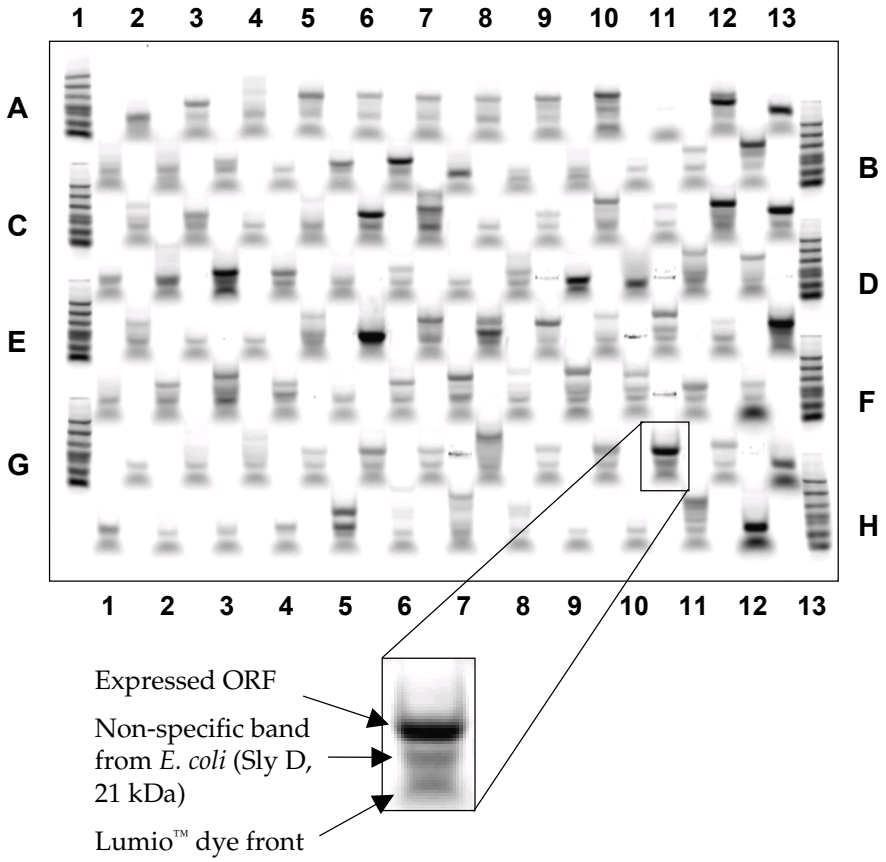
Lumio™-tagged proteins were analyzed directly in the E-PAGE™ *96* gel using a Typhoon™ 8600 Variable Mode Imager (see Figure 1, below).

Tip: The E-PAGE™ *96* gel was not removed from the cassette before visualizing the bands. Although the sensitivity of detection is higher when the gel is removed from the cassette, this step is not required. Gel removal is essential only for UV visualization.

Figure 1: In-gel Lumio™ detection of human kinase ORFs

BenchMark™ Fluorescent Protein Standard: Lanes A1, B13, C1, D13, E1, F13, G1, H13

pEXP3-GW/CAT control plasmid: Lane H12



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Sample Expressway™ HTP Cell-Free Experiment, continued

Accessory Products for High-Throughput Applications

The products listed below were used in the sample experiment provided in this section and are suitable for use for high-throughput applications. Ordering information is provided below. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 18).

Item	Quantity	Catalog no.
pEXP3-DEST Vector	6 µg	V960-03
LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
MultiShot™ TOP10 Chemically Competent *E. coli*		C400-05
Lumio™ Green Detection Kit	100 reactions	LC6090
E-PAGE™ *96* High-Throughput (HTP) Protein Electrophoresis System	1 Starter Kit	EPST96-06
E-PAGE™ *96* 6% Gels	8 gels	EP096-06
BenchMark™ Fluorescent Protein Standard	125 µl	LC5928

Troubleshooting

Introduction

The table below lists some potential problems and possible solutions that may help you troubleshoot your *in vitro* transcription and translation experiment.

Problem	Reason	Solution
Low or no yield of target protein (but control reaction produces protein)	DNA template not optimally configured	• Use one of the pEXP-DEST vectors (see page viii or follow the guidelines on pages 3-5 to clone your gene of interest into a T7 expression vector 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). • For linear DNA templates, make sure that at least 6-10 additional nucleotides are present upstream of the T7 promoter.
	DNA template not pure • Contaminated with ethanol, sodium salt, or ammonium acetate • Contaminated with RNase	• 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	We recommend using a commercial DNA purification kit to purify your DNA construct. See page 5 for additional guidelines.
	DNA concentration not optimal (generally more of a problem for linear DNA or PCR products)	• Determine the concentration of your DNA template and adjust the concentration to at least 200 ng/µl. • Titrate the amount of DNA construct used in the protein synthesis reaction to determine the optimal yield.
	Size of protein	• Protein yield may decrease as the size of the protein increases; optimize expression conditions. • Reduce incubation temperature to as low as 30°C for Step 5 of protocol on page 8. Extend incubation time for up to 4 hours.
	Samples incubated in a non-shaking incubator during protein synthesis reaction	For optimal results, use a floor shaker or thermomixer to shake samples (see protocol on page 8).

continued on next page

Troubleshooting, continued

Problem	Reason	Solution
Low or no yield of control protein	Components in the Expressway™ HTP Cell-Free *E. coli* Expression Kit have lost activity	• Store the Expressway™ HTP Expression Mix at -80°C. • Thaw the Expressway™ HTP Expression Mix **on ice**. Do not let the Expressway™ HTP Expression Mix undergo more than two freeze/thaw cycles.
	The Expressway™ HTP Cell-Free *E. coli* Expression Mix is contaminated	Wear gloves and use RNase-free reagents when working with the Expressway™ HTP Expression Mix to prevent contamination.
Protein has low biological activity	Improper protein folding	Reduce incubation temperature to as low as 30°C for Step 5 of protocol on page 8.
Multiple bands on the polyacrylamide gel	Proteins denatured for too long	Add 1X SDS-PAGE sample buffer to sample and heat at 70-80°C for 10-15 minutes before loading on gel.
	Not enough SDS in the 1X SDS-PAGE sample buffer	Prepare new 1X SDS-PAGE sample buffer according to standard instructions.
	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 **o**r change RBS sequences using point mutations.
Smearing on the gel	Samples not precipitated with acetone	Precipitate the proteins with acetone to remove background smearing. Follow the protocol provided on page 10.
	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 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

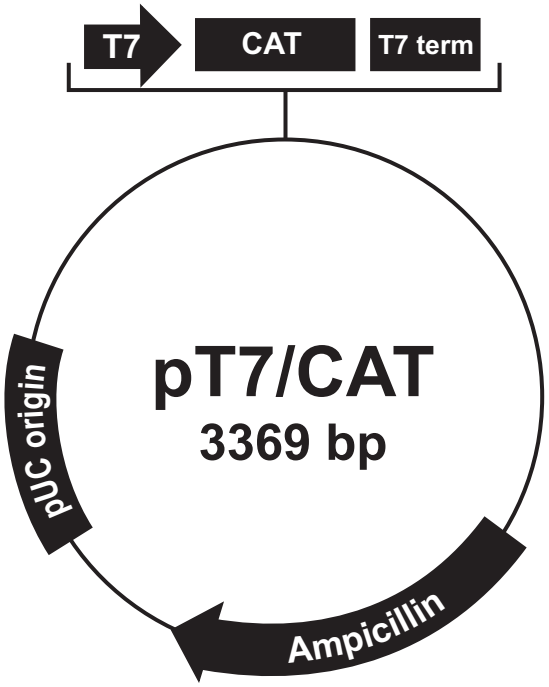
Map of pT7/CAT

Description

pT7/CAT is a 3369 bp control vector expressing the chloramphenicol acetyltransferase (CAT) protein. The CAT gene is cloned in optimal configuration for expression using the Expressway™ Plus System. The molecular weight of the CAT protein is approximately 25 kDa.

Map of pT7/CAT

The map below shows the elements of pT7/CAT. The complete sequence of the vector is available from our Web site (www.invitrogen.com) or by contacting Technical Service (page 18).



Comments for pT7/CAT
3369 nucleotides

T7 promoter: bases 31-50
Ribosome binding site: bases 96-101
CAT gene: bases 111-770
T7 transcription termination region: bases 811-939
bla promoter: bases 1050-1148
Ampicillin (*bla*) resistance gene: bases 1149-2009
pUC origin: bases 2154-2827

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
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- 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

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continued on next page

Technical Service, continued

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Introduction

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