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Promega

I. Introduction

Cell-free protein expression (in vitro transcription/translation) is a simplified and accelerated avenue for the transcription and/or translation of a specific protein in a quasi cell environment. This approach lends itself to specific protein labeling with fluorescence, biotin, radioactivity or heavy atoms, via modified charged tRNA's or amino acids. Cell-free protein expression systems provide quick access to proteins of interest and remain a staple in the collection of tools available for the elucidation of cellular pathways and mechanisms (Arduengo *et al.* 2007) as well as for high-throughput screening for drug discovery (Pratt *et al.* 2004; Galam *et al.* 2007). The advent of cell-free systems with higher expression levels has broadened the applications to include NMR-based structural proteomics and membrane protein purification. The open environment of the cell-free system grants flexibility, allowing addition of components during protein synthesis such as liposomes/detergents or microsomal membranes for membrane proteins, while being impervious to the synthesis of toxic proteins. Cell-free protein expression systems based on lysates from Eukaryotic (mammalian, plant and insect) and Prokaryotic cells are available. With these systems, the input templates can be either plasmid DNA, PCR DNA or mRNA.

Prokaryotic S30 transcription/translation systems rely on endogenous transcription machinery or may be supplemented with T7 RNA polymerases. Transcription and translation are typically coupled in prokaryotic systems; that is, they contain an endogenous or phage RNA polymerase, which transcribes mRNA from an exogenous DNA template. This RNA is then used as a template for translation. The DNA template may be either a gene cloned into a plasmid vector (cDNA) or a PCR generated template. With either template, a ribosome binding site (RBS) is required for translation in prokaryotic systems.

A. Template Considerations

DNA purified using purification methods such as the PureYield™ Plasmid Midiprep System (Cat.# A2492, A2495) is sufficiently pure for use in TNT® Rabbit Reticulocyte Lysate or Wheat Germ Extract reactions. A standard (50µl) TNT® translation reaction requires 1µg of plasmid DNA as a template. However, 0.2–2.0µg of DNA template can provide satisfactory levels of translation, and adding more than 2µg of plasmid does not necessarily increase the amount of protein produced. For simultaneous expression from two or more DNA templates, we recommend adding approximately 0.5–1.0µg of each template, keeping the total amount of DNA added to 2µg or less.

Two template elements that are very helpful for increasing the efficiency of in vitro translation are an optimal Kozak sequence and a synthetic poly(A) tail of at least 30 nucleotides. Neither of these elements is required for translation using the TNT® Systems, but each can help improve translation efficiency. The Kozak sequence (Kozak, 1986) serves to position the ribosome at the initiating AUG codon of the translated RNA. Poly(A)+ sequences have been reported to affect the stability and, therefore, the level of protein synthesized in Rabbit Reticulocyte Lysate (Jackson and Standart, 1990). Another important template consideration is the length of untranslated sequence between the transcription start site and the translation start site—a long 5′ untranslated region can form secondary structures, which may inhibit translation. In addition, there may be additional AUG sequences present in the untranslated region that could be recognized as a translation start site, resulting in fusion proteins or incorrect products. We recommend limiting the length of 5′ untranslated regions to less than 100bp.

Cell-free extracts of wheat germ and rabbit reticulocyte lysate support the in vitro translation of a wide variety of viral, prokaryotic and eukaryotic mRNAs. These RNA-driven systems are widely used to identify mRNA species and characterize their products. Starting with the DNA of interest, in vitro transcripts (5-80µg/ml) for translation can be generated with the RiboMAX™ Large Scale RNA Production Systems (Cat.# P1280, P1300). RNA from other standard transcription procedures may contain components at concentrations that inhibit translation. Therefore, a lower concentration, 5–20µg/ml of in vitro transcript, should be used with these systems. The presence of inhibitors can significantly reduce translation efficiency. The optimal RNA concentration should be determined before performing experiments. In addition, the presence of certain nucleic acid sequence elements can have profound effects on initiation fidelity and translation efficiency; 3′-poly(A)+ sequences, 5′-caps, 5′-untranslated regions and the sequence context around the AUG start, or secondary AUGs in the sequence (Kozak, 1990).

Additional Resources for Cell-Free Expression

Promega Publications

Innovative Applications for Cell-Free Expression

Non-Radioactive Detection of Proteins Expressed in Cell-Free Expression System

Cell-Free Expressed Protein in Fluorescent Gel Shift Assays

A Guide to Optimizing Protein Synthesis in the S30 T7 High-Yield Protein Expression System

II. Eukaryotic Cell-Free Expression

The eukaryotic cell-free expression systems (RRL , wheat germ or insect cell extract) are either translation systems that are primed with mRNA or coupled transcription/translation (TNT®) systems supplemented with the optimal phage RNA polymerases (T7, SP6 or T3) and primed with plasmid DNA or PCR DNA containing the T7, SP6 or T3 promoter. Coupled eukaryotic cell-free systems combine a prokaryotic phage RNA polymerase with eukaryotic extracts and utilize an exogenous DNA or PCR-generated templates with a phage promoter for in vitro protein synthesis (Figure 5.1).

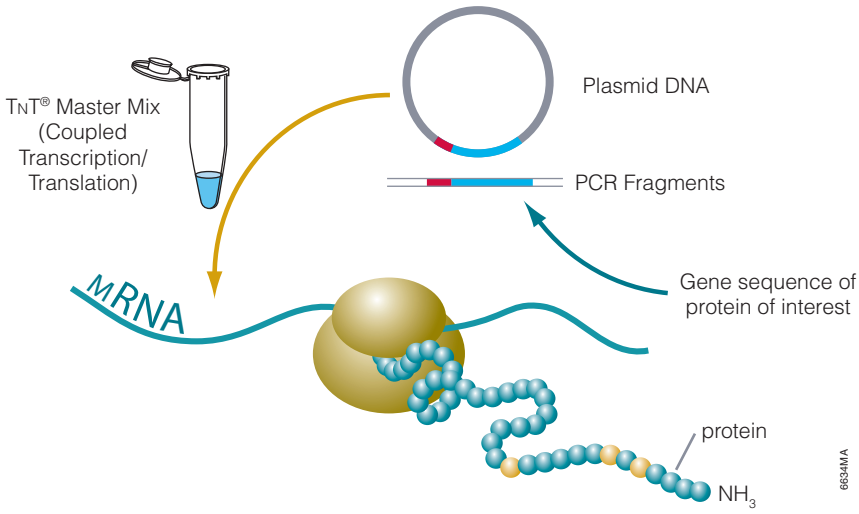

Figure 5.1. Cell-Free expression using the TNT® systems.

Cell-free transcription and /or translation systems offer considerable utility, especially in functional proteomics. In particular, the recent development of the higher yield expression systems has expanded their application (Hurst, 2011).

A. Cell-Free Translation Systems

The Rabbit Reticulocyte Lysate Translation Systems (Nuclease-treated and Untreated), and Wheat Germ Extract System are used for translation of mRNA. The Rabbit Reticulocyte Lysate, Nuclease-Treated, has been optimized for mRNA translation by adding several supplements. These include hemin, which prevents activation of the heme-regulated eIF-2a kinase (HRI); an energy-generating system consisting of pretested phosphocreatine kinase and phosphocreatine; and calf liver tRNAs, to balance the accepting tRNA populations, thus optimizing codon usage and expanding the range of mRNAs that can be translated efficiently. In addition both lysates are treated with micrococcal nuclease to eliminate endogenous mRNA, thus reducing background translation. The Flexi® Rabbit Reticulocyte Lysate System provides greater flexibility of reaction conditions than the Rabbit Reticulocyte Lysate, Nuclease-Treated, by allowing translation reactions to be optimized for a wide range of parameters, including Mg2+ and K+ concentrations, and offers the choice of adding DTT.

In contrast to treated RRL, the Rabbit Reticulocyte Lysate, Untreated, contains the cellular components necessary for protein synthesis (tRNA, ribosomes, amino acids, initiation, elongation and termination factors) but has not been treated with micrococcal nuclease. Untreated Rabbit Reticulocyte Lysate is not recommended for in vitro translation of specific mRNAs.

Finally, Wheat Germ Extract contains the cellular components necessary for protein synthesis (tRNA, ribosomes, initiation, elongation and termination factors). The extract is optimized further by the addition of an energy-generating system consisting of phosphocreatine and phosphocreatine kinase, spermidine to stimulate the efficiency of chain elongation and thus overcome premature termination, and magnesium acetate at a concentration recommended for the translation of most mRNA species. Only the addition of exogenous amino acids (including an appropriately labeled amino acid) and mRNA are necessary to stimulate translation. For further optimization, Potassium Acetate can be added for translation of a wide range of mRNAs.

B. Cell-Free Transcription/Translation Systems

Coupled transcription/translation systems offer researchers time saving alternative for eukaryotic in vitro transcription and translation by coupling transcription/translation into a one-tube system. Standard Rabbit Reticulocyte Lysate or Wheat Germ Extract translations (Pelham and Jackson, 1976) use RNA synthesized in vitro (Krieg and Melton, 1984) from SP6, T3 or T7 RNA polymerase promoters. The RNA is then used as a template for translation. Coupled systems like the TNT® Systems bypass many of these steps by incorporating the reagents needed for transcription directly in the translation mix.

In most cases, the TNT® System reactions produce significantly more protein (two- to sixfold) in a 1- to 2-hour reaction than standard in vitro Rabbit Reticulocyte Lysate or Wheat Germ Extract translations using RNA templates. In addition, TNT® Lysates also can be used with microsomal membranes to study processing events.

Microsomal vesicles are used to study co-translational and initial post-translational processing of proteins (Rando, 1996; Han and Martinage, 1992; Chow *et al.* 1992). Processing events such as signal peptide cleavage, (MacDonald *et al.* 1988), membrane insertion (Ray *et al.* 1995), translocation and core glycosylation (Bocco *et al.* 1988) can be examined by translation of the appropriate mRNA in vitro in the presence of microsomal membranes. Processing and glycosylation events may also be studied by transcription/translation of the appropriate DNA. For

a detailed protocol and background information about this Canine Pancreatic Microsomal Membranes, please see Technical Manual #TM231.

Alternatives to Rabbit Reticulocyte Lysate systems include wheat germ-based systems and systems using extracts from insect cell lines such as the commonly used *Spodoptera frugiperda* Sf21 cell line (Ezure *et al.* 2006) . Wheat germ extract-based cell-free protein synthesis provides unique advantages over other cell-free lysates. These include room temperature incubations, the ability to do high-throughput screening, flexibility to add auxiliary components, expression of proteins toxic to cells and screening of protein folding and function (Morita, E.H. et al. (2003) Protein Sci. 12, 1216–21; Vinarov, D.A. et al. (2004) Nature Methods 1, 149–53).

C. TNT® Quick Coupled Transcription/Translation Systems

The TNT® Quick Coupled Transcription/Translation Systems simplify the transcription/translation process by including all of the reaction components (RNA Polymerase, Nucleotides, salt and RNasin® Ribonuclease Inhibitor) together with the reticulocyte lysate solution in a single TNT® Quick Master Mix. The components of this Master Mix have been carefully adjusted to maximize both expression and fidelity for most gene constructs. When necessary, Magnesium Acetate and Potassium Chloride can be used to optimize in vitro translation reactions with the TNT® Quick Systems. The inclusion of RNasin® Ribonuclease Inhibitor directly in the Master Mix protects against potential disaster from the introduction of RNases carried over in the DNA solutions prepared using some miniprep protocols. The TNT® Quick System is available in two configurations for transcription and translation of genes cloned downstream from either the T7 or SP6 RNA polymerase promoters. For a detailed protocol and background information on this system, please see Technical Manual #TM045.

Protocol

- appropriate TNT® Quick Coupled Transcription/Translation System (Cat.# L1170, L1171, L2080, or L2081)
- Nuclease-Free Water (Cat.# P1193)
- radiolabeled amino acid (for radioactive detection) or Transcend™ tRNA (Cat.# L5061) or Transcend™ Colorimetric (Cat.# L5070) or Chemiluminescent (Cat.# L5080) Translation Detection System (for non-radioactive detection) or FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (for fluorescent detection; Cat.# L5001)

To use these systems, 0.2–2.0µg of circular plasmid DNA containing a T7 or SP6 promoter, or a linearized plasmid or PCR-generated fragment containing a T7 promoter, is added to the TNT® Quick Master Mix and incubated for 60–90 minutes at 30°C. The synthesized proteins are then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected (Figure 5.2).

The following is a general guideline for setting up transcription/translation reactions using plasmid or PCR-generated DNA as template. Examples of standard reaction setup using [35S]methionine, Transcend™ Non-Radioactive Detection System or FluoroTect™ $Green_{Lys}$ Systems are provided.

Plasmid DNA

Assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube. After addition of all the components, gently mix by pipetting. If necessary, centrifuge briefly to return the reaction to the bottom of the tube. For the control reaction, use 1µl of the Luciferase Control DNA supplied.

Note: We recommend also including a negative control reaction containing no added template to allow measurement of background incorporation of labeled amino acids.

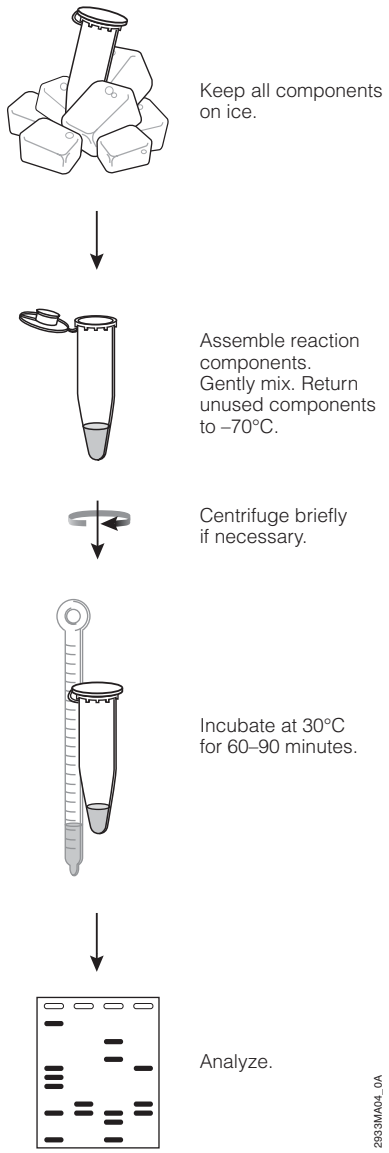

Figure 5.2. Flow chart illustrating the TNT® systems protocol.

Standard Reaction Conditions Using Plasmid DNA, [35S] Methionine, Transcend™ tRNA or FluoroTect™ Green$_{Lys}$ tRNA.

Components	[35S] methionine	Transcend™ tRNA	FluoroTect™ Green$_{Lys}$ tRNA
TNT® Quick Master Mix	40µl	40µl	40µl
Methionine, 1mM (mix gently prior to use)	–	1µl	1µl
[35S]methionine 1,000Ci/mmol at 10mCi/ml)	2µl	–	–
plasmid DNA template (0.5µg/µl)	2µl	2µl	2µl
Transcend™ Biotin-Lysyl-tRNA	–	1–2µl	–
FluoroTect™ Green$_{Lys}$ tRNA	–	–	1–2µl
Nuclease-Free Water to a final volume of	**50µl**	**50µl**	**50µl**

PCR-Generated DNA Templates

For PCR-generated templates, the T7 TNT® T7 Quick for PCR DNA System (Cat.# L5540) can be used for protein synthesis using PCR-generated DNA directly from the amplification reaction. No post-amplification purification is required. To use this system, the PCR fragment must contain a T7 promoter.

Assemble the reaction components (below) in a 0.5ml or 1.5ml microcentrifuge tube. After addition of all the components, gently mix by pipetting. If necessary, centrifuge briefly to return the reaction to the bottom of the tube. For the control reaction, use 1µl of the Luciferase Control DNA supplied.

Note: We recommend also including a negative control reaction containing no added template to allow measurement of background incorporation of labeled amino acids.

Standard Reaction Conditions Using PCR-Generated Templates with [35S]Methionine, Transcend™ tRNA or FluoroTect™ Green$_{Lys}$ tRNA.

Components	[35S] methionine	Transcend™ tRNA	FluoroTect™ Green$_{Lys}$ tRNA
TNT® Quick Master Mix	40µl	40µl	40µl
Methionine, 1mM (mix gently prior to use)	–	1µl	1µl
[35S]methionine 1,000Ci/mmol at 10mCi/ml)	2µl	–	–
PCR-generated DNA template	2.5–5µl	2.5–5µl	2.5–5µl
Transcend™ Biotin-Lysyl-tRNA	–	1–2µl	–
FluoroTect™ Green$_{Lys}$ tRNA	–	–	1–2µl
Nuclease-Free Water to a final volume of	**50µl**	**50µl**	**50µl**

Additional Resources for Eukaryotic Cell-Free Expression Systems

Technical Bulletins and Manuals

TM282 *TNT® SP6 High-Yield Protein Expression System Technical Manual*

TM305 *TNT® T7 Insect Cell Extract Protein Expression System Technical Manual*

TB165 *TNT® Coupled Wheat Germ Extract Systems Technical Bulletin*

TM045 *TNT® Quick Coupled Transcription/Translation Systems Technical Manual*

TM235 *TNT® T7 Quick For PCR DNA Technical Manual*

Promega Publications

The Role of Cell-Free Rabbit Reticulocyte Expression Systems in Functional Proteomics

A Guide to Optimizing Protein Synthesis in the S30 T7 High-Yield Protein Expression System

Cell-Free Protein Expression with the TNT® T7 Insect Cell Extract Protein Expression System

Express More Functional Protein: TNT® Quick Coupled Transcription/Translation Systems

Technically Speaking: TNT® Rabbit Reticulocyte Lysate Systems–Easy Protein Expression

TNT® SP6 High-Yield Protein Expression System: More Protein from a Coupled Transcription/Translation System

Citations

Zhao , L. *et al.* (2010) Engineering of a wheat germ expression system to provide compatibility with a high throughput pET-based cloning platform. *J. Struct. Genomics* **11**, 201–9.

The Northeast Structural Genomics Consortium (www.nesg.org) in their quest to express 5,000 eukaryotic proteins, investigated the use of wheat germ cell free system as a alternative to *E.coli.*. In this publication 59 human constructs were expressed in both *E.coli.* and the wheat germ cell free system. Only 30% of human proteins could be produced in a soluble form using *E.coli.* -based expression. Some 70% could be produced using the TNT® SP6 High-Yield Wheat Germ system. To further demonstrate the utility of expressing proteins that were suitable for structural studies from a wheat germ-based system, two of the proteins were isotope enriched and analyzed successfully by 2D NMR.

PubMed Number: 20574660

Citations

Shao, Y. *et al.* (2010) Involvement of histone deacetylation in MORC2-mediated down-regulation of carbonic anhydrase IX Bucleic. *Nucl. Acid Res.* **38**, 2813-24.

Carbonic anhydrase IX (CAIX) plays an important role in the growth and survival of tumor cells.The MORC proteins contain a CW-type zinc finger domain and are predicted to have the function of regulating transcription, but no MORC2 target genes have been identified. A DNA microarray hybridization was performed and CAIX mRNA was found to be down-regulated 8-fold when MORC2 was overexpressed. This result was further confirmed by northern and western blot analysis. The results also showed that the protected region 4 (PR4) was important for the repression function of MORC2. Moreover, MORC2 decreased the acetylation level of histone H3 at the CAIX promoter. Among the six HDACs tested, histone deacetylase 4 (HDAC4) had a much more prominent effect on CAIX repression. Assays showed that MORC2 and HDAC4 were assembled on the same region of the CAIX promoter. Interaction between MORC2 and HDAC 4 were confirmed by using cell free expression of MORC2 and GST-HDAC (GST pull-downs). Cell-free expression was also used to express MORC2 proteins to determine through gel shifts the binding location on the CAIX promoter region (gel shift experiments).

PubMed Number: 18187424

III. Prokaryotic Cell-Free Translation Systems

Typically, *E. coli* S30 fraction is used for prokaryotic expression. Although the choice of systems should not be determined just by the origin of the target protein, but also by the biological nature of the protein and the requirements of downstream applications. Yields from *E.coli*-based systems can be much greater than eukaryotic-based systems, as high as a few mg/mL depending on protein and reaction format.

A. E. coli S30 Extract Systems

The most common application of *E. coli* S30 Extract Systems is the synthesis of small amounts of radiolabeled protein. The synthesis of a protein of the correct size is a useful way to verify gene products. In addition, proteins expressed in the *E. coli* S30 Extract Systems may also be used for a variety of functional transcription and translation studies. Finally, *E. coli* S30 Extract Systems are useful to synthesis small amounts of radiolabeled protein for use as a tracer in protein purification and incorporation of unnatural amino acids into proteins for structural studies (Noren *et al.* 1989).

The S30 extracts in the *E. coli* S30 Extract Systems are prepared by modifications of the methods described by Zubay (Zubay, 1973; Zubay, 1980; Lesley *et al*. 1991). For expression using linear templates, the extract is prepared from *E. coli* B strains deficient in ompT endoproteinase and lon protease activity. This results in greater stability of expressed proteins, which would otherwise be degraded by proteases if expressed in vivo (Pratt, 1984; Studier and Moffatt, 1986). When circular template DNA is used, *E. coli* S30 Extract Systems can produce higher expression levels of proteins that are normally expressed at low levels in vivo due to the action of host-encoded repressors (Collins, 1979). For simplified transcription/translation of DNA sequences cloned in plasmid or lambda vectors containing a T7 promoter, an extract that contains T7 RNA Polymerase for transcription and all necessary components for translation can be used. The researcher only supplies the cloned DNA containing a T7 promoter and a ribosome binding site.

B. Template Considerations for Prokaryotic Expression

For expression using *E. coli* S30 extract-bassed systems, highly purified DNA templates (e.g., CsCl- or gel-purified) should be used. The activity of the *E. coli* S30 Systems may be inhibited by NaCl (≥50mM), glycerol (≥1%), and by small amounts of Mg^{2+} (1–2mM) or potassium salts (50mM). The DNA template should be ethanol-precipitated with sodium acetate rather than ammonium acetate. Protein yields from the *E. coli* S30 Extract Systems vary with the template and the conditions used. Typical protein yields range from 50–250ng per 50µl reaction.

Circular DNA
Expression of cloned DNA fragments in *E. coli* S30 Extract Systems for Circular DNA requires that the gene be under the control of a good *E. coli* promoter. Examples of such promoters include lambda PR, lambda PL, tac, trc and lacUV5. Expression levels from T7 promoters are typically higher than that from *E. coli* promoters in this extract. Additionally, expression from *E. coli* promoters can be inhibited when rifampicin is added to the extract; however, transcription by T7 RNA Polymerase is resistant to rifampicin.

Linear DNA or RNA
Expression of gene products from linear DNA containing supercoiling-sensitive promoters can be reduced in the S30 System by up to 100-fold (Chen and Zubay, 1983). Examples of good promoters that are supercoiling-insensitive include lacUV5, tac, λ and λPR. DNA from other prokaryotic species may not contain promoters that direct transcription in *E. coli* S30 Extract Systems. RNA generated in vitro from cloned genes lacking an *E. coli* promoter is also suitable. Larger templates, such as bacteriophage lambda DNA, can be used as well.

PCR-Generated Templates
PCR technology has introduced many methods for site-specific in vitro mutagenesis. Combining PCR with phage λ exonuclease treatments has produced mutated fragments larger than 2.5kb (Shyamala and Ames, 1991). To rapidly confirm the expected protein size or activity, PCR products can be added to *E. coli* S30 Extract Systems designed for linear DNA templates.

Care should be taken to avoid contaminating the S30 Extract reaction with the wrong PCR product or primer dimers. If agarose gel analysis indicates that your PCR reaction produced a unique band, any primer dimers present can be removed by ethanol precipitation with sodium acetate. Otherwise, PCR-amplified DNA should be gel purified before use.

Restriction Enzyme-Digested Templates
For restriction enzyme-digested DNA, 10–20µg of DNA should be digested in a 100–200µl reaction volume. Ethanol precipitate and 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. However, if the desired results are not obtained, the DNA should be further purified by phenol extraction followed by ethanol precipitation.

RNA Templates
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, P1300; RiboMAX™ Large Scale RNA Production Systems—SP6 and T7 Technical Bulletin, #TB166.

C. S30 T7 High-Yield Protein Expression System

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. This system can produce high levels of recombinant proteins (up to hundreds of micrograms of recombinant protein per milliliter of reaction) within an hour using a vector containing the sequence of interest, a T7 promoter and a ribosome-binding site (RBS).

The S30 T7 High-Yield Protein Expression System contains T7 S30 Extrac that is prepared by modifications of the method described by Zubay (Zubay, 1973 and Zubay, 1980) 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 (Studier and Moffatt, 1986; Pratt, 1984). An optimized S30 Premix Plus provides all other components required to express high levels of recombinant proteins.

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

The following protocol is designed for the S30 T7 High-Yeild Protein Expression System using circular plasmid DNA. For Linear DNA Templates, use the *E. coli* S30 T7 Extract System for Linear DNA. To obtain fluorescently labeled or biotinylated protein, use 2µl of FluoroTect™ or Transcend™ tRNA in 50µl of reaction. Titrate the tRNAs if necessary. For radioactive labeling, we recommend using the *E. coli* T7 S30 Extract System for Circular DNA. For positive control reactions, use 2µl of the Use the S30 T7 Control DNA provided. 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.

1. Set up the following reaction in a DNase- and RNase-free 1.5ml tube.

Component	Volume
DNA template	1µg
S30 Premix Plus (mix well prior to use)	20µl
T7 S30 Extract, Circular (mix gently prior to use)	18µl
Nuclease-Free Water to final volume	**50µl**

2. 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.

3. Quickly bring the reaction to 37°C, and incubate with vigorous shaking for 1 hour.
4. Stop the reaction by placing the tubes in an ice bath for 5 minutes.
5. Analyze the results.

Optimization

The amount of DNA added should be optimized. In general, reactions should not contain more than 4µg of DNA. Increasing the amount of DNA can result in higher incorporation of label but also can increase the number of internal translational starts or prematurely arrested translation products detected. We recomend a starting volume of 0.5–1µg/50µl reaction of plasmid DNA greater than 5kb in size that contains a T7 promoter. Higher DNA concentrations (such as 2µg/50µl reaction) can be used for large plasmids and vectors with an *E. coli* promoter.

For the T7 S30 Extract, transcription by the endogenous *E. coli* RNA polymerase can be inhibited by the addition of the antibiotic rifampicin, while transcription by the phage T7 RNA Polymerase is unaffected. To inhibit the endogenous RNA polymerase, add 1µl of a 50µg/ml solution of rifampicin in water prior to adding the DNA template to the reaction. Adding excess rifampicin is unnecessary and may decrease protein synthesis. The T7 S30 Extract contains nuclease activity, which prevents the use of linear DNA templates such as PCR products in the reaction.

Protein Analysis

The S30 T7 Control DNA synthesizes *Renilla* luciferase protein, which can be detected by a number of means such as Coomassie® blue staining following SDS-PAGE, fluorescent detection with the incorporation of FluoroTect™ tRNA, or detection by biotinylation using Transcend™ tRNA (Figure 5.3). Expression levels for the positive control reaction also can be measured using an enzymatic assay. For enzymatic assays, synthesize unlabeled synthetic *Renilla* luciferase. 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.

Reaction Temperature

The protein synthesis reaction may be incubated between 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.

Additional Resources for Prokaryotic Protein Expression Systems

Technical Bulletins and Manuals

TB306	*S30 T7 High-Yield Protein Expression Systems Technical Manual*
TB092	*E. coli S30 Extract System for Circular DNA Technical Bulletin*
TB102	*E. coli S30 Extract System for Linear Templates Technical Bulletin*
TB219	*E. coli T7 S30 Extract for Circular DNA Technical Bulletin*

Promega Publications

The S30 T7 High-Yield Protein Expression System

A Guide to Optimizing Protein Synthesis in the S30 T7 High-Yield Protein Expression System

Optimized Gene Expression with the T7 Sample System

Citations

Cameron, A.D. *et al.* (2008) RNA secondary structure regulates the translation of sxy and competence development in Haemophilus influenzae. *Nucleic Acids Res.* **36**, 10–20.

The *sxy (tfoX)* gene product is the central regulator of DNA uptake by naturally competent gamma-proteobacteria such as *Haemophilus influenzae*, *Vibrio cholerae* and probably *Escherichia coli*. However, the mechanisms regulating *sxy* gene expression are not understood despite being key to understanding the physiological role of DNA uptake. We have isolated mutations in *H. influenzae sxy* that greatly elevate translation and thus cause competence to develop in otherwise non-inducing conditions (hypercompetence). In vitro nuclease analysis confirmed the existence of an extensive secondary structure at the 5' end of *sxy* mRNA that sequesters the ribosome-binding site and start codon in a stem-loop. All of the hypercompetence mutations reduced mRNA base pairing, and one was shown to cause a global destabilization that increased translational efficiency. Conversely, mutations engineered to add mRNA base pairs strengthened the secondary structure, resulting in reduced translational efficiency and greatly reduced competence for genetic transformation. Transfer of wild-type cells to starvation medium improved translational efficiency of *sxy* while independently triggering the sugar starvation regulator (CRP) to stimulate transcription at the sxy promoter. Thus, mRNA secondary structure is responsive to conditions where DNA uptake will be favorable, and transcription of *sxy* is simultaneously enhanced if CRP activation signals that energy supplies are limited.

PubMed Number: 1798184

IV. Labeling and Detection

Detection of proteins expressed using cell-free systems is necessary for most applications such as protein:protein interaction and protein:nucleic acid interaction studies. Traditionally, radioactive [^{35}S]methionine has been added

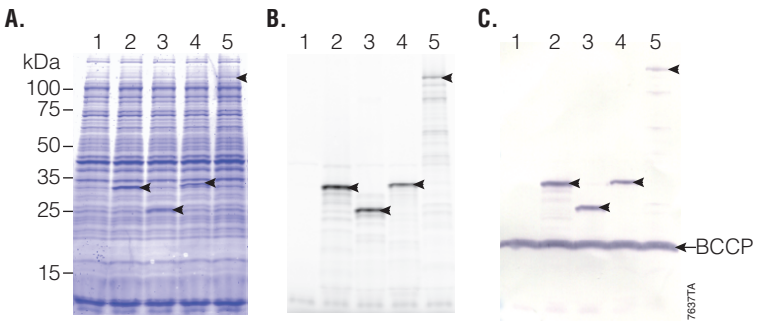

Figure 5.3. Coupled in vitro transcription/translation of circular DNA templates using the S30 T7 High-Yield Protein Expression System. The protein-coding sequences cloned into pFN6A (HQ) Flexi® Vector were expressed as described in the S30 T7 High-Yield Protein Expression System Technical Manual #TM306, resolved by SDS-polyacrylamide gel electrophoresis (PAGE; 4–20% Tris-glycine) and visualized by Coomassie® blue staining (**Panel A**), fluorescent scanning (**Panel B**), or transferred to PVDF membrane, treated with Streptavidin Alkaline Phosphatase and stained with Western Blue® Stabilized Substrate for Alkaline Phosphatase (**Panel C**). For each gel: lane 1, no DNA; lane 2, *Renilla* luciferase; lane 3, Monster Green® Fluorescent Protein; lane 4, HaloTag® protein; lane 5, β-galactosidase. (BCCP = *E. coli* biotin carboxyl carrier protein.)

to cell-free expression reactions, and the methionine is incorporated into the expressed protein, allowing detection by autoradiography. Many researchers are moving away from radioactivity due to high costs, regulations, radioactive exposure and waste disposal issues. Traditional Western blot analysis provided researchers with a non-radioactive method for detection but, if performed improperly, could result in high backgrounds. However, detection methods such as FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (Cat.# L5001) and the Transcend™ Chemiluminescent Non-Radioactive Translation Detection System (Cat.# L5080) allow Western blotting with sensitive detection and low backgrounds (Hook, 2011).

The FluoroTect™ System employs a tRNA charged with a lysine that is labeled at the ε position with the BODIPY®-FL fluorophore. These fluorescently labeled lysine residues are incorporated into synthesized proteins during in vitro translation. The Transcend™ System relies on incorporation of biotinylated lysine residues into nascent proteins during translation. The biotinylated lysine is added to the translation reaction as a charged ε-labeled biotinylated-lysine:tRNA complex (Transcend™ tRNA) rather than a free amino acid. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting, biotinylated proteins can be visualized by binding Streptavidin-AP or Streptavidin-HRP, followed by colorimetric or chemiluminescent detection, respectively. Typically, these methods can detect 0.5–5ng of protein, with a sensitivity equivalent to that achieved with [35S]methionine incorporation and autoradiographic detection.

A. Transcend™ Non-Radioactive Translation Detection Systems

The Transcend™ Non-Radioactive Translation Detection Systems enable non-radioactive detection of proteins synthesized in vitro. Using this system, biotinylated lysine residues are incorporated into nascent proteins during translation, eliminating the need for labeling with [35S]methionine or other radioactive amino acids.

Biotinylated lysine is added to the translation reaction as a pre-charged ε-labeled biotinylated lysine-tRNA complex (Transcend™ tRNA) rather than a free amino acid. After SDS-PAGE and electroblotting, the biotinylated proteins can be visualized by binding either Streptavidin-Alkaline Phosphatase (Streptavidin-AP) or Streptavidin-Horseradish Peroxidase (Streptavidin-HRP), followed either by colorimetric or chemiluminescent detection. Typically, 0.5–5ng of protein can be detected within 3–4 hours after gel electrophoresis. This sensitivity is equivalent to that achieved with [35S]methionine incorporation and autoradiographic detection 6–12 hours after gel electrophoresis. For a detailed protocol and background information, please see Technical Bulletin #TB182.

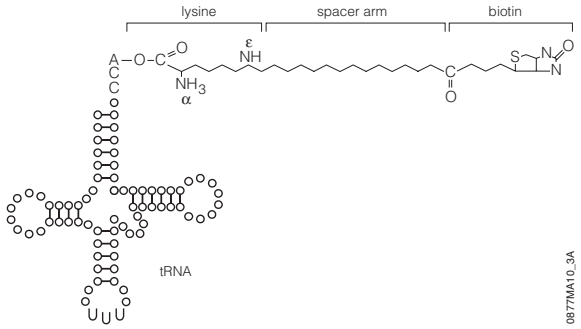

Figure 5.4. Schematic representation of Transcend™ tRNA structure.

The use of Transcend™ tRNA offers several advantages:

- No radioisotope handling, storage or disposal is needed.
- The biotin tag allows detection (0.5–5ng sensitivity).
- The biotin tag is stable for 12 months, both as the Transcend™ tRNA Reagent and within the labeled proteins. It is not necessary to periodically resynthesize biotin-labeled proteins, unlike [35S]-labeled proteins, whose label decays over time.
- Labeled proteins are detected as sharp gel bands, regardless of the intensity of labeling or amount loaded

on the gel, thus allowing the detection of poorly expressed gene products.
- Results can be visualized quickly, using either colorimetric or chemiluminescent detection.

The precharged *E. coli* lysine tRNAs provided in this system have been chemically biotinylated at the ε-amino group using a modification of the methodology developed by Johnson *et al.* (1976). The biotin moiety is linked to lysine by a spacer arm, which greatly facilitates detection by avidin/streptavidin reagents (Figure 5.6). The resulting biotinylated lysine tRNA molecule (Transcend™ tRNA) can be used in either eukaryotic or prokaryotic in vitro translation systems such as the TNT® Coupled Transcription/Translation Systems, Rabbit Reticulocyte Lysate, Wheat Germ Extract or *E. coli* S30 Extract (Kurzchalia *et al.* 1988). Lysine is one of the more frequently used amino acids. On average, lysine constitutes 6.6% of a protein's amino acids, whereas methionine constitutes only 1.7% (Dayhoff, 1978).

Effects of Biotinylated Lysine Incorporation on Expression Levels and Enzyme Activity

Lysine residues are common in most proteins and usually are exposed at the aqueous-facing exterior. The presence of biotinylated lysines may or may not affect the function of the modified protein. In gel shift experiments, c-Jun synthesized in TNT® Reticulocyte Lysate reactions and labeled with Transcend ™ tRNA performed identically to unlabeled c-Jun (Crowley *et al.* 1993).

Estimating Incorporation Levels of Biotinylated Lysine

Incorporation of radioactively labeled amino acids into proteins typically is quantitated as percent incorporation of the label added. This value can include incorporation of radioactivity into spurious gene products such as truncated polypeptides. Thus, percent incorporation values provide only a rough estimate of the amount of full-length protein synthesized and do not provide any information on translation fidelity. With Transcend™ tRNA reactions, it is difficult to directly determine the percent incorporation of biotinyl-lysines into a translated protein. An alternative means of estimating translation efficiency and fidelity in Transcend™ tRNA reactions is to determine the minimum amount of products detectable after SDS-PAGE. In all cases tested, we detected translation products in 1µl of a 50µl translation reaction using as little as 0.5µl of Transcend ™ tRNA (Figure 5.7). The amount of biotin incorporated increases linearly with the amount of Transcend™ tRNA added to the reaction, up to a maximum at approximately 2µl.

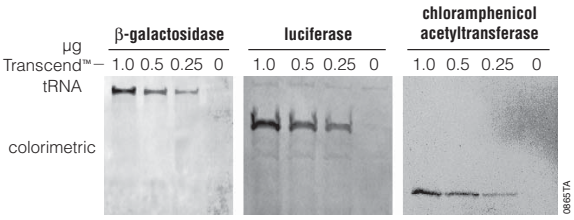

Figure 5.5. Effects of Transcend™ tRNA concentration on detection of proteins synthesized in vitro. Coupled transcription/translation reactions were performed as described in Section II. The indicated amounts of Transcend™ tRNA (equivalent to 2.0, 1.0, 0.5 or 0µl) were added to the translation reactions prior to incubation at 30°C for 1 hour. One microliter of the reaction was used for SDS-PAGE. The separated proteins were transferred to PVDF membrane (100V for 1 hour). The membrane was blocked in TBS + 0.5% Tween® 20 for 15 minutes, probed with Streptavidin-AP (45 minutes), washed twice with TBS + 0.5% Tween® 20 and twice with TBS, and incubated with Western Blue® Substrate for 2 minutes.

Capture of Biotinylated Proteins

Biotinylated proteins can be removed from the translation reaction using biotin-binding resins such as SoftLink™ Soft Release Monomeric Avidin Resin. Nascent proteins containing multiple biotins bind strongly to SoftLink™ Resin and cannot be eluted using "soft-release" nondenaturing conditions. SoftLink™ Resin is useful, however, as a substitute for immunoprecipitation.

Colorimetric and Chemiluminescent Detection of Translation Products

Biotin-containing translation product can be analyzed in either of two ways. The product can be resolved directly by SDS-PAGE, transferred to an appropriate membrane and detected by either a colorimetric or chemiluminescent reaction (Figure 5.6). Alternatively, biotinylated protein can be captured from the translation mix using a biotin-binding resin such as SoftLink™ Resin. This approach is useful as a replacement for immunoprecipitation of protein complexes.

B. FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System

The FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System uses a charged lysine tRNA molecule labeled with the fluorophore BODIPY®-FL at the epsilon (ε) amino acid position of lysine (Figure 5.7). For the FluoroTect™ System, lysine was chosen as the labeled amino acid because it is one of the more frequently used amino acids, comprising, on average, 6.6% of a protein's amino acids. Detection of the labeled proteins is accomplished in 2–5 minutes directly "in-gel" by use of a laser-based fluorescent gel scanner. This eliminates any requirement for protein gel manipulation such as fixing/drying or any safety, regulatory or waste disposal issues such as those associated with the use of radioactively labeled amino acids. The convenience of non-isotopic "in-gel" detection also avoids the time-consuming electroblotting and detection steps of conventional non-isotopic systems. For a detailed protocol and background information about this system, please see Technical Bulletin #TB2852.

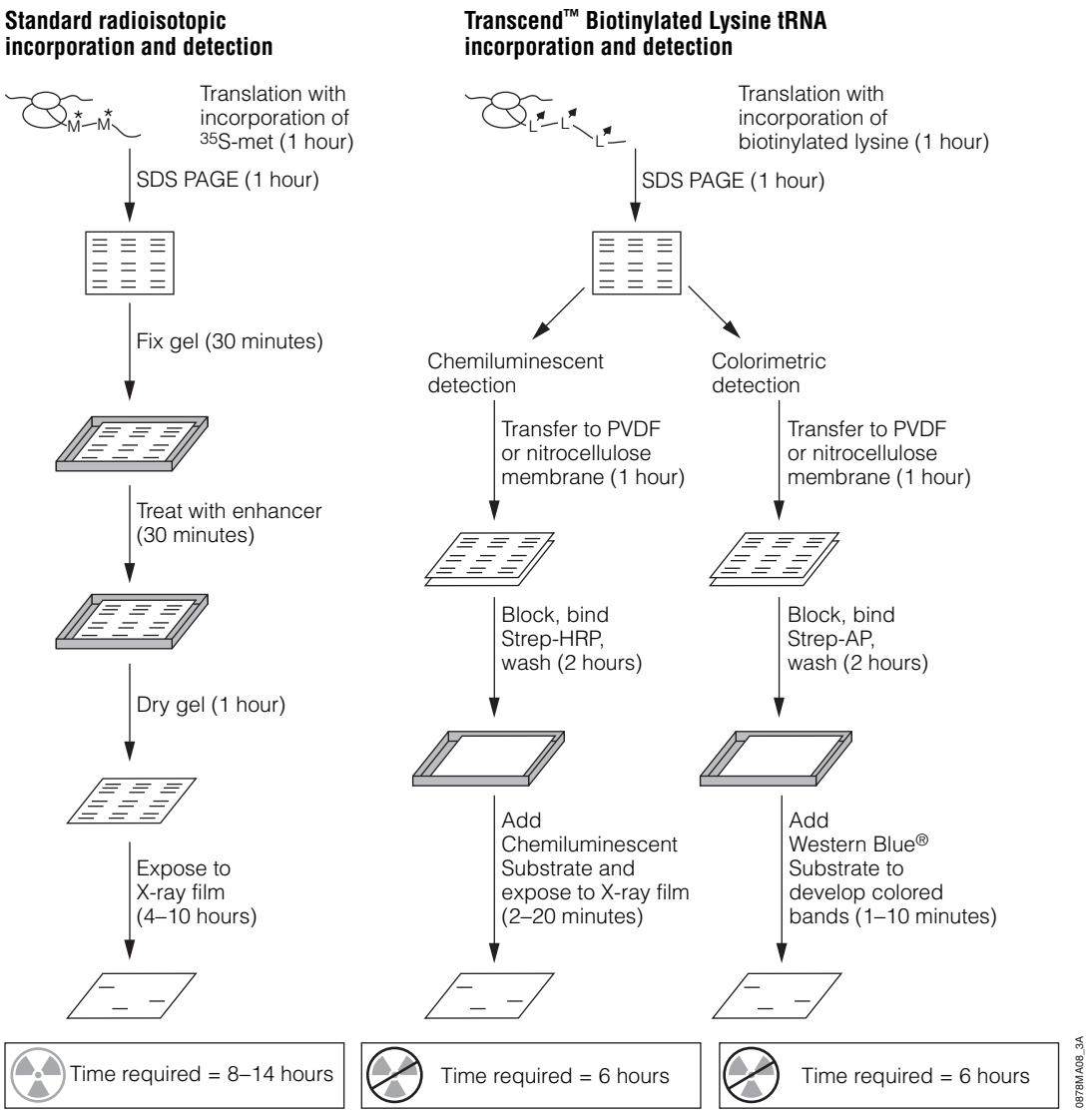

Figure 5.6. Schematic of colorimetric and chemiluminescent detection of translation products.

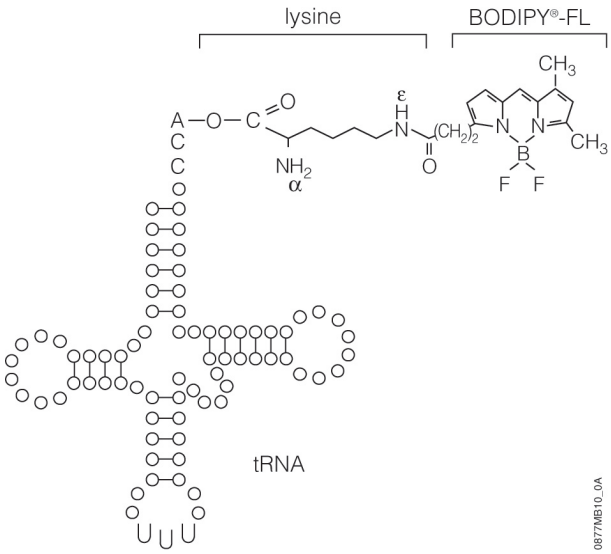

Figure 5.7. Structure of FluoroTect™ Green$_{Lys}$ tRNA.

Additional Resources for Protein Labeling and Detection

Technical Bulletins and Manuals

TB285 *FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System Technical Bulletin*

Promega Publications

Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin

A General Method for Isolating Targets of RNA and DNA Binding Proteins

FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System

Citations

Naoe, H. *et al.* (2010) The anaphase-promoting complex/cyclosome activator Cdh1 modulates Rho GTPase by targeting p190 RhoGAP for degradation. *Mol. Cell Biol.* **30**, 3994-4005.

Cdh1 is one of the coactivators of the anaphase-promoting complex/cyclosome that functions as an E3 ubiquitin ligase for various cell cycle proteins from anaphase to end of the G1 phase of the cell cycle. Other data suggest that Cdh1 is active in processes other than cell cycle such as the regulation of cell shape. By expressing p190 in the TNT® system labeling with Transcend™ tRNA and including components required for ubiquitation, it was determined that Cdh1 targeted p190 for degradation.

PubMed Number: 17098985

Shibuya, N. and Nakashima, N. (2008) Characterization of the 5′ internal ribosome entry site of *Plautia stali* intestine virus *J. Gen. Virol.* **87**, 3679–3686 .

The *Plautia stali* virus contains two open reading frames and includes a 5′ internal ribosome entry site (IRES) and an intergenic IRES region. These authors showed that the 5′ IRES was functional and initiated translation in insect cell lysate but not in rabbit reticulocyte lysate or wheat germ extract. The efficiency of translation mediated by the 5′ IRES region was tested with and without cap analog using various firefly and *Renilla* luciferase reporter constructs. They also used deletion mutants to identify the specific regions required for translation initiation.

PubMed Number: 17098985

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