SELECTING THE CELL-FREE PROTEIN EXPRESSION SYSTEM THAT MEETS YOUR EXPERIMENTAL GOALS

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Here we present criteria for selecting a cell-free protein expression system based on template type, desired yield and intended downstream application.

Introduction

Cell-free protein expression offers unique advantages when compared to cell-based protein expression including time savings (Figure 1) and increased overall yields of functional, soluble, full-length proteins. Additionally, cell-free protein synthesis systems are not as sensitive to toxic proteins such as kinases; modified tRNAs can be used for labeling, and unnatural amino acids can be incorporated at specific sites. Also, these systems are adaptable to high-throughput experiments (1). The wide variety of cell-free expression systems available offer researchers several options for characterizing proteins, including immunoprecipitation, pull-down experiments and enzymatic assays. The primary considerations for selecting a cell-free protein expression system include the source of the extract or lysate, the template, and the desired protein yield. Here we present information designed to help researchers choose a cell-free protein expression system for their experimental system and downstream applications.

Template Considerations

There are several things to consider when engineering an insert sequence or vector for expression in an eukaryotic system: (i) the ATG start codon in the sequence should be the first ATG encountered following the transcription start site; (ii) ideally, following the promoter, the ATG is included in a Kozak consensus sequence; (iii) a stop codon should be included at the 3´ terminus of the sequence; and (iv) a synthetic poly(A) tail should be included following the stop codon (2). Additionally vectors used in the TnT® T7 Coupled Wheat Germ System should contain a T7 terminator sequence or be linearized.

Online Resources for Promega Cell-Free Expression Systems

For additional assistance in selecting a cell-free expression system that best meets your needs, visit the TnT® Product Selector at: **www.promega.com/selectors/tnt/** or the Protein Interaction Product Selector at: **www.promega.com/selectors/pulldown/**

Also, you can learn how other researchers have used Promega cell-free protein expression systems in their work. Visit the citations database at: **www.promega.com/citations/** and search by product number, name or application.

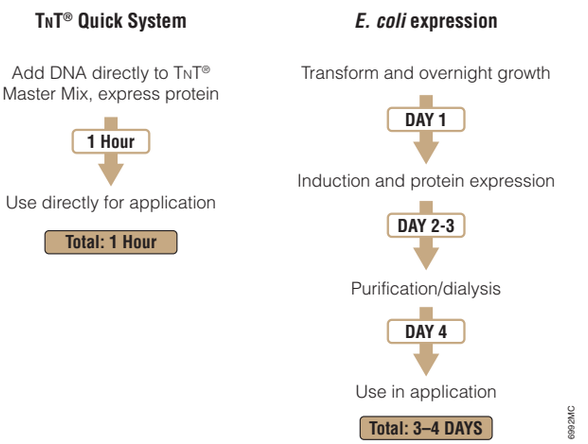

Figure 1. Cell-free protein expression systems can provide significant time savings over cell-based protein expression.

In prokaryotic systems, the selection of a start codon almost invariably depends on the presence of a ribosomal binding site (RBS), which contains a signal that marks the start of the reading frame (2). The presence of an optimal RBS can greatly increase expression in prokaryotic systems. The prokaryotic system does not recognize ATGs upstream of the ATG start codon unless they contain a properly positioned RBS.

DNA-directed synthesis has some advantages over mRNA-primed synthesis in Rabbit Reticulocyte Lysate (RRL) including the elimination of mRNA handling while maintaining high levels of protein synthesis.

Eukaryotic RNA-Based Translation

During the 1950s and 60s researchers showed that RRL could be manipulated for exogenously directed mRNA protein synthesis so that only the protein of interest was synthesized (1). Both the Nuclease-Treated and Flexi® Rabbit Reticulocyte Lysates are optimized for mRNA translation by adding several supplements. These include hemin, which prevents activation of the heme-regulated eIF-2α 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. 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 Mg^{2+} and K^{+} concentrations and the presence or absence of DTT.

Wheat Germ Extract (WGE) contains the cellular components necessary for protein synthesis (tRNA, ribosomes, initiation, elongation and termination factors). The extract is optimized further by adding 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. Finally, potassium acetate is supplied as a separate component so that the translational system may be optimized for a wide range of mRNAs.

Wheat Germ Extract is useful for expressing small proteins or for expressing proteins expected to be abundant in RRL. It is also useful for RNA preparations that have low amounts of dsRNA or thiols, which can inhibit translation in RRL. Researchers expressing proteins from plants or yeasts or other fungi also may find WGE preferable to RRL.

Eukaryotic DNA-Based Transcription and Translation

In the 1990s the development of coupled transcription/translation (i.e., TnT® Systems) in which either RRL or WGE were supplemented with T7, T3 or SP6 RNA polymerases allowed DNA-directed protein synthesis.

The TnT® Coupled Reticulocyte Lysate Transcription/Translation Systems and the TnT® Quick Coupled Transcription/Translation Systems transcribe and translate proteins from plasmid templates using a single-tube format (Figure 2). The TnT® Coupled Systems provide all the reaction components separately, including three separate amino acid mixtures: minus methionine, minus cysteine, or minus leucine. The TnT® Quick Coupled System provides a master mix containing all the reaction components (including a minus methionine amino acid mix), saving time by requiring fewer pipetting steps. TnT® T7 Quick for PCR DNA is specially formulated for transcription/translation of linear, PCR-generated templates, which often require higher potassium and magnesium concentrations than plasmid DNA.

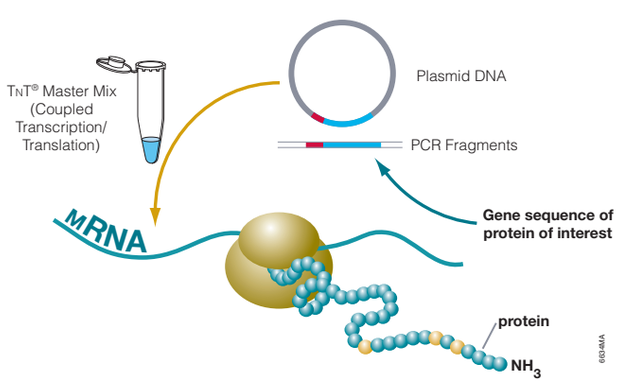

Figure 2. The TnT® Coupled Reticulocyte Lysate Transcription/Translation Systems transcribe and translate proteins from plasmid templates in a single-tube format, and TnT® Quick for PCR DNA works well with PCR templates.

The TnT® Coupled Wheat Germ Extract System offers an alternative to the RRL systems for eukaryotic coupled transcription/translation in a single-tube format. Unlike standard WGE translations, which commonly use RNA synthesized in vitro from SP6, T3 or T7 RNA polymerase promoters, the TnT® Coupled Wheat Germ Extracts incorporate transcription directly in the translation mix.

Prokaryotic DNA-Based Transcription and Translation

The *E. coli* S30 Extract Systems are prepared from *E. coli* B strains deficient in *omp*T endoproteinase and lon protease activity. This results in greater stability of expressed proteins, which would otherwise be degraded by proteases if expressed in vivo. The *E. coli* S30 Extract Systems allow higher expression levels of proteins that are normally expressed at low levels in vivo due to the action of host-encoded repressors. The *E. coli* B strain used to produce the S30 Extract for Linear Templates also is deficient in exonuclease V (the *rec*BCD enzyme). The S30 Extract for Linear Templates is less active than the S30 Extract System for Circular DNA and T7 S30 Extract. The investigator needs only to supply the cloned DNA containing the appropriate prokaryotic promoter and ribosome binding sites when using these systems.

The *E. coli* T7 S30 Extract System for Circular DNA simplifies transcription/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. The researcher only supplies the cloned DNA containing a T7 promoter and a ribosome binding site.

Considerations of Protein Yield

Most in vitro systems produce picomole or nanogram amounts of proteins per 50 µl reaction. This yield usually is sufficient for most types of radioactive, fluorescent and antibody analyses, such as polyacrylamide gel separation, Western blotting, immunoprecipitation or, depending on the protein of interest, enzymatic or biological activity assays. For radioactive detection, a radioactive amino acid is added to the translation reaction, and after incorporation the gene product is identified by autoradiography following SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively, non-radioactive labeling methods such as fluorescent, chemiluminescent or colorimetric detection may be used (e.g., Transcend™ and

FluoroTect™ Systems). If antibodies to the protein are available, then techniques such as immunoblotting or immunoprecipitation can be used. The functional activity of in vitro-translated products often can be detected directly in the reaction mixture. If protein purification is necessary, fusion of the protein to a purification tag allows the protein to be isolated from the in vitro translation reaction and subsequently studied.

High-Yield Cell-Free Protein Synthesis

As noted, most cell-free expression systems express limited amounts of protein. The eukaryotic WGE is modified further to enhance protein levels for a variety of functional and structural proteomic applications. The SP6 TNT® High-Yield System uses a high-yield extract supplemented with SP6 RNA polymerase and other components. In batch mode the system supports the synthesis of 100 µg/ml and in dialysis mode 200–400 µg/ml of protein (Figure 3). Furthermore, enzyme activities can be assayed directly in the extract without purification (Figure 4, reference 3). Simple purification with affinity tags can be achieved in one-step, and with minor modifications, labeling can be accomplished with the system.

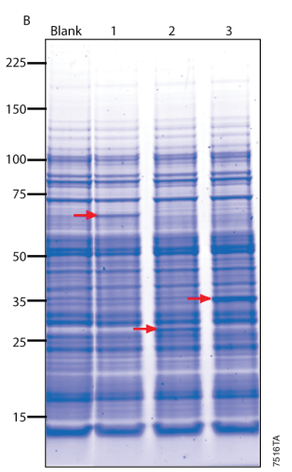

Figure 3. Coomassie®-stained SDS PAGE. Proteins were expressed in dialysis mode using 8 µg of plasmid and 60 µl of TNT® SP6 High-Yield Extract in 100 µl reactions. The reactions were incubated for 18 hours at 25 °C in dialysis cups (MWCO 12,000 BioTec International, sold by Daiichi Pure Chemicals DBC Code 212956) and 2.5 ml of dialysis buffer in a Uniplate (Whatman®).The dialysis buffer consisted of 12 mM HEPES, 0.5 mM spermidine, 5 mM DTT, 80 µM amino acids, 70 mM KOAc, 1.7 mM ATP, 0.6 mM GTP, 0.6 mM CTP, 6 mM UTP, 20 mM CP and 3.5 mM $Mg(OAc)_2$. Lane 1: firefly luciferase, MW 62 KDa; lane 2: Monster Green® GFP, MW 28 KDa; lane 3: humanized *Renilla* luciferase, MW 36 KDa.

Summary

Cell-free expression systems allow you to express proteins quickly and with sufficient yield for downstream applications. They are less sensitive than live cells to toxic proteins; they can be used with accessories like microsomal membranes to express properly folded and modified membrane proteins, and they allow the you to incorporate labels or tags for purification. The Promega platform of cell-free expression products gives you the options you need for your protein of interest.

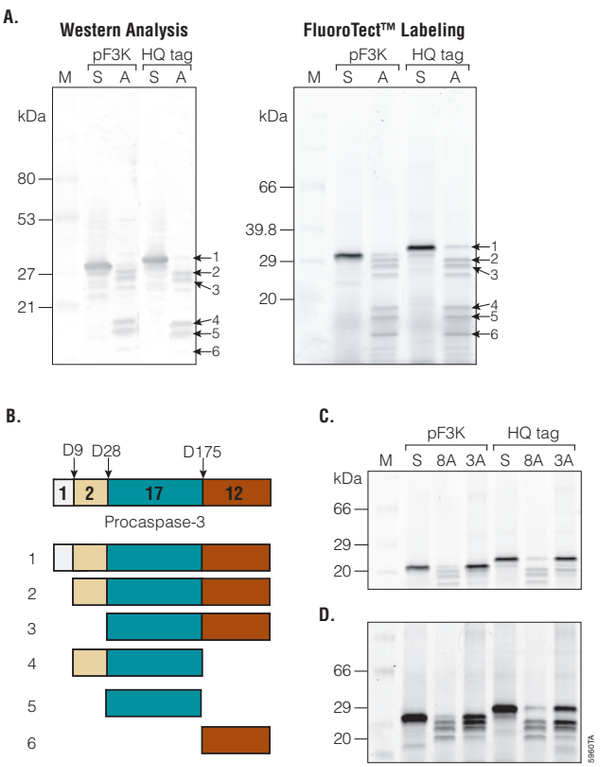

Figure 4. Detection of procaspase-3 processing in batch mode with FluoroTect™ $Green_{Lys}$ labeling. Procaspase-3 with or without an N-terminal HQ tag (in pF3K WG (BYDV) Flexi® Vector) was synthesized in batch mode with FluoroTect™ $Green_{Lys}$ labeling. Procaspase-3 was activated (Panel A) by treating 10 µl of lysate with 100 U (1 µl) caspase-8 or caspase-3 (BioMol) for 1 hour at 37°C. Mock-treated, matched lysate supernatants (S) received 1 µl of 10 mM HEPES (pH 7.5) and were incubated for 1 hour at 37 °C. Each lane is equivalent to 1 µl of the original reaction. **Panel A.** Activation patterns of procaspase-3 are shown as detected by fluorescent labeling or Western analysis. Lanes S, supernatant without caspase-8 treatment; lanes A, supernatant after caspase-8 treatment. Lane M for fluorescent labeling was the Fluorescent Molecular Weight Marker, (Sigma); lane M for Western analysis was ProSieve® color protein markers (Cambrex). **Panel B.** Tentative assignments of the cleaved bands at residues D9, D28 (for removal for prodomain) and D175 (for initial activation) are shown. Numbers inside the bars are rough estimations of fragment molecular weight (kDa); p17 is the protein fragment that is approximately between D28 and D175, and p12 is the carboxy-terminal fragment of the protein. **Panel C.** Gels show extensive processing of procaspase-3 by caspase- 8 (lanes 8A) and the lack of processing of procaspase-3 by caspase-3 (lanes 3A) for 1 hour at 37 °C. **Panel D.** Limited processing of procaspase-3 by its own mature form (lanes 3A) was observed if the reactions from Panel C were incubated for another 24 hours at 4 °C. Lanes M for Panels C and D, Fluorescent Molecular Weight Marker (Sigma).

Table 1. Comparison of Promega Cell-Free Expression Systems: Template Considerations, Primary Applications and Yield.

	Template				Application				Yield
Promega Cell-Free Expression System	**RNA**	**DNA**	**RBS Required**	**Kozak Preferred**	**Transcriptional Analysis**	**Eukaryotic Protein Analysis (e.g., pulldown, coimmuno-precipitation)**	**Prokaryotic Protein Analysis**	**Structural Analysis**	**(Standard reaction volume of 50 µl with system control)**
Rabbit Reticulocyte Lysate or Wheat Germ Extract Systems	Yes	No	–	Yes	Yes	Yes	No	No	RRL 50–200 ng; WGE 30–150 ng; WGE Plus 10–80 µg
TnT® Coupled Transcription/ Translation Systems	Yes	Yes	–	Yes	No	Yes	No	No	150–350 ng (depending on the system)
TnT® SP6 High-Yield Protein Expression System	Yes	Yes	–	Yes	No	Yes	No	Yes	10–100 µg
S30 *E. coli* Protein Expression Systems	Yes	Yes	Yes	–	No	No	Yes	No	100–300 ng (depending on the system)

References

1. Arduengo, M., Schenborn, E. and Hurst, R. (2007) The Role of Cell-Free Rabbit Reticulocyte Expression Systems in Functional Proteomics. *In: Cell-Free Expression* Kudlicki, W., Katzen, F. and Bennett, R., eds. Landis Bioscience, Austin, TX.
2. Brouette, C., Betz, N. and Kobs, G. (2002) *Promega Notes* **80**, 10–3.
3. Zhao, K. *et al.* (2006) *Promega Notes* **94**, 31–5

Protocols

TnT® SP6 High-Yield Protein Production System Technical Manual #TM282 (www.promega.com/tbs/tm282/tm282.html)

TnT® Quick Coupled Transcription/Translation Systems (in vitro protein expression) Technical Manual #TM045 (www.promega.com/tbs/tm045/tm045.html)

TnT® Coupled Reticulocyte Lysate Systems Technical Bulletin #TB126 (www.promega.com/tbs/tb126/tb126.html)

TnT® Coupled Wheat Germ Extract System #TB165 (www.promega.com/tbs/tb165/tb165.html)

TnT® T7 Quick for PCR DNA Technical Manual #TM235 (www.promega.com/tbs/tm235/tm235.html)

Rabbit Reticulocyte Lysate System, Nuclease Treated Technical Manual #TM232 (www.promega.com/tbs/tm232/tm232.html)

Flexi® Rabbit Reticulocyte Lysate System Technical Bulletin #TB127 (www.promega.com/tbs/tb127/tb127.html)

Wheat Germ Extract Plus Technical Manual #TM066 (www.promega.com/tbs/tm066/tm066.html)

Wheat Germ Extract Technical Manual #TM230 (www.promega.com/tbs/tm230/tm230.html)

T7 Sample System Technical Bulletin #TB293 (www.promega.com/tbs/tb293/tb293.html)

Rabbit Reticulocyte Lysate, Untreated Technical Manual #TM232 (www.promega.com/tbs/tm232/tm232.html)

E. coli *T7 S30 Extract System for Circular DNA Technical Bulletin* #TB219 (www.promega.com/tbs/tb219/tb219.html)

E. coli *S30 Extract System for Linear Templates Technical Bulletin* #TB102 (www.promega.com/tbs/tb102/tb102.html)

E. coli *S30 Extract System for Circular DNA Technical Bulletin #TB092* (www.promega.com/tbs/tb092/tb092.html)