TECHNICAL BULLETIN

E. coli S30 Extract System for Linear Templates

Instructions for Use of Product
L1030

Revised 6/15
TB102

Promega

E. coli S30 Extract System for Linear Templates

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 Bulletin.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The *E. coli* S30 Extract System for Linear Templates[a] is prepared using minor modifications of the protocol described by Lesley and colleagues (1) and allows successful transcription/translation of linear DNA templates (Figure 1). The investigator only needs to provide linear DNA containing a prokaryotic *E. coli*-like promoter (see Section 3.A) and sequence for a ribosomal binding site. In vitro-generated RNA from DNA templates lacking an *E. coli* promoter may also be used in this system, but protein yields will be decreased to 1–10% of those produced from linear DNA templates.

The S30 Extract for Linear Templates is prepared from an *E. coli* B strain (SL119), which is deficient in OmpT endoproteinase, lon protease and exonuclease V (*rec*BCD). The absence of protease activity results in greater stability of expressed proteins. The *rec*D mutation of the SL119 strain produces a more active S30 Extract for Linear DNA than the previously described nuclease-deficient, *rec*BC-derived S30 extracts (1–3). However, the S30 Extract for Linear Templates is less active than the S30 Extract System for Circular DNA (Cat.# L1020).

The S30 System contains an S30 Premix Without Amino Acids that is optimized for each lot of S30 Extract and contains all other required components, including NTPs, tRNAs, an ATP-regenerating system, IPTG and appropriate salts. Amino Acid Mixtures lacking cysteine, methionine or leucine are provided to facilitate radiolabeling of translation products. This system also includes pBEST*luc*™ DNA, a control DNA template that contains the eukaryotic firefly luciferase gene positioned downstream from the *tac* promoter and a ribosome binding site (see Figure 3). An easy-to-perform, non-radioactive positive control reaction (Section 5) using the provided Luciferase Assay Reagent allows detection of luciferase gene expression in the S30 System for Linear Templates (4–6). The assay reaction produces high light output for several minutes, allowing the researcher to choose from several methods of detection (5–8), including simple visual observation of luminescence.

Applications of the *E. coli* S30 Extract System for Linear Templates include:

- Identification of cloned or synthesized genes from linear or covalently closed circular DNA.
- Rapid, fine-structure mapping of genes without additional cloning steps.
- Rapid verification of in vitro PCR-generated mutations.
- Expression of toxic or otherwise unobtainable, in vivo-expressed gene products.
- Synthesis of truncated gene products from PCR-generated or restriction enzyme-digested DNA, for functional domain mapping and epitope mapping studies.
- Expression from in vitro-generated RNA for genes lacking an appropriate *E. coli* promoter.

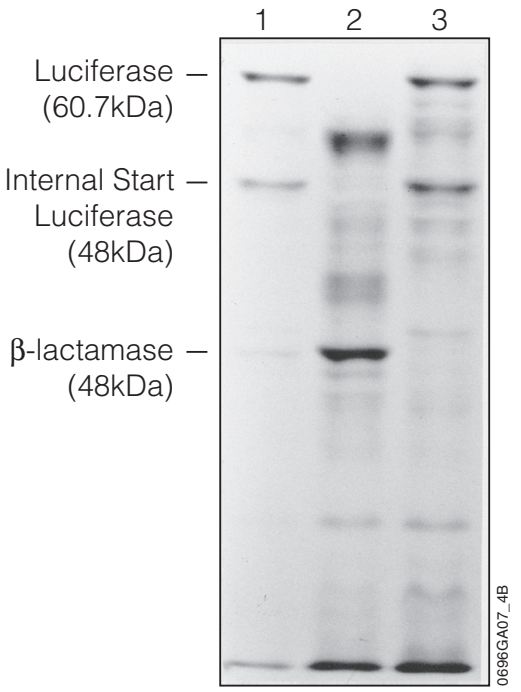

Figure 1. Coupled in vitro transcription/translation of linear DNA templates using the *E. coli* S30 Extract System for Linear Templates. Protein analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent fluorography (45-minute exposure). Five microliters of each 50µl reaction mix was loaded into each lane. Lane 1 shows protein products synthesized from 2µg of the pBEST*luc*™ DNA, which is linearized with *Xho* I. Full-length luciferase migrates at 60kDa, and β-lactamase migrates at 31.5kDa. An apparent internal translation start results in a second major gene product of 48kDa. Lane 2 shows protein products from an S30 reaction containing 4µg of *Cla* I-digested pBEST*luc*™ DNA. A truncated luciferase polypeptide lacking 10kDa of native luciferase carboxy terminus is expected. Also note that all the faint proteins resulting from putative internal translation starts migrate at positions that are 10kDa less than in Lane 3. Lane 3 shows protein products synthesized from PCR-generated DNA (primers were used to generate a PCR product comprising the *Hin*d III to *Xho* I region of pBEST*luc*™ DNA). The faint bands in lanes 2 and 3 (absent in lane 1) apparently result from internal translation start sites, which can appear when excess DNA is added to the S30 System. These bands have been shown to be truncated luciferase by Western blot analysis.

2. Product Components and Storage Conditions

PRODUCT	CAT.#
E. coli S30 Extract System for Linear Templates	L1030

Each system contains sufficient reagents for 30 × 50µl coupled reactions. Includes:

- 175µl Amino Acid Mixture Minus Cysteine, 1mM
- 175µl Amino Acid Mixture Minus Methionine, 1mM
- 175µl Amino Acid Mixture Minus Leucine, 1mM
- 450µl S30 Extract, Linear (3 × 150µl)
- 750µl S30 Premix Without Amino Acids
- 250µl Luciferase Assay Reagent
- 1ml Luciferase Dilution Reagent
- 20µl pBEST*luc*™ DNA, Linear (0.5µg/µl)

Please see Related Products (Section 12.B) for information on Amino Acid Mixture, Complete (Cat.# L4461), and Amino Acid Mixture Minus Methionine and Cysteine (Cat.# L5511).

Bulk quantities (over 50ml) of the S30 Extract and Premix are available from Promega.

Stability/Storage: Store all components at –70°C. Product is sensitive to CO_2 (avoid prolonged exposure) and multiple freeze-thaw cycles, which may have an adverse effect on activity/performance. Luciferase Assay Reagent is stable for at least 12 months if stored and handled properly.

3. General Considerations

3.A. Template Considerations

Template Purity

Use highly purified DNA templates (e.g., CsCl- or gel-purified). Avoid adding excessive salts or glycerol with the DNA template. The activity of the S30 System may be inhibited by NaCl (≥50mM), glycerol (≥1%), or by very small amounts of magnesium or potassium salts. Precipitate the DNA template with sodium acetate rather than ammonium acetate. Protein yields from the S30 System vary depending on the template and the conditions of use. Typical yields range from 50–250ng per reaction.

Large Templates

Larger DNA templates, such as Lambda DNA, may be used in the *E. coli* S30 Extract System for Linear Templates (Figure 2).

Promoter Choice

Expression of gene products from linear DNA containing supercoiling-sensitive promoters (e.g., the *lac* promoter) can be reduced in the S30 System by up to 100-fold of that of other methods (3). Examples of good supercoiling-insensitive promoters include *lac*UV5, *tac*, λP_L and λP_R. DNA from other prokaryotic species may not contain promoters that direct transcription in the *E. coli* S30 Extract System for Linear Templates.

The context in which a gene resides may account for large differences in expression. Changes in the gene position relative to the ribosomal binding site (RBS) will affect expression levels (9). The RBS is generally located approximately seven bases upstream of the AUG start codon. In addition, many eukaryotic genes contain sequences within the 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 is seen in the expression of the firefly luciferase gene in the *E. coli* S30 Extract System. The firefly luciferase gene contains 14 methionine codons, several of which are preceded by potential RBS sequences and produce truncated translation products (see Figure 1).

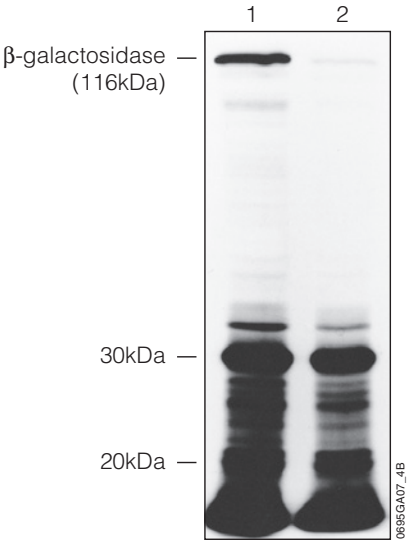

Figure 2. Coupled in vitro transcription/translation of a λ DNA template using the *E. coli* S30 Extract System for Linear Templates. Protein analysis by SDS-PAGE with subsequent fluorography (45-minute exposure). Lane 1 shows protein products synthesized from 5µg of λgt11 *Sfi-Not* DNA. The λgt11 *Sfi-Not* DNA, when added to the extract, rapidly circularizes at the cos sites and becomes supercoiled. Lane 2 shows protein products synthesized from λgt11 *Sfi-Not Eco*R I/*Not* I "arms" (or linear DNA). Five microliters of each 50µl reaction mix was loaded into both lanes. Comparing lanes 1 and 2, all the λ gene products are expressed at similar levels from either supercoiled or linear DNA, while β-galactosidase from the arms is dramatically reduced. The decrease in β-galactosidase synthesis probably results from reduced transcription from the supercoiling-sensitive *lac* promoter.

PCR-Generated Templates

PCR technology has introduced many methods for site-specific in vitro mutagenesis. Advances combining PCR with phage λ exonuclease treatments produce mutated fragments larger than 2.5kb (10). The mutated PCR product may be added to the *E. coli* S30 Extract System for Linear Templates for rapid confirmation of expected protein size or activity.

Avoid contaminating the S30 reaction with the wrong PCR product or primer dimers. If agarose gel analysis indicates that your PCR produced a unique band, primer dimers can be removed by ethanol precipitation with sodium acetate. Otherwise, PCR-amplified DNA should be gel-purified before using it in the *E. coli* S30 Extract System for Linear Templates.

Restriction Enzyme-Digested Templates

For restriction enzyme-digested DNA, digest 10–20µg of DNA in a 100–200µl volume. After ethanol precipitation, resuspend the DNA at a concentration of 1µg/µl in TE buffer or water. Add 2–4µg of this DNA directly to the S30 reaction. If the desired results are not obtained using this method, the DNA may be purified further by phenol:chloroform extraction followed by ethanol precipitation.

RNA Templates

For optimal results, the amount of in vitro RNA added to the extract can vary from 10–100µg. For synthesizing milligram quantities of highly pure, "translatable" RNA, we recommend using one of the RiboMAX™ Large Scale RNA Production Systems (Cat.# P1280 and P1300).

3.B. Detection Methods

The amino acid mixtures provided are compatible with the use of radiolabeled cysteine, methionine and leucine. Several amino acid mixtures are also available separately (see Section 12.B). Radiolabeled proteins can be detected by standard methods.

The Transcend™ Non-Radioactive Translation Detection Systems (Cat.# L5070 and L5080) provide an alternative to the use of radiolabeled amino acids. Using these systems, biotinylated lysine residues are incorporated into nascent proteins during translation, eliminating the need for labeling with [^{35}S]methionine or other radioactive amino acids. After SDS-PAGE and electroblotting, the biotinylated proteins are visualized by binding streptavidin alkaline phosphatase or streptavidin-horseradish peroxidase, followed by colorimetric 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 within 3–4 hours after gel electrophoresis using the Transcend™ method. This sensitivity is equivalent to that of [^{35}S] methionine incorporation and autoradiographic detection 6–12 hours after gel electrophoresis. For more information, request the *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin* #TB182.

3.C. pBEST*luc*™ Control DNA

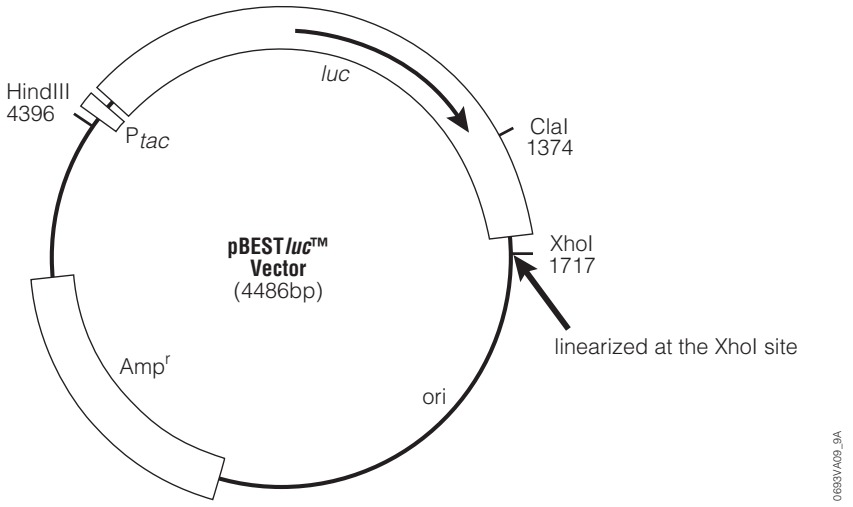

Figure 3. Vector circle map of pBEST*luc*™ Vector. The pBEST*luc*™ DNA supplied in the *E. coli* S30 Extract System for Linear Templates is linearized with Xho I

4. Coupled Transcription/Translation Procedure

4.A. Standard Protocol

An example of a standard reaction using [^{35}S]methionine is provided. [^{35}S]cysteine, [^{3}H]leucine or [^{14}C]leucine can also be used. In general, add 1µl of the radiolabeled amino acid to a 50µl reaction.

If radiolabeled products are not required, omit the radiolabeled amino acid and use a complete amino acid mixture. To create a complete amino acid mixture, combine equal volumes of any two Minus Amino Acid Mixtures. Add 5µl of the complete amino acid mixture per 50µl reaction (see Note 3).

For multiple reactions, create a master mix by combining the appropriate volumes of Amino Acid Mixture Minus Methionine (or Cysteine or Leucine), S30 Premix Without Amino Acids, radiolabeled amino acid (optional), S30 Extract and Nuclease-Free Water. Aliquot the master mix into 1.5ml microcentrifuge tubes, and initiate the reactions by adding the DNA template to the tubes.

Materials to Be Supplied by the User

- Nuclease-Free Water (Cat.# P1193)
- [^{35}S]methionine (1,200Ci/mmol at 15mCi/ml) or other radiolabeled amino acid [optional]

1. Set up the following reactions

Component	Standard	Positive Control (See Note 3)
DNA or RNA template (see Note 1)	≤4µg	8µl
Amino Acid Mixture Minus Methionine (mix before use)	5µl	5µl
S30 Premix Without Amino Acids (mix gently before use)	20µl	20µl
[^{35}S]methionine (1,200Ci/mmol at 15mCi/ml) (optional, see Notes 2 and 3)	1µl	1µl
S30 Extract, Linear (mix gently before use)	15µl	15µl
Nuclease-Free Water to a final volume of	50µl	50µl

2. Vortex gently, then centrifuge in a microcentrifuge for 5 seconds to bring the reaction mixture to the bottom of the tube.
3. Incubate the reaction(s) at 37°C for 1–2 hours (see Note 4).
4. Stop the reaction by placing the tubes in an ice bath for 5 minutes.
5. Analyze the results of the reaction. See Sections 6–10 for incorporation assays and gel analysis of proteins.

Template DNA and water purity are extremely important. If efficiencies are low, examine the quality of the template DNA and water used.

Notes:

1. Optimize the amount of DNA added. In general, reactions should not contain more than 4µg of DNA. An increased amount of linearized DNA can result in higher incorporation of label but also can increase the number of internal translational starts or prematurely arrested translation products. Use the supplied pBEST*luc*™ DNA (Figure 3) for the positive control reaction. Refer to Section 3.A for a discussion of templates.
2. We recommend using EasyTag™ L-[^{35}S]methionine (PerkinElmer Cat.# NEG709A). This [^{35}S]methionine may be stored at 4°C without dispensing into aliquots. Other types of ^{35}S-labeled amino acids may be oxidized easily to translation-inhibiting sulfoxides and should be stored in aliquots at –70°C in buffer containing DTT.
3. Use pBEST*luc*™ DNA (Figure 3) to synthesize luciferase. Luciferase migrates at 60kDa. An apparent internal translation start results in a second major gene product of 48kDa. The control plasmid also contains the gene for ampicillin resistance (β-lactamase). β-lactamase may appear as a faint band migrating at 31.5kDa. See Figure 1 for typical results.

   Unlabeled luciferase is used in a luminescence assay to monitor the efficiency of the S30 reaction (Section 5). To generate unlabeled luciferase, use a complete amino acid mixture rather than a Minus Amino Acid Mixture and omit the radiolabeled amino acid (see Section 5).

Perform a negative control reaction with each set of translation reactions. For negative controls, omit the DNA from the reaction. Use the negative control to determine background radiolabel incorporation (see Section 6.A).

3. Enhanced expression at lower temperatures for longer times appears to be gene/protein-specific and may be tested if the standard reaction at 37°C for 1 hour does not produce the desired results.

4.B. Large-Scale Dialysis Reactions Using the *E. coli* S30 Extract System

Continuous systems have been described in which inhibitory translation byproducts are removed through a membrane by pumping a feed solution containing amino acids and ribonucleotides through the reaction vessel during the course of a 20–30 hour translation reaction (11). This type of dialysis system can be used to increase protein synthesis by 10- to 20-fold over standard batch reactions (9). Refer to reference 9 for protocol details.

5. Synthesis and Assays of Luciferase Control

The gene encoding firefly luciferase has proven highly effective for the study of gene function and cellular events. The assay of luciferase activity is extremely sensitive, rapid, easy to perform, and relatively inexpensive. For more information, see the *Luciferase Assay System Technical Bulletin* #TB281.

A total luciferase assay volume of 60–70µl (Section 5.A, Step 5) is convenient for most light measurement devices. For some methods, such as when using film for activity detection, a larger volume may be desired. Conversely, many scintillation counters and luminometers may operate well when using a smaller volume. Changes in the total volume of the assay, while maintaining the ratio of sample volume to assay reagent volume, should not affect the properties of the assay.

5.A. Synthesis of Luciferase Control

1. Synthesize unlabeled luciferase using the following reaction:

Component	Volume
pBEST*luc*™ DNA	8µl
Complete Amino Acid Mixture* (mix before use)	5µl
S30 Premix Without Amino Acids (mix gently before use)	20µl
S30 Extract, Linear (mix gently before use)	15µl
Nuclease-Free Water to a final volume of	50µl
*To create a complete amino acid mixture, combine equal volumes of any two Minus Amino Acid Mixtures.	

! Water purity is extremely important. If efficiencies are low, examine the quality of the water used.

2. Vortex gently, then centrifuge in a microcentrifuge for 5 seconds to bring the reaction mixture to the bottom of the tube.
3. Incubate the reaction at 37°C for 1–2 hours (see Section 4.A, Note 4).
4. Stop the reaction by placing the tubes in an ice bath for 5 minutes.
5. Prepare a dilution series as follows:

a. At room temperature, add 50µl of Luciferase Dilution Reagent to each of 4 microcentrifuge tubes.

b. Add 50µl of the luciferase S30 reaction to the first tube (this results in a twofold dilution), mix and pipet 50µl from first tube to second tube. Mix and continue the series of twofold dilutions in the remaining 2 tubes.

Note: If the samples are to be quantitated in a scintillation counter, further dilution (five- to tenfold) using Luciferase Dilution Reagent may be required at Step 5, as these instruments experience signal saturation at high light intensities.

6. A 10–20µl aliquot of each dilution can be placed either into a separate microcentrifuge tube or spotted into the well of a white 96-well plate. The remaining dilutions may be stored at –20°C for several months with little loss of luciferase activity.
7. Measure luminescence by luminometry or scintillation counting (Section 5.B), photography (Section 5.C), or visual detection (Section 5.D).

5.B. Standard Luciferase Assay (Luminometer or Scintillation Counter)

Luminometer

Because of the constant light output with the Luciferase Assay System, an automated injection device is not required. In many luminometers, the photomultiplier tube requires 1–2 seconds to stabilize after a sample is introduced. Therefore, allow an initial delay of at least 3 seconds, then measure luminescence for 10 seconds to 5 minutes.

Promega offers several luminometers in single-tube and microplate format. The features of these luminometers make them an excellent choice for measuring luminescence both in cell extracts and in living cells expressing luciferase.

Scintillation Counter

Scintillation counters should be used in the manual mode because light generated by the luminescent reaction decays slowly ($t_{1/2}$ is approximately 5 minutes). Samples should be introduced into the counting chamber shortly after light production is initiated. Because the enzymatic reaction produces light at all wavelengths, samples to be quantitated in a scintillation counter should be measured with all channels open (“open window”). Measure light produced for a period of 10 seconds to 6 minutes. The light intensity of the reaction is nearly constant for about 20 seconds and then decays slowly, with a half-life of about 5 minutes.

Ideally, the coincidence circuit of the scintillation counter should be turned off. Usually this is achieved through an option of the programming menu or by a switch on the instrument. If the circuit cannot be turned off, a linear relationship between luciferase concentration and cpm can be produced by calculating the square root of the measured cpm minus background cpm (i.e., [sample – background]$^{1/2}$). To measure background cpm, read a water blank. To measure activity in your sample, place the sample in a microcentrifuge tube, then place the tube in the scintillation vial. Do not add scintillant; it will inactivate the luciferase.

It may be necessary to make a significant dilution of the sample (for a first check, dilute the sample 1:10,000 in water containing 1mg/ml BSA). If the photomultiplier tubes are saturated by too much light output, the scintillation counter may either produce no reading or identical readings from dilution to dilution.

Protocol

Fully equilibrate the Luciferase Assay Reagent to 25°C before beginning measurements. This can be accomplished by placing the tube containing the Luciferase Assay Reagent into a container of room-temperature water for 30 minutes prior to use.

1. Add 50µl of **room-temperature** Luciferase Assay Reagent (LAR) to each aliquot, and mix quickly by pipetting. It is recommended that the sample to be assayed also be at room temperature.

   Note: Light intensity is a measure of the rate of catalysis by luciferase and is dependent upon temperature. Reproducible luciferase assay readings will result using assay temperatures of 20–25°C.

2. Place the reaction in the luminometer or scintillation counter (place the microcentrifuge tube inside the scintillation vial). Consult the appropriate operator's manual for operation of the luminometer or scintillation counter.

5.C. Photographic Luciferase Assay

1. Prepare a Polaroid® camera for a 6-minute exposure. Either a hand-held (IBI Quickshooter model QSP) or an overhead-positioned Polaroid® camera is acceptable. Position the camera over a white 96-well plate and focus on the top rim of the well. Open the aperture as wide as possible (e.g., f4.5), and set the shutter speed to the bulb, or B, setting. Make sure the camera is loaded with Polaroid® 667 (ISO 3,000) film.
2. Add 50µl of **room-temperature** Luciferase Assay Reagent to each sample, and mix quickly by pipetting.
3. Immediately turn off all lights (including red darkroom lights), and set the camera for a 6-minute exposure.
4. The photographic assay is sensitive in the 1–10ng luciferase range using these conditions. The sensitivity of this assay is directly related to the size, depth and "whiteness" of the reaction chamber.

5.D. Qualitative Visual Detection of Luciferase Activity

1. Add 50µl of **room-temperature** Luciferase Assay Reagent to each sample, and mix quickly by pipetting.
2. Turn off all lights. For qualitative determination of luciferase activity, the reactions may be visualized by eye in a dark room after acclimation to the dark.

   Note: Most people should be able to see the reaction after a minute or two of acclimation, although individuals may differ in their ability to detect these low light levels. The visual intensity of the luciferase reaction is increased by using a white reaction chamber.

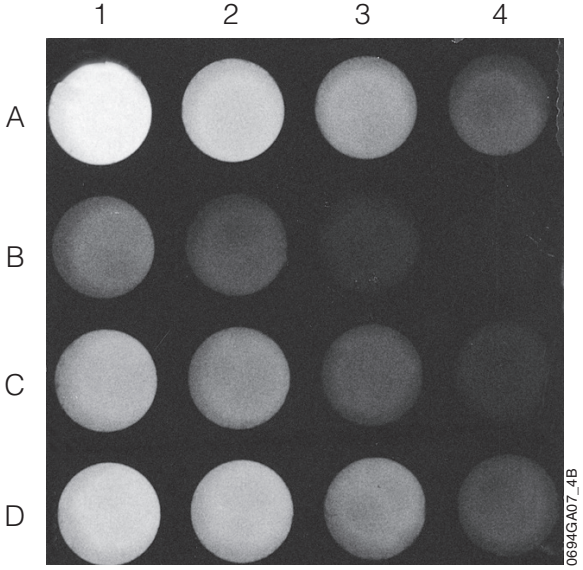

Figure 4. Photographic luciferase assay. Row A. Twofold dilutions of purified luciferase from adult fireflies: lane 1, 2.5ng; lane 2, 1.25ng; lane 3, 0.625ng; lane 4, 0.312ng. **Rows B, C and D.** Twofold dilutions of 50µl in vitro reactions containing: S30 Extract, Linear, and 2µg of linear pBEST*luc*™ DNA (Row B), 2µg of supercoiled pBEST*luc*™ DNA (Row C) or 4µg linear pBEST*luc*™ DNA (Row D). The columns represent dilutions of: lane 1, twofold; lane 2, fourfold; lane 3, eightfold; lane 4, sixteenfold. The S30 reactions were incubated at 37°C for 1 hour. All dilutions were in Luciferase Dilution Reagent (5). Ten microliters of each dilution were added to wells of a white 96-well plate. Fifty microliters of Luciferase Assay Reagent (5) were simultaneously added to each well. The reactions were incubated at room temperature while exposing a Polaroid® 667 (ISO 3,000) film for 6 minutes.

6. TCA Protein Precipitation Assay for Amino Acid Incorporation

6.A. TCA Precipitation Procedure

Use the following protocol for determining the amount of radiolabeled amino acid incorporated into protein during a typical coupled transcription/ translation reaction.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 12.A.)

- 1N NaOH
- 25% TCA/2% casamino acids (ice-cold)
- 5% TCA (ice-cold)
- acetone
- Whatman® GF/A glass microfiber filters (Whatman® Cat.# 1820 021)

1. Vortex the translation reaction gently. Remove a 5µl aliquot and add it to 245µl of 1N NaOH in a 1.5ml microcentrifuge tube. Mix and incubate at 37°C for 10 minutes. The NaOH hydrolyzes aminoacyl tRNAs and prevents labeled tRNA from being included in the incorporation calculation.

   To determine background counts, remove 5µl from the negative control reaction (see Section 4.A, Note 3), and proceed with Steps 2–5.

2. After 10 minutes, add 1.0ml of ice-cold 25% TCA/2% casamino acids to precipitate the translation products. (The casamino acids act as carrier molecules.) Incubate on ice for 30 minutes.

3. Collect the precipitate by filtering under vacuum on Whatman® GF/A glass microfiber filters. Wet the filter with a small amount of ice-cold 5% TCA. Filter the sample and rinse the filter 3 times with 3ml of ice-cold 5% TCA. Rinse once with 1–3ml of acetone. Dry the filter completely at room temperature or at 75°C for 10 minutes.

4. To determine ^{35}S or ^{14}C incorporation, put the filter in 1–3ml of an appropriate scintillation mixture, invert to mix and count.

   To measure ^{3}H incorporation, put the filter in 1–3ml of an appropriate scintillation mixture, invert to mix, then leave the scintillation vials in the dark for 30 minutes at room temperature prior to counting.

5. To determine total counts present in the translation reaction, spot 5µl of the reaction mix directly onto a Whatman® GF/A glass microfiber filter and allow it to dry. Count in a liquid scintillation counter as in Step 4.

6.B. Sample Calculations to Determine Translation Efficiency

1. Calculate total counts in the translation reaction (typical volume is 50µl):

$$\frac{\text{cpm from Section 6.A, Step 5}}{\text{volume spotted on filter from Section 6.A, Step 5}} \times \text{total reaction volume} = \text{total counts in reaction}$$

Example:

$$\frac{1 \times 10^7 \text{cpm}}{5\mu\text{l}} \times 50\mu\text{l} = 1 \times 10^8 \text{cpm}$$

2. Calculate the total number of counts incorporated into protein in a standard reaction:

$$\frac{\text{cpm of TCA precipitate collected on filter (Section 6.A, Step 4)}}{\text{volume of reaction used for TCA precipitation}} \times \text{total volume} = \text{total TCA-precipitable counts}$$

Example:

$$\frac{1 \times 10^6 \text{cpm}}{5\mu\text{l}} \times 50\mu\text{l} = 1 \times 10^7 \text{cpm}$$

3. Calculate percent incorporation:

$$\frac{\text{total TCA-precipitable counts}}{\text{total counts in reaction}} \times 100 = \%\ \text{incorporation}$$

Example:

$$\frac{1 \times 10^7 \text{cpm}}{1 \times 10^8 \text{cpm}} \times 100 = 10\%\ \text{incorporation}$$

7. SDS-PAGE Analysis of Translation Products

The most widely applicable and versatile method for analysis of cell-free translation products is polyacrylamide slab gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) and a discontinuous buffer system. A 15% acrylamide separating gel gives good separation of peptide mixtures between 20,000 and 100,000 Daltons (molecular weight), with peptides between 55,000 and 60,000 Daltons migrating halfway down the length of the gel.

7.A. Sample Preparation

Materials to Be Supplied by the User

(Solution compositions are provided in Section 12.A.)

- acetone
- SDS-PAGE sample buffer

1. Once the S30 extract reaction is complete (or at any desired time point), remove a 5µl aliquot, add it to 20µl of acetone in a microcentrifuge tube, and place it on ice for 15 minutes (an acetone precipitation is required to remove PEG from the extract; PEG will result in background staining). The unused portion of the reaction may be stored at –20°C.
2. Centrifuge the acetone-precipitated S30 sample at 12,000 × *g* for 5 minutes.
3. Remove the supernatant and dry the pellet for 15 minutes under vacuum.
4. When the pellet is dry, add 20µl of SDS-PAGE sample buffer and heat at 100°C for 2–5 minutes. A small aliquot of the sample may be loaded onto an SDS-PAGE gel, or the sample may be stored at –20°C.

7.B. Preparation and Running of SDS-PAGE Gels

For instructions on the preparation and running of SDS-PAGE gels, consult references 12 and 13.

1. Load 10µl of the heated sample into the bottom of the wells.
2. Typically, electrophoresis is performed at a constant current of 15mA in the stacking gel and 30mA in the separating gel, or 30mA for gradient gels. Electrophoresis is usually performed until the bromophenol blue dye front has run off the bottom of the gel.

   Note: Gel banding patterns may be improved by loading unlabeled samples of S30 Extract in the lanes adjacent to the radioactive sample lanes.

7.C. Staining of SDS-PAGE Gels

Materials to Be Supplied by the User
(Solution compositions are provided in Section 12.A.)

- staining solution
- destaining solution

After electrophoresis, protein bands may be visualized by staining with Coomassie® blue dye. Coomassie® staining, however, is usually not sensitive enough to detect translation products, and thus does not need to be performed before analyzing gel results by fluorography. The staining and destaining steps help wash out unincorporated labeled amino acids at the dye front.

1. Incubate in staining solution with gentle agitation until the dye has penetrated the gel (15–30 minutes).
2. Transfer gel to destaining solution. Dye that is not bound to protein is removed in this step. Laboratory tissues can be added to absorb excess stain. Gently agitate in destaining solution until bands are clearly visible (changing the destaining solution may be required).

8. Fluorography

Following electrophoresis, labeled protein bands in gels may be visualized by autoradiography or fluorography. Fluorography dramatically increases the sensitivity of detection of ^{35}S-, ^{14}C- and ^{3}H-labeled proteins and is recommended for the analysis of in vitro translation products.

The increased detection sensitivity of fluorography is obtained by infusing an organic scintillant into the gel. The scintillant converts the emitted energy of the isotope to visible light and so increases the proportion of energy that may be detected by X-ray film. Commercial reagents are available that can conveniently be used for fluorographic enhancement of signal. Follow the manufacturer's recommended procedure. After the gel is dried, a 1–4 hour exposure to film (Kodak® X-OMAT®) at –70°C will detect the ^{35}S-labeled translation products.

9. Gel Drying

Following staining and the optional treatment for fluorography, dry the gel as follows: Cut a sheet of Whatman® 3MM paper a little larger than the gel itself. Place this under the gel once destaining or fluorography has been completed. Transfer the gel and 3MM paper to a vacuum gel drier. Place plastic wrap over the gel and dry for 1 hour at 60°C followed by 1 hour at room temperature.

Alternatively, the gel may be air-dried using Gel Drying Frames (Cat.# V7120). Soak the gel in 10% glycerol for 30 minutes to prevent the gel from cracking during drying. Place the gel between two sheets of thoroughly moistened cellulose gel drying film and clamp in the frames. Allow the gel to dry overnight.

10. Autoradiography

Following electrophoresis and drying, labeled protein bands in gels may be visualized by autoradiography. Autoradiography is sufficiently sensitive to detect ^{35}S-labeled translation products using an overnight exposure to film (Kodak® X-OMAT® AR).

11. References

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12. Appendix

12.A. Composition of Buffers and Solutions

destaining solution (per liter)

70ml	glacial acetic acid
930ml	deionized water

Store at room temperature.

1M NaOH

0.4g	NaOH

Bring to a final volume of 10ml with deionized water.

SDS-PAGE sample buffer (per 10ml)

2.0ml	glycerol
2.0ml	10% SDS
0.25mg	bromophenol blue
2.5ml	stacking gel 4X buffer
0.5ml	β-mercaptoethanol

Bring to a final volume of 10ml with deionized water. Store at room temperature.

stacking gel 4X buffer (per 100ml)

6.06g	Tris-base
4ml	10% SDS

Adjust to pH 6.8 with 12N HCl, and add deionized water to a final volume of 100ml. Store at room temperature.

staining solution (per liter)

250ml	isopropanol
100ml	glacial acetic acid
650ml	deionized water
2.5g	Coomassie® brilliant blue R250

Store at room temperature.

25% TCA/2% casamino acids

25% (w/v)	trichloroacetic acid (TCA)
2% (w/v)	casamino acids (Difco® Cat.# 0231-17-2)

Prepare 500ml and store at 4°C.

Luciferase Dilution Reagent

25mM	Tris-phosphate (pH 7.8)
2mM	DTT
2mM	1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid
10%	glycerol
1%	Triton® X-100
1mg/ml	BSA

12.B. Related Products

Bulk quantities of Extract and Premix are available from Promega.

Product	Size	Cat.#
E. coli S30 Extract System for Circular DNA	30 reactions	L1020
E. coli T7 S30 Extract System for Circular DNA	30 reactions	L1130

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

Product	Size	Cat.#
Amino Acid Mixture Minus Leucine (1mM)	175µl	L9951
Amino Acid Mixture Minus Methionine (1mM)	175µl	L9961
Amino Acid Mixture Minus Cysteine (1mM)	175µl	L4471
Amino Acid Mixture Minus Methionine and Cysteine (1mM)	175µl	L5511
Amino Acid Mixture Complete (1mM)	175µl	L4461

Product	Size	Cat.#
Streptavidin-Alkaline Phosphatase	0.5ml	V5591

Product	Size	Cat.#
TnT® T7 Quick Coupled Transcription/Translation System	40 reactions	L1170
TnT® SP6 Quick Coupled Transcription/Translation System	40 reactions	L2080
TnT® T3 Coupled Reticulocyte Lysate System	40 reactions	L4950
TnT® T7 Coupled Reticulocyte Lysate System	40 reactions	L4610
TnT® SP6 Coupled Reticulocyte Lysate System	40 reactions	L4600
TnT® T7/SP6 Coupled Reticulocyte Lysate System	20 reactions of each	L5020
TnT® T7/T3 Coupled Reticulocyte Lysate System	20 reactions of each	L5010

Additional Sizes Available.

Product	Size	Cat.#
TnT® SP6 High-Yield Wheat Germ Protein Expression System	300µl	L3261
TnT® T3 Coupled Wheat Germ Extract System	40 reactions	L4120
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
TnT® T7/T3 Coupled Wheat Germ Extract System	40 reactions	L5040

Promega

12.B. Related Products (continued)

Product	Size	Cat.#
TnT® T7 Insect Cell Extract Protein Expression Syste	40 reactions	L1102

Product	Cat.#
Transcend™ Colorimetric Translation Detection System	L5070
Transcend™ Chemiluminescent Translation Detection System	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 are sufficient for 30 × 50µl translation reactions.

Product	Cat.#
Gel Drying Kit, 17.5 × 20cm capacity	V7120
Gel Drying Film, 25 × 28cm	V7131

13. Summary of Changes

The following changes were made to the 6/15 revision of this document:

1. The patent information was updated to remove expired statements.
2. The document design was updated.

[a]U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289 and other patents.