TECHNICAL MANUAL

# S30 T7 High-Yield Protein Expression System

Instructions for Use of Products
**L1110 and L1115**


Revised 5/16
TM306

# S30 T7 High-Yield Protein Expression System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com



## 1. Description

*E. coli*-based protein expression systems that use bacteriophage T7 RNA polymerase and T7 promoters are popular for high-level expression of recombinant proteins in vivo (1–3 and references within). These expression systems require the presence of T7 RNA polymerase, which is typically supplied by inducing a recombinant T7 polymerase gene contained on a lambda lysogen in the host strain (4).

The S30 T7 High-Yield Protein Expression System is an *E. coli* extract-based cell-free protein synthesis system. It simplifies the transcription and translation of DNA sequences cloned in plasmid or lambda vectors containing a T7 promoter by providing an extract that contains T7 RNA polymerase for transcription and all necessary components for translation (Figure 1). This system can produce high levels of recombinant proteins (up to hundreds of micrograms of recombinant protein per milliliter of reaction) within an hour. The investigator supplies only cloned DNA containing a T7 promoter and a ribosome-binding site (RBS). Tested vectors suitable for expressing recombinant proteins in this cell-free expression system are listed in Table 1, Section 3.A.

The S30 T7 High-Yield Protein Expression System contains the T7 S30 Extract, Circular, which is prepared by modifications of the method described by Zubay (5–7) from an *E. coli* strain B deficient in OmpT endoproteinase and lon protease activity. This results in greater stability for translated proteins that would otherwise be degraded by proteases if expressed in vivo (4,8). An optimized S30 Premix Plus provides all other required components, including amino acids, rNTPs, tRNAs, an ATP-regenerating system, IPTG and appropriate salts to express high levels of recombinant proteins.

This system also includes a control DNA template, the S30 T7 Control DNA (Section 10.B). This vector is based on the pFN6A (HQ) Flexi® Vector and contains the *Renilla reniformis* luciferase gene with humanized codon usage (*hRluc*) downstream from the T7 promoter, a gene10 sequence, an RBS and an N-terminal $(HQ)_3$ tag for metal affinity purification. Expression of this *hRluc* gene in the S30 T7 High-Yield Protein Expression System can be detected on a Coomassie® blue-stained polyacrylamide gel (Figure 2, Panel A).

The synthesis of a protein of the correct size is a useful way to verify the gene product of a particular DNA sequence. The reaction can be performed with smaller volumes (as low as 5µl) for high-throughput screening in a 96-well plate format. The amount of protein synthesized increases proportionally with reaction volume (up to 250µl). This system can be used to produce proteins toxic to *E. coli* cells. Small amounts of target protein can be purified using affinity tags (e.g., metal-affinity tag) for downstream analysis. Proteins expressed in the cell-free system also may be used for a variety of functional studies, such as enzymatic analysis, protein interactions as well as transcription and translation studies (9). Additional applications of the S30 T7 High-Yield Protein Expression System include incorporation of unnatural amino acids into proteins and screening of compounds that affect translation (10,11).

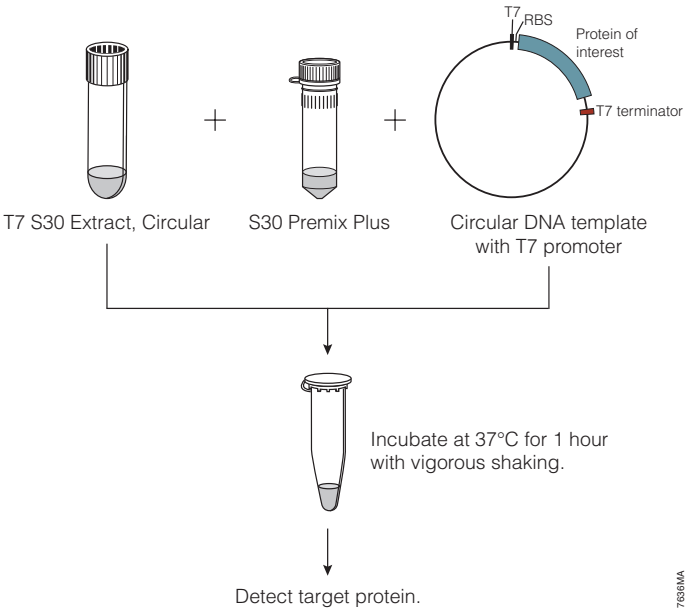


**Figure 1. Schematic for the S30 T7 High-Yield Protein Expression System.**

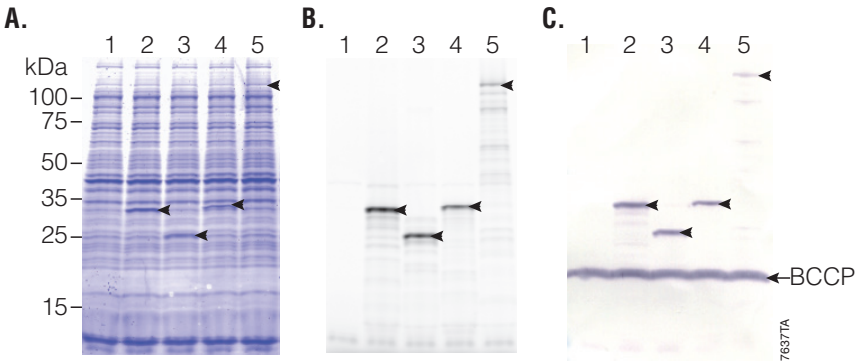


**Figure 2. Coupled in vitro transcription/translation of circular DNA templates using the S30 T7 High-Yield Protein Expression System.** The protein-coding sequences cloned into pFN6A (HQ) Flexi® Vector were expressed as described in Section 4, resolved by SDS-polyacrylamide gel electrophoresis (PAGE; 4–20% Tris-glycine) and visualized by Coomassie® blue staining (**Panel A**), fluorescent scanning (**Panel B**), or transferring to PVDF membrane, treating with Streptavidin Alkaline Phosphatase (Cat.# V5591) and staining with Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841; **Panel C**). For each gel: lane 1, no DNA; lane 2, S30 T7 Control DNA (*Renilla* luciferase); lane 3, Monster Green® Fluorescent Protein; lane 4, HaloTag® protein; lane 5, β-galactosidase. (BCCP = *E. coli* biotin carboxyl carrier protein.)

## 2. Product Components and Storage Conditions

| PRODUCT | CAT.# |
|---|---|
| **S30 T7 High-Yield Protein Expression System** | **L1110** |

Each system contains sufficient reagents for 24 × 50µl reactions. Includes:

- 450µl T7 S30 Extract, Circular (3 × 150µl)
- 600µl S30 Premix Plus (3 × 200µl)
- 20µl S30 T7 Control DNA (0.5µg/µl)
- 1,250µl Nuclease-Free Water

| PRODUCT | CAT.# |
|---|---|
| **S30 T7 High-Yield Protein Expression System** | **L1115** |

Each system contains sufficient reagents for 8 × 50µl reactions. Includes:

- 150µl T7 S30 Extract, Circular
- 200µl S30 Premix Plus
- 20µl S30 T7 Control DNA (0.5µg/µl)
- 1,250µl Nuclease-Free Water

**Storage Conditions:** Store all components at –70°C. Avoid prolonged exposure of this product to $CO_2$ and multiple freeze-thaw cycles, which may have an adverse affect on activity and performance of this product.

## 3. General Considerations

### 3.A. Template Considerations

Expression of cloned DNA fragments in the S30 T7 High-Yield Protein Expression System requires that a protein-coding sequence be under the control of either a T7 promoter or a strong *E. coli* promoter. Vectors used for successful protein expression with this system are listed in Table 1. Expression from T7 promoters is typically higher than from *E. coli* promoters using this system. Expression from *E. coli* promoters can be inhibited by adding rifampicin to the extract; transcription by T7 RNA polymerase is resistant to rifampicin.

Use a DNA template with high purity and concentration (such as 500ng/µl) to minimize the possibility of introducing factors that might reduce protein synthesis.

**Table 1. Vectors Used for Successful Protein Synthesis in S30 T7 High-Yield Protein Expression System (Listed Proteins can be Detected by SDS-PAGE and Coomassie® Blue Staining).**

| Vector | Promoter | Proteins Tested |
|---|---|---|
| pFN6A/K | T7 | firefly luciferase, HaloTag® protein, *Renilla* luciferase, Monster Green® fluorescent protein, cPKA, β-galactosidase |
| pFN18A/K | T7 | cPKA, firefly luciferase, *Renilla* luciferase, Id |
| pIVEX | T7 | elongation factor Ts (EF-Ts) |
| pIX | T7 | green fluorescent protein (GFP) |
| pExp5 | T7 | calmodulin3 (CALM3) |
| pET32a | T7 | Thioredoxin |
| pET43a | T7 | NusA |
| pET3a | T7 | *Renilla* luciferase |
| pET15b | T7 | *Renilla* luciferase |
| pQE30 | T5 | *Renilla* luciferase, firefly luciferase |

It is important to emphasize that large differences in the level of gene expression can result due to the size of the protein, gene of interest and the context in which the gene resides. Changes in the position of the protein-coding sequence relative to the RBS will affect expression levels (12). The RBS generally is located approximately seven bases upstream of the AUG start codon. In addition, many eukaryotic genes contain sequences within the protein-coding region that can function as ribosomal-binding sites when they precede a methionine codon. The presence of such internal sequences can result in internal translation initiation and the synthesis of potentially undesired truncated proteins in the prokaryotic system. An example of this can be seen in the expression of the firefly luciferase gene in the E. coli *T7 S30 Extract System for Circular DNA Technical Bulletin* #TB219, available online at: **www.promega.com/protocols/**. The firefly luciferase gene contains 14 methionine codons, several of which are preceded by potential RBS sequences and produce truncated translation products.

Other factors have been reported to affect protein synthesis in S30 systems, such as:

- 5´ and 3´ untranslated regions (UTRs; 13,14)
- N- (15) or C-terminal (16) fusion tags
- codon usage (17)
- mRNA secondary structure (18,19)
- mRNA stability (20)

To achieve optimal protein yield, use only highly purified plasmid DNA templates with concentrations at 500ng/µl or higher, and avoid adding high concentrations of salts or glycerol with the DNA template. The activity of the S30 T7 High-Yield Protein Expression System may be inhibited by NaCl at >50mM, glycerol at >1%, and by small amounts of $Mg^{2+}$ (1–2mM) or potassium salts (50mM).Ethanol precipitate the DNA template with sodium acetate rather than ammonium acetate to minimize changes to the ammonium concentration. Protein yields from the S30 T7 High-Yield Protein Expression System vary with the template and conditions used. Typical yields can reach tens of micrograms per 50µl reaction (up to 500ng/µl).

### 3.B. Creating a Ribonuclease-Free Environment

The in vitro transcription/translation system is very sensitive to nuclease contamination. To reduce the chance of DNase and RNase contamination, gloves should be worn when setting up experiments, and reaction tubes and pipette tips should be DNase- and RNase-free.

### 3.C. Detecting Recombinant Proteins on Coomassie® Blue-Stained Polyacrylamide Gels

After protein synthesis, several detection methods can be applied. Proteins synthesized at high levels (up to 500ng/µl) in S30 T7 High-Yield Protein Expression System can be detected on a Coomassie® blue-stained SDS-polyacrylamide gel by following manufacturer's suggested protocols.

### 3.D. Detecting Biotinylated Proteins

Biotinylation of the target protein can be achieved with the Transcend™ Non-Radioactive Translation Detection Systems (Cat.# L5070, L5080), which provide an alternative to the use of radiolabeled amino acids. Using these systems, biotinylated lysine residues are incorporated into nascent proteins during translation. This biotinylated lysine is added to the translation reaction as a precharged, labeled biotinylated lysine-tRNA complex (Transcend™ tRNA) rather than a free amino acid. After SDS-PAGE and electroblotting, the biotinylated proteins are visualized by binding streptavidin alkaline phosphatase or streptavidin horseradish peroxidase, followed by either colorimetric (Figure 2, Panel C) or chemiluminescent detection. The biotin tag allows both detection and capture of the translated protein. As little as 0.5–5ng of protein can be detected using the Transcend™ method within 3–4 hours after gel electrophoresis. This sensitivity is equivalent to that achieved with [$^{35}$S]methionine incorporation and autoradiographic detection 6–12 hours after gel electrophoresis. For more information on the Transcend™ System, see the *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin* #TB182, available online at: **www.promega.com/protocols/**

### 3.E. Detecting Fluorescently Labeled Proteins

Another non-radioactive detection method is the FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (Cat.# L5001), which enables fluorescent labeling and detection of proteins synthesized in vitro. The system is based on a lysine-charged tRNA that is labeled at the epsilon position of the lysine with the fluorophore BODIPY®-FL. Fluorescent lysine residues will be incorporated into synthesized proteins during in vitro translation reactions, eliminating the need for radioactivity. For details, see the *FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System Technical Bulletin* #TB285, available online at: **www.promega.com/protocols/**

## 4. Coupled Transcription/Translation Procedure

### 4.A. Standard Protocol

**Note:** For radioactive labeling, we recommend using the *E. coli* T7 S30 Extract System for Circular DNA (Cat.# L1130).

For multiple reactions, create a master mix by combining the appropriate volumes of S30 Premix Plus, T7 S30 Extract, Circular, and Nuclease-Free Water immediately before use. Divide the master mix into microcentrifuge tubes, PCR strip tubes or 96-well PCR plates, and initiate the reactions by adding the DNA template to the tubes.

**Materials to Be Supplied by the User**

- DNase- and RNase-free 1.5ml microcentrifuge tubes
- plasmid DNA encoding the protein of interest
- floor incubator shaker or thermomixer

1. Remove the reagents from the freezer, and allow them to thaw on ice or in an ice-water bath.
2. Set up the following reactions on ice in a DNase- and RNase-free 1.5ml microcentrifuge tube:

| Component | Standard Reaction | Negative Control (see Note 10) | Positive Control (see Note 11) |
|---|---|---|---|
| DNA template (see Notes 7–9) | 1µg | 0 | 2µl |
| S30 Premix Plus (mix well prior to use) | 20µl | 20µl | 20µl |
| T7 S30 Extract, Circular (mix gently prior to use) | 18µl | 18µl | 18µl |
| Nuclease-Free Water to a final volume of | 50µl | 50µl | 50µl |

3. Mix thoroughly by pipetting several times or vortexing gently, then centrifuge in a microcentrifuge for 5 seconds to force the reaction mixture to the bottom of the tube.

### 4.A. Standard Protocol (continued)

4. Quickly bring reaction to 37°C, and incubate the reactions with vigorous shaking for 1 hour (see Notes 12 and 13).
5. Stop the reaction by placing the tubes in an ice-water bath for 5 minutes.
6. Analyze the results of the reaction. See Section 6 for gel analysis of proteins. To evaluate the results of the positive control reaction, see Section 7.

**Notes:**

1. The reaction is very sensitive to nuclease contamination. Wear gloves, and use RNase- and DNase-free reagents and reaction tubes, and filtered pipette tips.
2. Higher protein expression levels can be achieved by increasing the amount of T7 S30 Extract, Circular, in the reaction (up to 45% of the reaction volume) while keeping the total volume constant. However, increasing the volume of the extract will reduce the total number of reactions.
3. Template DNA and water purity are critical. If protein synthesis efficiencies are low, examine the quality of the template DNA and water. Read the discussion concerning DNA templates in Section 3.A.
4. If necessary, transcription by the endogenous *E. coli* RNA polymerase can be inhibited by the antibiotic rifampicin, while transcription by the phage T7 RNA polymerase is unaffected. To inhibit the endogenous RNA polymerase, add 1µl of 50µg/ml of rifampicin in water (Section 10.A) prior to adding the DNA template to the reaction. Adding excess rifampicin is unnecessary and may decrease protein synthesis levels.
5. To obtain fluorescently labeled or biotinylated protein, use 2µl of FluoroTect™ or Transcend™ tRNA in 50µl of reaction. Titrate the tRNAs if necessary. For incorporation of radioactive amino acids, we recommend using the *E. coli* T7 S30 Extract System for Circular DNA (Cat.# L1130).
6. The reaction volume can be scaled down to as little as 5µl without reducing protein yields. For reactions less than 25µl, we recommend assembling the reactions in PCR strip tubes or 96-well PCR plates; for reactions of 25–50µl, we recommend using 0.5ml microcentrifuge tubes; for reactions of 50–250µl, we recommend using 1.5ml microcentrifuge tubes.
7. The amount of DNA required might be template-dependent. We recommend 0.5–1µg/50µl reaction of <5kb plasmid DNA with a T7 promoter. Higher DNA concentrations (such as 2µg/50µl reaction) can be used for large plasmids and vectors with an *E. coli* promoter. If necessary, optimize the amount of DNA added. In general, reactions should not contain more than 4µg of DNA. An increased amount of DNA can increase the incidence of internal translational initiation or the number of prematurely arrested translation products.

8. Refer to Section 3.A for a discussion on suitable DNA templates for this system.

9. The T7 S30 Extract, Circular, contains nuclease activity, preventing the use of linear DNA templates, such as PCR products. PCR products containing a T7 promoter and a ribosome binding site can be expressed using TnT® T7 Quick for PCR DNA (Cat.# L5540).

10. For a negative control, omit the DNA from the reaction. Use the negative control to determine the protein expression background in downstream analysis.

11. Use the S30 T7 Control DNA to synthesize *Renilla* luciferase, which has been codon optimized for use with mammalian cells. The expressed *hRluc* protein (37kDa) can be detected by Coomassie® blue staining of polyacrylamide gels following electrophoresis (Figure 2, Panel A, lane 2). The calculated molecular weight (MW) of *hRluc* protein is 37kDa, but it migrates with an apparent MW of ~33kDa.

12. The reaction may be incubated at 24–37°C. The fastest rate occurs at 37°C for approximately 1 hour, although the reaction will continue for several hours at a slower rate. Lower temperatures produce a slower rate of translation but often extend the time to several hours. Enhanced expression at lower temperatures for longer times appears to be gene- or protein-specific and may be tried if the standard reaction at 37°C for 1 hour does not produce the desired results.

13. To achieve maximum protein expression, quickly bring the reactions to the target temperature (e.g., place the reactions in a heat block preset to target reaction temperature), and shake the reactions vigorously at 300rpm with a floor incubator shaker or in a thermomixer at 1,200rpm.

### 4.B. Synthesis and Assay of the S30 T7 Control Protein

Protein synthesis from the S30 T7 Control DNA can be detected by a number of means such as those described in Section 6. The recombinant *Renilla* luciferase can be detected by Coomassie® blue staining following SDS-PAGE, by fluorescent detection with the incorporation of FluoroTect™ tRNA, or detection by biotinylation using Transcend™ tRNA. A negative-control reaction (i.e., no DNA) is useful to identify background protein levels, such as fluorescence and endogenous biotinylated proteins in the extract. The results comparing the controls are shown in Figure 2, lanes 1 and 2.

Protein expression levels for the positive control reaction also can be measured using an enzymatic assay (Section 7). For enzymatic assays, synthesize unlabeled synthetic *Renilla* luciferase protein as described in Section 4.A. The reaction is diluted with *Renilla* Luciferase Assay Buffer and assayed with *Renilla* Luciferase Assay System (Cat.# E2810, E2820).

## 5. Purification of His- or HQ-Tagged Recombinant Protein Using MagneHis™ Ni-Particles

### Materials to Be Supplied by the User

- magnetic stand (see Section 10.C for options)
- MagneHis™ Protein Purification System (Cat.# V8500, V8550)
- solid NaCl or 5M NaCl solution

1. Add 50µl of Binding/Wash Buffer to a 50µl reaction containing the His-tagged protein. [For HQ-tagged protein, also add NaCl (500mM final concentration) to improve binding of HQ tag to MagneHis™ Ni-Particles.]
2. Vortex the MagneHis™ Ni-Particles to obtain a uniform suspension.
3. Add 30µl of MagneHis™ Ni-Particles.

   **Note:** You may need to titrate the amount of MagneHis™ Ni-Particles used for proteins expressed at higher or lower levels.
4. Invert tube to mix (approximately 10 times), and incubate for 2 minutes at room temperature. Make sure the MagneHis™ Ni-Particles are well mixed.
5. Place the tube in the appropriate magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles. Using a pipette, carefully remove the supernatant.
6. Remove the tube from the magnetic stand. Add 150µl of MagneHis™ Binding/Wash Buffer with 500mM NaCl to the MagneHis™ Ni-Particles, and pipet to mix. Make sure that particles are resuspended well.
7. Place the tube in the appropriate magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles. Using a pipette, carefully remove the supernatant.
8. Repeat the wash step twice for a total of three washes.
9. Remove the tube from the magnetic stand. Add 100µl of MagneHis™ Elution Buffer, and pipet to mix.

   **Note:** HQ-tagged proteins may elute at a lower concentration of imidazole (50–100mM) compared to polyhistidine-tagged proteins. The MagneHis™ Elution Buffer, which contains 500mM imidazole, can be diluted with MagneHis™ Binding/Wash Buffer or water to decrease the imidazole concentration, if desired.
10. Incubate for 1–2 minutes at room temperature. Place tube in a magnetic stand to capture the MagneHis™ Ni-Particles. Using a pipette, remove the supernatant containing the purified protein. Analyze the samples by SDS-PAGE or by functional assay.

    **Note:** Steps 8–10 can be omitted, and the sample containing the MagneHis™ Ni-Particles can be used directly for SDS-PAGE analysis. Add 30µl of 1X SDS gel-loading buffer to resuspend the MagneHis™ Ni-Particles. Load the sample directly onto an SDS-polyacrylamide gel.

## 6. SDS-Polyacrylamide Gel Analysis of Translation Products

The most widely applicable and versatile method for analysis of in vitro translation products is polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) and a discontinuous buffer system. Precast gels are available from a number of manufacturers. In addition to convenience and safety, precast gels provide consistent results.

### Materials to Be Supplied by the User

(Solution compositions are provided in Section 10.A.)

- 2X SDS-polyacrylamide sample buffer
- Precast SDS polyacrylamide gels (e.g., NOVEX® 14% Tris-Glycine gels [Invitrogen Cat.# EC64852]
- or 4–20% Tris-Glycine gradient gels [Invitrogen Cat.# EC60252])
- RNase ONE™ Ribonuclease (Cat.# M4261, M4265) or RNase A Solution (Cat.# A7973)
- Coomassie® blue gel-staining solution (e.g., SimplyBlue™ SafeStain [Invitrogen Cat.# LC6060])
- 1X TBS

**Note:** Before starting, dilute RNase ONE™ Ribonuclease or RNase A solution by mixing 1µl of RNase with 9µl of 1X TBS.

1. Once the transcription/translation reaction is complete (or at any desired time point), remove a 5µl aliquot, and add to it to a microcentrifuge tube containing 14µl of 1X TBS and 1µl of diluted RNase solution. Incubate at room temperature for 5 minutes. Store unused portion of the reaction at –20°C.

   **Note:** Ribonuclease treatment of the sample can reduce the background generated by unincorporated fluorescent and biotinylated tRNAs when used in the reaction.

2. Add 20µl of 2X SDS-polyacrylamide sample buffer.

3. Close the tube, and heat at 90–100°C for 2 minutes to denature the proteins.

   **Note:** The FluoroTect™ tRNA is sensitive to extreme heating. Do **not** exceed 70°C for more than 2–3 minutes. In some cases, high-molecular-weight complexes are formed at 100°C, and denaturation may need to be performed at lower temperatures (e.g., 20 minutes at 60°C or 3–4 minutes at 80–85°C).

4. Load 8µl of the prepared sample on an SDS-PAGE gel. (This is equivalent to 1µl of the original reaction.)

   **Note:** Sample loading volume can be increased or decreased. To load larger volumes, remove a 5µl aliquot, add it to 20µl of acetone in a microcentrifuge tube, and place it on ice for 15 minutes. Centrifuge the acetone-precipitated S30 sample at 12,000 × *g* for 5 minutes. Remove the supernatant, dry the pellet for 15 minutes under vacuum and follow instructions from Step 2.

## 6. SDS-Polyacrylamide Gel Analysis of Translation Products (continued)

5. Perform electrophoresis using standard conditions for your apparatus. Typically, electrophoresis is carried out at a constant current of 20mA until the bromophenol blue dye has run to the bottom of the gel.

6. Detect the expression of target protein by the following methods:

   a. Coomassie® blue staining following manufacturer's suggested methods.

   b. Fluorescent detection when labeling proteins with FluoroTect™ tRNA as directed in the *FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling Systems Technical Bulletin* #TB285.

   c. Detection of biotinylated protein when labeling proteins with Transcend™ tRNA as directed in the *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin* #TB182. A single endogeneous biotinylated protein, *E. coli* biotin carboxyl carrier protein (BCCP), is present in the S30 extract.

## 7. Positive Control *Renilla* Luciferase Assays

### Materials to Be Supplied by the User

- luminometer
- siliconized polypropylene tube or small glass vial
- 96-well white multiwell plate [Luminometer Plates (Case of 50; Cat.# Z3291)]
- *Renilla* Luciferase Assay System (Cat.# E2810, E2820)

*Renilla* Luciferase Assay Reagent is best when prepared before each use. The reagent is stable for 12 hours at room temperature. If necessary, *Renilla* Luciferase Assay Reagent may be stored at –20°C for 2 weeks or at –70°C for up to 1 month. It may be thawed at room temperature up to five times without appreciable activity loss. (See the Renilla *Luciferase Assay System Technical Manual* #TM055 for details).

The reagent should be fully equilibrated to room temperature and mixed thoroughly by vortexing before beginning measurements. The samples to be assayed also should be at ambient temperature and diluted in the 1X *Renilla* Luciferase Assay Lysis Buffer.

The luminescent signal generated by the assay should be linear and within the detection range of the instrument. Please consult your instrument operator's manual for general operating instructions.

The following protocol is designed for use with manual luminometers or other luminometers fitted with one reagent injector, and it is modified from the standard protocol found in the Renilla *Luciferase Assay System Technical Manual* #TM055, available online at: **www.promega.com/protocols/**

### Preparation of *Renilla* Luciferase Assay Reagent for 10 Assays

Add 10µl of 100X *Renilla* Luciferase Assay Substrate (RLAS) to 1ml of *Renilla* Luciferase Assay Buffer contained in either a glass vial or siliconized polypropylene tube. This will prepare sufficient *Renilla* Luciferase Assay Reagent to perform 10 assays.

### Preparation of *Renilla* Luciferase Assay Reagent for 100 assays

Transfer 10ml of *Renilla* Luciferase Assay Buffer into a glass vial or siliconized polypropylene tube. Alternatively, measure 10ml of *Renilla* Luciferase Assay Buffer in a 10ml pipette, discard the residual buffer in the bottle and return the 10ml of *Renilla* Luciferase Assay Buffer to the bottle. Add 100µl of 100X RLAS to 10ml of *Renilla* Luciferase Assay Buffer. Rinse the pipette tip used to transfer the RLAS in the newly prepared reagent to wash any residual substrate from the pipette tip.

Prepare an adequate volume to perform the desired number of *Renilla* Luciferase Assays (100µl of reagent per assay). Add 1 volume of 100X RLAS to 100 volumes of *Renilla* Luciferase Assay Buffer in a glass or siliconized polypropylene tube. If the entire volume of *Renilla* Luciferase Assay Buffer is to be made into *Renilla* Luciferase Assay Reagent, the *Renilla* Luciferase Assay Buffer bottle may be used.

### Protocol for a Manual Luminometer

1. Add 97.5µl of *Renilla* Luciferase Assay Lysis Buffer to 2.5µl of the transcription/translation reaction prepared in Section 4.A, and mix thoroughly by vortexing. This results in a 1:40 dilution of the reaction.
2. In a separate tube, add 50µl of the diluted reaction, then add 50µl of *Renilla* Luciferase Assay Reagent as prepared above.
3. Mix quickly by flicking the tube with a finger, or vortex to thoroughly mix (1–2 seconds).
4. Place tube in luminometer, and initiate measurement. Luminescence is normally integrated over 10 seconds with a 2-second delay. Other integration times also may be used.
5. If the luminometer is not connected to a printer or computer, manually record the *Renilla* luciferase activity measurement.

## 7. Positive Control *Renilla* Luciferase Assays (continued)

### Protocol for a Luminometer Fitted with One or Two Reagent Injectors

1. Format the luminometer so that the injector dispenses 50µl. Prime the injector with *Renilla* Luciferase Assay Reagent. Priming the assay reagent through an empty injector system prevents dilution and contamination of the primed reagent. (See the Renilla *Luciferase Assay System Technical Manual* #TM055 for details.)
2. Add 97.5µl of *Renilla* Luciferase Assay Lysis Buffer to 2.5µl of the transcription/translation reaction prepared in Section 4.A, and mix thoroughly by vortexing. This results in a 1:40 dilution of the reaction.
3. For each reaction, carefully add 50µl of the diluted reaction to an individual luminometer tube or well of a white multiwell plate.
4. Place the sample in a luminometer.
5. Initiate measurement. This action will initiate injection of the *Renilla* Luciferase Assay Reagent into the reaction vessel and subsequently measure the *Renilla* luciferase activity. Luminescence is normally integrated over 10 seconds with a 2-second delay. Other integration times also may be used.
6. If the luminometer is not connected to a printer or computer, manually record the *Renilla* luciferase activity measurement.

## 8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

| Symptoms | Possible Causes and Comments |
|---|---|
| Positive control reaction produces no protein | System was not stored at –70ºC. The T7 S30 Extract, Circular, will lose activity if stored improperly. Always store at –70ºC. |
| | The T7 S30 Extract, Circular, and S30 Premix Plus were frozen and thawed multiple times. Freezing and thawing the T7 S30 Extract, Circular, and S30 Premix Plus more than once will reduce protein synthesis and should be avoided. |
| | RNase contamination in the reaction. Follow recommendations in Section 3.B. |
| | Reaction was set up improperly. Mix all reagents well after thawing, and set up the reactions on ice. Further suggestion in Section 4.A, Note 13. Repeat the experiment. |
| Low or no target protein expression, but positive control reaction produces protein | DNA quality was poor, or wrong DNA quantity was used:<br>• Use only highly purified plasmid DNA.<br>• Check DNA concentration and quality by $A_{260}$ measurement and ratio of $A_{260}/A_{280}$ (ratio should be ≥1.8).<br>• Check DNA integrity and purity on an agarose gel.<br>• Prepare new DNA template.<br>• Ethanol precipitate the DNA template (use sodium acetate and avoid ammonium acetate). See the *PureYield*™ *Plasmid Midiprep System Technical Manual* #TM253 for details.<br>• DNA template is contaminated with ethanol, sodium salt or ammonium acetate (see Section 3.A).<br>• Do not use agarose gel-purified DNA.<br>• Perform DNA titration reactions to determine the optimal DNA concentration. |
| | Gene of interest was not in proper context for efficient transcription or translation. Transfer the target protein-coding sequence to a vector that has optimal configuration for protein expression (Table 1 and Section 3.A). |

## 8. Troubleshooting (continued)

| Symptoms | Possible Causes and Comments |
|---|---|
| Low or no target protein expression, but positive control reaction produce protein (continued) | Reaction was not incubated with vigorous shaking. Incubate the reaction in a thermomixer or floor shaker (Section 4.A). |
| | Protein was expressed with a fusion tag that reduces its level of synthesis. Use a different DNA construct for optimal protein expression. |
| | PCR DNA was used in the reaction. PCR templates produce less protein than plasmid DNA in this system. For optimal protein yield, use a circular DNA template. |
| | Protein-coding region has several internal translational initiation sites. Modify the coding sequence to remove these sites. |
| | Protein not compatible with the cell-free expression system. The target protein might not be expressed in this system; change to a different cell-based or cell-free expression system (e.g., TnT® Quick Coupled Transcription/Translation System). |
| | Protein is large. Protein yield can be affected by protein size. Perform reaction at a lower temperature (24–30°C) to increase yield of larger proteins. |
| | Plasmid contains an *E. coli* promoter. *E. coli* promoters generally produce less protein than the T7 promoter. Use T7 promoter-based plasmids for transcription/translation. See Table 1. |
| | Protein was degraded by protease. The extract in this system is deficient in OmpT endoproteinase and lon protease activity. Use protease inhibitors in the reaction to reduce other protease activities that might be present. |
| | Clone is without gene of interest or has a mutation that interferes with transcription or translation. Perform DNA sequencing to confirm correct insert. If sequence is wrong, use the correct construct in the reaction. |

## 8. Troubleshooting (continued)

| Symptoms | Possible Causes and Comments |
|---|---|
| Protein is produced but has low activity | Protein folding was not correct. Perform reaction at a lower temperature (24–30°C) to try to improve protein folding. |
| | Disulfide bond is required for protein activity. The system contains the reducing agent DTT; thus, disulfide bonds might not form properly. |
| | Posttranslational modification is required for activity. The system is based on *E. coli* extract, which lacks posttranslational modifications, such as glycosylation and phosphorylation. |
| | Protein was not soluble. We recommend performing reactions at 37ºC for optimal yield. To improve solubility, perform reactions at a lower temperature (24–30ºC). |
| | Protein activity requires a cofactor:<br>• Add cofactors to the protein synthesis reaction, or assay protein activity with added cofactors.<br>• If the cofactor is another gene product, cotranslation might enhance protein activity. |
| Low level of fluorescence or biotinylation (or biotin incorporation) | The template lacks AAA codon for lysine. Check sequence to make sure the template contains lysine (AAA) codons in the protein-coding region. |
| | The sample was heated at temperatures >70ºC for a long period of time. The FluoroTect™ tRNA fluorophore is sensitive to extreme heating. If heating to denature the proteins, do **not** exceed 70°C for more than 2–3 minutes. |
| | The amount of labeled tRNA was not sufficient. Increase the amount of FluoroTect™ and Transcend™ tRNA used in the reaction to determine the optimal tRNA levels. |
| | Detection of biotinylated lysine residues using blotting was not performed optimally. Please see the troubleshooting section in the *Transcend™ Non-Radioactive Translation Detection System Technical Bulletin* #TB182 for details. |

## 8. Troubleshooting (continued)

| Symptoms | Possible Causes and Comments |
|---|---|
| High background using fluorescent/biotinylation detection | Unincorporated tRNAs present in the sample during gel analysis. Following the recommended procedure in Section 6, treat samples with RNase ONE™ Ribonuclease or RNase A before denaturing polyacrylamide gel analysis. |
| | Protein might be truncated or has internal initiation sites:<br>• Perform reaction at a lower temperature (24–37°C).<br>• Modify the protein-coding sequence to remove internal initiation sites. |
| | A ~17kDa band was detected when using Transcend™ tRNA while following the procedure in Section 6. There is an endogenous *E. coli* biotin carboxyl carrier protein (BCCP) in the T7 S30 Extract, Circular. |

## 9. References


1. Structural Genomics Consortium *et al.* (2008) Protein production and purification. *Nat. Methods* **5**, 135–46.
2. Studier, F.W. *et al.* (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89.
3. Terpe, K. (2006) Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.* **72**, 211–22.
4. Studier, F.W. and Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–30.
5. Nevin, D.E. and Pratt, J.M. (1991) A coupled in vitro transcription-translation system for the exclusive synthesis of polypeptides expressed from the T7 promoter. *FEBS Lett.* **291**, 259–63.
6. Zubay, G. (1973) In vitro synthesis of protein in microbial systems. *Annu. Rev. Genet.* **7**, 267–87.
7. Zubay, G. (1980) The isolation and properties of CAP, the catabolite gene activator. *Methods Enzymol.* **65**, 856–77.
8. Pratt, J.M. (1984) In: *Transcription and Translation: A Practical Approach*, Hames, B. D. and Higgins, S. J., eds., IRL Press, New York, 179.
9. *Promega Notes* (1990) *E. coli* S30 coupled transcription/translation system. **26**, 1–2.
10. Noren, C.J. *et al.* (1989) A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244**, 182–8.

11. Liu, M. *et al.* (2008) Bacterial addiction module toxin Doc inhibits translation elongation through its association with the 30S ribosomal subunit. *Proc. Natl. Acad. Sci. USA* **105**, 5885–9.

12. Baranov, V.I. and Spirin, A.S. (1993) Gene expression in cell-free system on preparative scale. *Methods Enzymol.* **217**, 123–42.

13. Ahn, J.H. *et al.* (2005) Cell-free synthesis of recombinant proteins from PCR-amplified genes at a comparable productivity to that of plasmid-based reactions. *Biochem. Biophys. Res. Commun.* **338**, 1346–52.

14. Son, J.M. *et al.* (2006) Enhancing the efficiency of cell-free protein synthesis through the polymerase-chain-reaction-based addition of a translation enhancer sequence and the in situ removal of the extra amino acid residues. *Anal. Biochem.* **351**, 187–92.

15. Torizawa, T. *et al.* (2004) Efficient production of isotopically labeled proteins by cell-free synthesis: A practical protocol. *J. Biomol. NMR* **30**, 311–25.

16. Palmer, E. *et al.* (2006) Enhanced cell-free protein expression by fusion with immunoglobulin Ckappa domain. *Protein Sci.* **15**, 2842–6.

17. Chumpolkulwong, N. *et al.* (2006) Translation of 'rare' codons in a cell-free protein synthesis system from *Escherichia coli*. *J. Struct. Funct. Genomics* **7**, 31–6.

18. Paulus, M., Haslbeck, M. and Watzele, M. (2004) RNA stem-loop enhanced expression of previously non-expressible genes. *Nucleic Acids Res.* **32**, e78.

19. Voges, D. *et al.* (2004) Analyzing and enhancing mRNA translational efficiency in an *Escherichia coli* in vitro expression system. *Biochem. Biophys. Res. Commun.* **318**, 601–14.

20. Ahn, J.H., Kang, T.J. and Kim, D.M. (2008) Tuning the expression level of recombinant proteins by modulating mRNA stability in a cell-free protein synthesis system. *Biotechnol. Bioeng.* **101**, 422–7.

## 10. Appendix

### 10.A. Composition of Buffers and Solutions

**1X TBS**

| | |
|---|---|
| 100mM | Tris-HCl (pH 7.6) |
| 150mM | NaCl |

**2X SDS-polyacrylamide sample buffer**

| | |
|---|---|
| 100mM | Tris-HCl (pH 6.8) |
| 4% | SDS |
| 0.2% | bromophenol blue |
| 20% | glycerol |
| 200mM | DTT |

2X SDS polyacrylamide sample buffer lacking DTT can be stored at room temperature. DTT should be added from 1M stock solution just before the buffer is used.

**4X SDS gel-loading buffer**

| | |
|---|---|
| 0.24M | Tris-HCl (pH 6.8) |
| 2% | SDS |
| 3mM | bromophenol blue |
| 50.4% | glycerol |
| 0.4M | DTT |

SDS gel-loading buffer lacking DTT can be stored at room temperature. DTT should be added from a 1M stock just before the buffer is used.

**MagneHis™ Binding/Wash Buffer (pH 7.5)**

| | |
|---|---|
| 100mM | HEPES |
| 10mM | imidazole |

**MagneHis™ Elution Buffer (pH 7.5)**

| | |
|---|---|
| 100mM | HEPES |
| 500mM | imidazole |

**rifampicin stock solution (10mg/ml)**

| | |
|---|---|
| 50mg | rifampicin |

Add DMSO to a final volume of 5ml. Dispense into aliquots, and store at –20°C. For a working solution, dilute the stock to 50µg/ml in water.

**SDS-PAGE gel 10X running buffer (per liter)**

| | |
|---|---|
| 30g | Tris base |
| 144g | glycine |
| 10ml | 10% SDS |

Add deionized water to a final volume of 1L. Store at room temperature.

## 10.B. S30 T7 Control DNA Circular Map

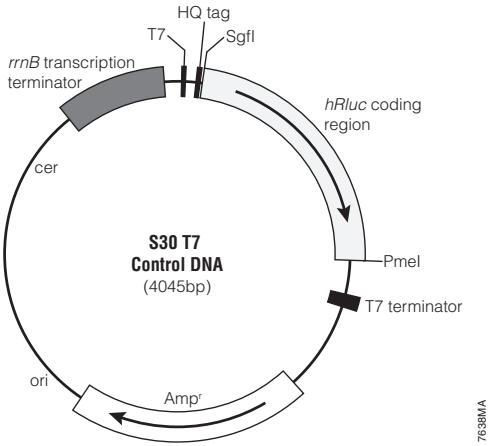


**Figure 3. S30 T7 Control DNA circular map.**

**Sequence reference points:**

| | |
|---|---|
| T7 RNA polymerase promoter | 21–38 |
| $(HQ)_3$ tag | 76–93 |
| *Renilla* luciferase (*hRluc*) coding region | 98–1039 |
| T7 RNA polymerase terminator region | 1163–1210 |
| β-lactamase ($Amp^r$) coding region | 1544–2404 |
| *rrnB* transcription terminater | 3602–4003 |

## 10.C. Related Products

| Product | Size | Cat.# |
|---|---|---|
| *E. coli* S30 Extract System for Linear Templates | 30 reactions | L1030 |
| *E. coli* S30 Extract System for Circular Templates | 30 reactions | L1020 |

Each system contains sufficient reagents to perform 30 × 50µl coupled reactions.

| Product | Size | Cat.# |
|---|---|---|
| Transcend™ Colorimetric Translation Detection System | 30 reactions | L5070 |
| Transcend™ Chemiluminescent Translation Detection System | 30 reactions | L5080 |

Each system contains sufficient reagents to label 30 × 50µl translation reactions and perform detection of biotinylated proteins on 6 blots (7 × 9cm).

| Product | Size | Cat.# |
|---|---|---|
| Transcend™ tRNA | 30µl | L5061 |

Thirty microliters of Transcend™ tRNA is sufficient for 30 × 50µl translation reactions.

## 10.C. Related Products (continued)

| Product | Size | Cat.# |
|---|---|---|
| FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System | 40 reactions | L5001 |

| Product | Size | Cat.# |
|---|---|---|
| TnT® SP6 Quick Coupled Transcription/Translation System | 40 reactions | L2080 |
| | 5 reactions | L2081 |
| TnT® T7 Quick Coupled Transcription/Translation System | 40 reactions | L1170 |
| | 5 reactions | L1171 |
| TnT® T7 Quick for PCR DNA | 40 reactions | L5540 |
| TnT® T3 Coupled Reticulocyte Lysate System | 40 reactions | L4950 |
| TnT® T7 Coupled Reticulocyte Lysate System | 40 reactions | L4610 |
| | 8 reactions | L4611 |
| TnT® SP6 Coupled Reticulocyte Lysate System | 40 reactions | L4600 |
| | 8 reactions | L4601 |
| TnT® T7/SP6 Coupled Reticulocyte Lysate System | 40 reactions | L5020 |
| TnT® T7/T3 Coupled Reticulocyte Lysate System | 40 reactions | L5010 |
| TnT® T7 Coupled Wheat Germ Extract System | 40 reactions | L4140 |
| TnT® SP6 Coupled Wheat Germ Extract System | 40 reactions | L4130 |
| TnT® T7/SP6 Coupled Wheat Germ Extract System | 40 reactions | L5030 |

| Product | Size | Cat.# |
|---|---|---|
| *Renilla* Luciferase Assay System | 100 assays | E2810 |
| | 1,000 assays | E2820 |
| MagneHis™ Protein Purification System | 65 reactions | V8500 |
| | 325 reactions | V8550 |
| RNase ONE™ Ribonuclease | 1,000u | M4261 |
| | 5,000u | M4265 |

**MagneSphere® Technology Magnetic Separation Stands**

| Product | Size | Cat.# |
|---|---|---|
| MagneSphere® Technology Magnetic Separation Stand (two-position) | 0.5ml | Z5331 |
| | 1.5ml | Z5332 |
| | 12 × 75mm | Z5333 |
| MagneSphere® Technology Magnetic Separation Stand (twelve-position) | 0.5ml | Z5341 |
| | 1.5ml | Z5342 |
| | 12 × 75mm | Z5343 |

## 11. Summary of Change

The following change was made to the 5/16 revision of this document:

Corrected notes for protocol in Section 6.