Instruction Manual

Expressway™ *In Vitro* Protein Synthesis System

For high-yield, *in vitro* protein synthesis from plasmid or linear templates

Catalog nos. K9600-01, K9600-02, K9605-01, K9605-02

Version D
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Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the Expressway™ *In Vitro* Protein Synthesis System. If you are a first time user of the Expressway™ System, we recommend following the detailed protocols in the manual.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Generate the DNA Template</td><td>For optimal expression in the Expressway™ System, clone your gene of interest into a pEXP-DEST or other T7 expression vector or use PCR to generate a DNA template containing the elements discussed on page 3.</td></tr>
<tr><td>Purify the DNA Template</td><td>Use your method of choice or a commercially available kit to purify your plasmid or linear DNA template. Resuspend purified DNA in 1X TE Buffer or water such that the final concentration is greater than 500 ng/µl. Take care to avoid contamination of your DNA template with ethanol, salt, or RNases.</td></tr>
<tr><td>Perform the Protein Synthesis Reaction</td><td>1. Prepare the following IVPS Reaction Cocktail on ice for each reaction to be performed. Remember to include a positive control (supplied with the kit) in your experiment.
DNase/RNase-free water 4 µl
2.5X IVPS E. coli Reaction Buffer 20 µl
T7 RNA polymerase 1 µl
Total Volume 25 µl
Note: To generate radiolabeled proteins, add 2 µl ^{35}S Methionine to the IVPS Reaction Cocktail and decrease the volume of DNase/RNase-free water to 2 µl.
2. For each reaction, thaw one 20 µl tube of IVPS E. coli Extract on ice and add the 25 µl IVPS Reaction Cocktail from Step 1.
3. Add DNA (1 µg of plasmid DNA or 2-3 µg of linear DNA) to the reaction tube.
4. Bring the final reaction volume to 50 µl with DNase/RNase-free water.
5. Vortex for 3 seconds to mix. Place tubes in a microcentrifuge and briefly centrifuge.
6. Shake tubes at 1,400 rpm in a thermomixer at 37°C for 2 hours.
Alternative: Transfer the 50 µl reaction to a 2 ml screw-cap tube (supplied with the kit) and incubate at 37ºC for 2 hours in a standard shaking incubator (275-325 rpm) or in a water bath.
7. Add 5 µl of RNase A to the 50 µl reaction and vortex briefly.
8. Place tubes in a microcentrifuge and briefly centrifuge.
9. Shake tubes at 1,400 rpm in a thermomixer at 37°C for 15 minutes.
Alternative: Incubate tubes at 37ºC for 15 minutes in a standard shaking incubator (275-325 rpm) or in a water bath.
10. Place tubes in a microcentrifuge and briefly centrifuge. Place the reaction on ice and proceed to analyze samples.</td></tr>
<tr><td>Analyze Sample</td><td>Use any method of choice including 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, precipitate the proteins with acetone prior to performing polyacrylamide gel electrophoresis (see page 9).</td></tr>
</table>

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Amount	Catalog no.
Expressway™ *In Vitro* Protein Synthesis System		
with pEXP1-DEST	20 reactions	K9600-01
	8 reactions	K9605-01
with pEXP2-DEST	20 reactions	K9600-02
	8 reactions	K9605-02

Shipping/Storage

The Expressway™ *In Vitro* Protein Synthesis System is shipped on dry ice. Upon receipt, store the kit at -80°C.

Kit Contents

The following reagents are supplied in the Expressway™ *In Vitro* Protein Synthesis System. **Store at -80°C.**

Item	Composition	Amount/ 8 Rxn Kit	Amount/ 20 Rxn Kit
IVPS *E. coli* Extract	Proprietary	8 x 20 µl	20 x 20 µl
2.5X IVPS *E. coli* Reaction Buffer	Proprietary	400 µl	
DNase/RNase-Free Distilled Water	---	400 µl	
RNase A	1 mg/ml in DNase/RNase-free water	100 µl	
T7 RNA Polymerase	Proprietary	20 µl	
2 ml screw-cap tubes	--	8	20

Product Qualification

Each lot of the Expressway™ *In Vitro* Protein Synthesis System is functionally tested for protein generation by incorporation of ^{35}S-methionine. Using a control plasmid in a 50 µl Expressway™ protein synthesis reaction, greater than 18 µg recombinant protein must be obtained.

Accessory Products

Accessory Products

DNase/RNase-free water and other reagents suitable for use with the Expressway™ *In Vitro* Protein Synthesis System are available separately from Invitrogen. Ordering information is provided below.

Item	Quantity	Catalog no.
DNase/RNase-Free Distilled Water	500 ml	10977-015
S.N.A.P.™ MiniPrep Kit	25 reactions	K1900-01
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
Coomassie Brilliant Blue R®-250 Protein Stain	10 g	15528-011

Coomassie Brilliant Blue R® is a registered trademark of Imperial Chemical Industries PLC

Introduction

Overview

Introduction

The Expressway™ *In Vitro* Protein Synthesis System ("Expressway™ System") is designed for T7-based, *in vitro* transcription and translation of target DNA to protein in a single tube. The Expressway™ System uses extract from an *E. coli* strain to drive the reaction, and a transcription/translation mix that allows for optimal protein production (up to 50 µg protein per 50 µl reaction, depending on the protein of interest) in about 2 hours. For higher yields, the reaction can be extended up to 4 hours.

Components of the System

The major components of the Expressway™ System include:

- Single-use tubes of S30 *E. coli* extract (Zubay, 1973)
- T7 RNA polymerase (Studier *et al.*, 1990)
- A reaction buffer composed of all the required amino acids and an ATP regenerating system (Kim *et al.*, 1996; Lesley *et al.*, 1991; Pratt, 1984)

Applications

The Expressway™ System is suitable for use in the following applications:

- Characterizing proteins
- Analyzing mutants
- Verifying cloned gene products
- Producing analytical quantities of protein or radiolabeled protein
- Producing proteins which are toxic to cells

Experimental Outline

The table below describes the major steps necessary to synthesize your recombinant protein of interest using the Expressway™ *In Vitro* Protein Synthesis System. Refer to the specified pages for details to perform each step.

Step	Action	Pages
1	Generate the DNA template.	2-4
2	Purify your DNA template.	5
3	Perform the protein synthesis reaction.	6-8
4	Analyze your sample using polyacrylamide gel electrophoresis, Western blot analysis, or activity assay.	9-12

Methods

Generating the DNA Template

Introduction

Successful use of the Expressway™ *In Vitro* Protein Synthesis System 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. The Expressway™ System includes an optimally configured pEXP-DEST vector from which to express your gene of interest. If you wish to use a different T7 expression vector, guidelines are provided to produce your DNA template in an optimal configuration for protein expression in the Expressway™ System.

Factors Affecting Protein Yield

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

- The size of the protein
- The sequence of the gene of interest
- The spacing of the T7 promoter and the gene of interest in the DNA template
- The 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™ System.

- Supercoiled plasmid DNA (recommended for most applications for highest protein yields)
- Linear DNA
- PCR product

For proper expression in the Expressway™ System, 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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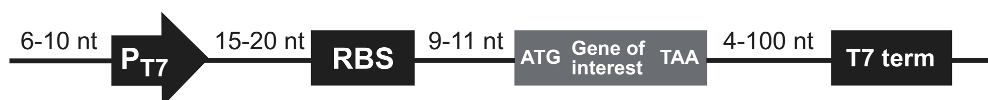
Generating the DNA Template, continued

Optimal Configuration of DNA Template

Optimized expression constructs are provided with the Expressway™ System, however, other expression constructs 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). 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 9-11 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 page 4.



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 the Expressway™ System. 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. Examples of T7-based expression vectors containing this configuration include:

- pEXP1-DEST or pEXP2-DEST (supplied with the kit, as specified)
- pCR®T7/CT-TOPO® (Invitrogen, Catalog no. K4210-01)
- pCR®T7/NT-TOPO® (Invitrogen, Catalog no. K4200-01)
- pRSET (Invitrogen, Catalog no. V351-20)

Note: T7 expression vectors that contain the T7*lac* promoter (*e.g.* pET101/D-TOPO® available from Invitrogen™) are suitable for use in the Expressway™ System 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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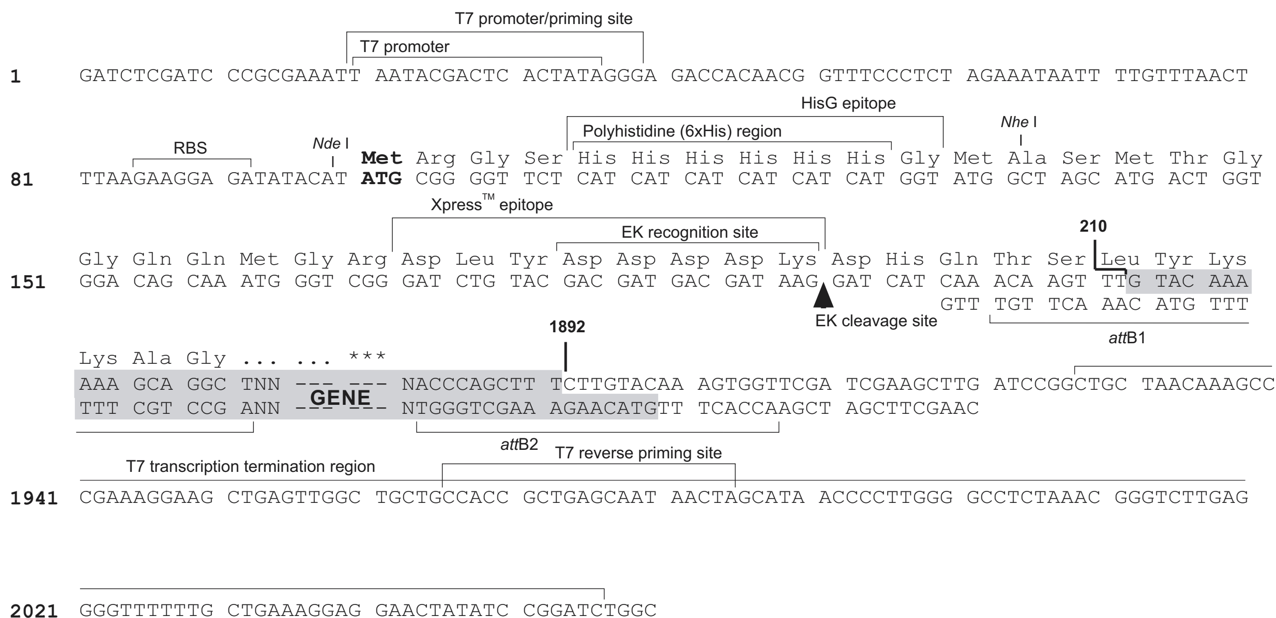
Generating the DNA Template, continued

Example

The T7-based pEXP1-DEST or pEXP2-DEST vector supplied with the kit allows you to generate an expression clone containing your gene of interest in an optimal configuration for expression in the Expressway™ System. To illustrate the points discussed on the previous page, the region surrounding the gene of interest after generating an expression clone in pEXP1-DEST is diagrammed below.



Generating an Expression Clone

You may generate an expression clone by recombination of an entry clone containing your gene of interest with the pEXP1-DEST or pEXP2-DEST vector using the Gateway® Technology. For more information about the pEXP1-DEST and pEXP2-DEST vectors, refer to the pEXP1-DEST and pEXP2-DEST Gateway® Vector Kits manual supplied with the vectors. For more information about the Gateway® Technology, creating an entry clone, and performing the LR recombination reaction, refer to the Gateway® Technology manual, which is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 16).

continued on next page

Generating the DNA Template, continued

Purifying the DNA Template

Once you have generated the DNA template, you must purify the DNA before proceeding to the protein synthesis reaction. Depending on the type of DNA template (*i.e.* supercoiled plasmid, linear DNA, or PCR product), you may use any method of choice to purify your DNA template including commercial DNA purification kits (see below) or CsCl gradient centrifugation. For protocols to purify DNA, refer to published reference sources (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989). When purifying your DNA template, keep the following in mind:

- **Do not** gel-purify your DNA template. Purified DNA solution obtained from agarose gels significantly inhibits the protein synthesis reaction. For PCR products or linear DNA, we recommend using a suitable purification kit.
- Ammonium acetate is not recommended for use in 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).
- Purified DNA should be free of excess ethanol or salt as both can inhibit translation.

  Note: Ethanol precipitated DNA should be carefully washed with 70% ethanol to remove excess salt and dried.
- Purified DNA should be resuspended in 1X TE Buffer or water such that the final concentration is at a minimum of 500 ng/µl.

Reminder: 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, we recommend using a suitable purification kit.

For rapid, resin-based isolation of purified plasmid DNA, we recommend using the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) available from Invitrogen. Other commercial DNA purification kits are suitable.

Performing the Protein Synthesis Reaction

Introduction

Once you have obtained purified template DNA, you are ready to synthesize your recombinant protein using the Expressway™ System. General guidelines and instructions to produce your recombinant protein are provided in this section.

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.

Incubation Conditions

To obtain the optimal yield of protein, we recommend using an Eppendorf Thermomixer (Fisher, Catalog no. 05-400-200) to shake your sample(s) at 37ºC during the protein synthesis reaction (see Steps 8-11 in the protocol on page 8).

If a thermomixer is unavailable, you may use one of the following:

- Standard shaking incubator
- Standard shaking water bath
- Non-shaking water bath

We do not recommend using a non-shaking incubator because it produces a less stable and less consistent temperature environment.

2 ml Tubes

If you will not using a thermomixer, we recommend that you transfer the reaction mixture to a 2 ml screw-cap tube (supplied with the kit) before incubating your samples. Using one of the thermomixer alternatives listed above and incubating your samples in 2 ml tubes will result in protein yields that are comparable to that obtained using a thermomixer.

Positive Control

The pEXP1-GW/*lacZ* or pEXP2-GW/*lacZ* vector is provided in the kit as appropriate for use as a positive control for protein expression. pEXP1-GW/*lacZ* and pEXP2-GW/*lacZ* were constructed using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pEXP1-DEST or pEXP2-DEST, respectively. For a map and a description of the features of each vector, see the pEXP1-DEST and pEXP2-DEST Gateway® Vector Kits manual.

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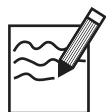
Materials to Have on Hand

You should have the following materials on hand before beginning:

- DNA Template (purified; resuspended in TE or water at a concentration greater than 500 ng/µl)
- IVPS *E. coli* Extract (supplied with the kit; keep on ice)
- 2.5X IVPS *E. coli* Reaction Buffer (supplied with the kit; thaw on ice)
- T7 RNA polymerase (supplied with the kit; keep on ice)
- RNase A (supplied with the kit; do not thaw until needed)
- DNase/RNase-free water (supplied with the kit)
- ^{35}S Methionine (3,000 Ci/mmol; 15 µCi/µl; if needed)
- Microcentrifuge tubes and microcentrifuge
- Thermomixer (recommended), standard shaking incubator (set to 37ºC), or water bath (set to 37°C)
- 2 ml screw-cap tubes, optional (supplied with the kit)
- Vortex mixer



Note

Upon thawing the 2.5X IVPS *E. coli* Reaction Buffer, you may notice some precipitate in the bottom of the tube. Gently flick the tube several times with your finger to mix and allow the precipitate to go back into solution. **Do not** pipette the buffer up and down or place the tube in a warm (greater than 37°C) water bath as this may result in loss of activity of the Reaction Buffer. Note that the solution may remain cloudy, but is suitable for use.

Important

The 2.5X IVPS *E. coli* Reaction Buffer is supplied in a single tube. Depending on the number of protein synthesis reactions performed, you may not use all of the 2.5X IVPS *E. coli* Reaction Buffer in a single experiment. In this case, make sure that you thaw the 2.5X IVPS *E. coli* Reaction Buffer on ice, mix (see **Note** above), remove the amount needed, and return the reaction buffer to a -80°C freezer. **Do not** store the 2.5X IVPS *E. coli* Reaction Buffer at -20°C or room temperature as this may result in loss of activity. To prevent contamination, use RNase-free, sterile pipette tips and wear gloves when removing the 2.5X IVPS *E. coli* Reaction Buffer from the tube.

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Performing the Protein Synthesis Reaction

Use the protocol below to synthesize your protein from the DNA template.

1. For each sample, prepare the following IVPS Reaction Cocktail on ice. If you are performing multiple reactions, scale up the volume of each reagent accordingly.

DNase/RNase-free water	4 µl
2.5X IVPS *E. coli* Reaction Buffer	20 µl
T7 RNA polymerase	1 µl
Total Volume	25 µl

   Note: If you wish to produce radiolabeled proteins, add 2 µl ^{35}S Methionine to the IVPS Reaction Cocktail and decrease the volume of DNase/RNase-free water to 2 µl. Include a negative control (no DNA) to determine background.

2. For each reaction to be performed, thaw one tube of IVPS *E. coli* Extract on ice.
3. To each tube of extract, add the 25 µl IVPS Reaction Cocktail from Step 1.
4. Add the following amount of DNA to the reaction tube.
   - For plasmid DNA templates, add 1 µg
   - For linear DNA templates or PCR products, add 2-3 µg
   - For control DNA, add 1 µg.
5. Bring the final reaction volume to 50 µl with DNase/RNase-free water. For no-DNA controls, add 5 µl of DNase/RNase-free water only.
6. Vortex for 3 seconds to mix.
7. Place tubes in a microcentrifuge and briefly centrifuge.
8. Shake tubes at 1,400 rpm in a thermomixer at 37°C for 2 hours.
   Alternative: Transfer the 50 µl reaction to a 2 ml screw-cap tube (supplied with the kit) and incubate at 37ºC for 2 hours in a standard shaking incubator (275-325 rpm) or in a water bath.

   Note: It is possible to shake tubes for up to 4 hours to obtain as much as 50% greater protein yield.
9. Add 5 µl of RNase A to the 50 µl reaction and vortex briefly.
10. Place tubes in a microcentrifuge and briefly centrifuge.
11. Shake tubes at 1,400 rpm in a thermomixer at 37°C for 15 minutes.
    Alternative: Incubate tubes at 37ºC for 15 minutes in a standard shaking incubator (275-325 rpm) or in a water bath.
12. Place tubes in a microcentrifuge and briefly centrifuge. Place the reaction on ice and proceed to **Analyzing Samples**, next page.

Analyzing Samples

Introduction

Once you have performed the protein synthesis reaction, you may use any method of choice to analyze your sample. Generally, sufficient protein is produced for analysis on a Coomassie®-stained protein gel, by Western blot analysis, by enzymatic activity, or by affinity purification (if affinity tag is present), however, expression levels may vary depending on the nature of your protein and the configuration of the DNA template (see page 3 for more details). If you plan to analyze your sample using polyacrylamide gel electrophoresis, note that you should first precipitate the proteins with acetone to remove background smearing. A protocol for acetone precipitation and other general guidelines for gel electrophoresis are provided in this section.

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 (see pages 11-12).

Materials Needed

You should have the following materials on hand before proceeding:

- Acetone (room temperature)
- Microcentrifuge
- SpeedVac® concentrator (Thermo Savant)
- 1X SDS-PAGE sample buffer (see page 15 for a recipe)
- Appropriate polyacrylamide gel to resolve your protein of interest (see the next page)
- Coomassie® blue stain

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 12, page 8 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. Dry the sample under vacuum for 15 minutes. **Note:** Alternatively, you may air dry the sample for 1 hour at room temperature.
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, samples may be stored at -20°C until needed.

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Polyacrylamide Gels Available from Invitrogen™

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. In addition, Invitrogen carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 16).

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 staining	Stain gel with Coomassie® blue stain. **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.

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Determining Total Counts Incorporated

For radiolabeled reactions, we recommend using TCA precipitation to determine the amount of ^{35}S methionine incorporated. A protocol is provided below.

Determining Total Counts

1. Mix and spot 5 µl of each radiolabeled reaction from Step 12, page 8 on an individual 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

1. Mix and spot 5 µl of each radiolabeled reaction from Step 12, page 8 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.
6. Count samples in a scintillation counter.
7. Proceed to **Calculating the Yield of Protein**, below to determine your protein yield.

Calculating the Yield of Protein

If you generate radiolabeled proteins using 2 µl of ^{35}S methionine (3,000 Ci/mmol, 15 µCi/µl) in the protein synthesis reaction (see page 8), you may calculate the yield of protein as shown below. For a sample calculation, see the next page.

Before beginning, you will need to determine the total counts incorporated using TCA precipitation (see above).

Total pmoles of methionine (includes radiolabeled and non-radiolabeled methionine) = 1,240 pmoles/µl (or 62,000 total pmoles for a 50 µl reaction)

Total counts:	Total cpm per 5 µl spotted x total reaction volume/5
Specific activity:	$\frac{\text{Total counts}}{\text{pmoles of methionine}}$
pmoles methionine incorporated:	$\frac{(\text{TCA precipitable counts} - \text{background}) \times 50/5}{\text{specific activity}}$
pmoles of protein:	$\frac{\text{pmoles of methionine incorporated for protein}}{\text{number of methionines in protein}}$
Yield of protein:	pmoles of protein x molecular weight of protein

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Sample Calculation for Protein Yield

The sample calculation below illustrates how to use the data obtained from the TCA precipitation procedure, page 11 to calculate the yield of protein.

Example: We wish to calculate the yield of chloramphenicol acetyltransferase (CAT) protein obtained from a typical protein synthesis reaction using a T7-based plasmid containing the CAT gene.

1. We know the following:
   - The CAT protein has a molecular weight of 24 kDa and contains 9 methionines (0.024 µg/pmole)
   - There are 62,000 total pmoles of methionine in a 50 µl reaction
2. Using the TCA precipitation protocol on page 11, we obtain the following values after scintillation counting:

Sample	Radioactive counts (cpm)
Total counts (5 µl spotted):	4×10^6 cpm
Background TCA precipitable counts (No DNA; 5 µl spotted):	1.3×10^4 cpm
CAT TCA precipitable counts (5 µl spotted):	6.2×10^5 cpm

3. Determine total counts in the reaction:

$$4 \times 10^6 \text{ cpm in } 5\,\mu\text{l} \times \frac{50}{5} = 4 \times 10^7 \text{ cpm in } 50\,\mu\text{l reaction}$$

4. Calculate the specific activity:

$$\frac{4 \times 10^7 \text{ cpm}}{62000} = 661 \text{ cpm/pmoles methionine}$$

5. Calculate the pmoles methionine incorporated:

$$\frac{\left[\left(6.2 \times 10^5 \text{ cpm} - 1.3 \times 10^4 \text{ cpm}\right) \times \frac{50}{5}\right]}{661} = 9183 \text{ pmoles methionine}$$

6. Calculate the pmoles of protein obtained:

$$\frac{9183 \text{ pmoles}}{9} = 1020 \text{ pmoles protein}$$

7. Calculate the yield of protein obtained:

   1020 pmoles x 0.024 µg/pmole = 24 µg protein

Troubleshooting

Introduction

The table below and on the next page lists some potential problems and possible solutions that you may use to 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 or follow the guidelines on pages 2-3 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 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	We recommend using a commercial DNA purification kit to purify your DNA template. 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 500 ng/µl. • Titrate the amount of DNA template used in the protein synthesis reaction to determine the optimal yield.
	Sample incubated in a non-shaking incubator during protein synthesis reaction	• Use a thermomixer and shake sample at 1,400 rpm (see protocol on page 8). • Incubate samples in a standard shaking incubator (275-325 rpm) or in a water bath (see recommend alternatives on page 6).
	Expression time too short	Extend expression time up to 4 hours.

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Troubleshooting, continued

Problem	Reason	Solution
	Size of protein	Protein yield may decrease as the size of the protein increases; optimize expression conditions.
	Sample not mixed before spotting on filter for TCA precipitation (radiolabeled samples only)	Mix sample before spotting on filter for TCA precipitation.
Control reaction produces no protein	Reagents have lost activity	• Store reagents at -80°C. • Use care when freeze thawing the 2.5X IVPS *E. coli* Reaction Buffer.
	2.5X IVPS *E. coli* Reaction Buffer contaminated	Wear gloves and use RNase-free reagents when working with 2.5X IVPS *E. coli* Reaction Buffer to prevent contamination.
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.
	Old ^{35}S methionine	Use fresh ^{35}S methionine.
	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 **or** change RBS sequence(s) using point mutation(s).
Smearing on the gel	Samples not precipitated with acetone	Precipitate the proteins with acetone to remove background smearing. Follow the protocol provided on page 9.
	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

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.

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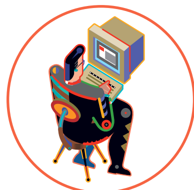

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Corporate Headquarters:

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA

Tel: 1 760 603 7200

Tel (Toll Free): 1 800 955 6288

Fax: 1 760 602 6500

E-mail:
tech_service@invitrogen.com

Japanese Headquarters:

Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park Bldg. 4F
2-35-4, Hama-Cho, Nihonbashi

Tel: 81 3 3663 7972

Fax: 81 3 3663 8242

E-mail: jpinfo@invitrogen.com

European Headquarters:

Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK

Tel: +44 (0) 141 814 6100

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United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, California 92008
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 603 7229
Email: tech_service@invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
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International Offices:
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