Expressing Mammalian Proteins Using Insect and Rabbit Cell-Free Lysates

ABSTRACT | Eukaryotic cell-free protein expression systems, such as the TNT® systems, provide a rapid method for protein expression that avoids the solubility problems associated with bacterial expression. Soluble, functional protein can be produced from plasmid DNA in one to four hours. Here we demonstrate the advantages of eukaryotic cell-free expression using ten difficult-to-express proteins and the TNT® T7 Insect Cell Extract and TNT® Quick Coupled Transcription/Translation Systems.

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Eukaryotic cell-free lysates can synthesize functional, soluble protein in hours, in contrast to days needed for bacterial, baculovirus or mammalian culture expression.

INTRODUCTION

Coupled transcription and translation systems (TNT® systems) using cell-free lysates are a rapid method for testing protein production from coding sequences. Many eukaryotic proteins are difficult to express in bacterial systems. Differences in codon usage, the need for helper proteins and inclusion product formation all can interfere with target protein recovery. Eukaryotic culture systems avoid these problems but are time-consuming and not amenable to protein labeling. Eukaryotic cell-free lysates can synthesize functional, soluble protein in hours, in contrast to days needed for bacterial, baculovirus or mammalian culture expression. To demonstrate the advantages of eukaryotic cell-free lysates, we expressed ten challenging proteins in the new TNT® T7 Insect Cell Extract (ICE) Protein Expression System[a] and in the well established TNT® Quick Coupled Transcription/Translation System[b,c], which uses rabbit reticulocyte lysate (RRL).

EXPRESS SOLUBLE PROTEINS EASILY

We chose ten human proteins based on their lack of solubility when expressed in the *E. coli* strain BL21 (1). The proteins covered a range of sizes from 9,658 to 254,630 kDa (Table 1). The coding regions for these proteins were cloned into Flexi® vector pF1A or pF1K for RRL expression. A simple transfer reaction (2) was used to move the coding regions into pF25A or pF25K ICE T7 Flexi® Vectors. These vectors add polyhedrin 5′- and 3′-untranslated regions and a synthetic polyA sequence to the messenger RNA of the gene of interest to improve expression in insect cell extracts. Both pF1 and pF25 Flexi® vectors have T7 promoter and terminator sequences but do not add any affinity or solubility tags to the native amino acid sequence. Plasmid DNA was purified with the PureYield™ Plasmid Miniprep and Midiprep Systems. The DNA for the TNT® T7 ICE reactions was concentrated by ethanol precipitation.

Table 1. Human Proteins Tested for Expression and Solubility. ACCN indicates GenBank® accession number.

	Description	Abbreviation	GenBank® Accession Number	Molecular Weight (Da)	Number of Lysines
1	Small muscular protein	SMPX	BC005948	9,658	9
2	Melanoma antigen recognized by T cells (MLANA)	MAR1	BC014423	13,256	6
3	B-cell translocation gene 1 anti-proliferative	BTG1	NM_001731	19,308	8
4	Caspase-6, apoptosis-related cysteine protease	CASP6	BC000305	33,409	20
5	Mitogen-activated protein kinase 14	MAPK14	NM_001315	41,393	16
6	Cytochrome P450, family 3, subfamily A, polypeptide 4	CYP3A4	NM_017460	57,542	38
7	Protein kinase C, gamma	PRKCG	NM_002739	78,547	37
8	Minichromosome maintenance deficient 5 cell division cycle 46	MCM5	NM_006739	82,385	47
9	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	NM_004448	138,026	39
10	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	NM_002851	254,630	109

TNT® Systems allow you to incorporate labeled amino acids into the synthesized protein. This makes it easier to track the newly synthesized protein by SDS-PAGE as well as in downstream applications.

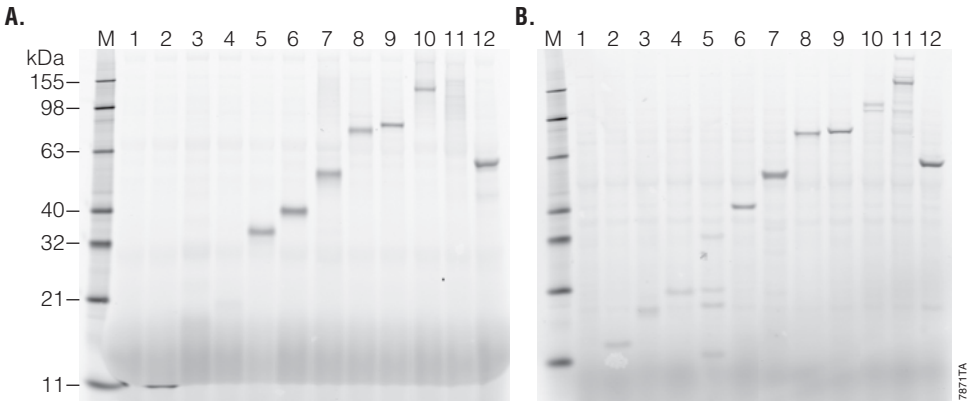

Figure 1. Eukaryotic proteins of various sizes expressed in TNT® extracts. Reactions were performed as recommended in TNT® Quick Coupled Transcription/Translation System (Cat.# L1170) (**Panel A**) or TNT® T7 Insect Cell Extract Protein Expression System (Cat.# L1102; **Panel B**) with the addition of 1 µl of FluoroTect™ $Green_{Lys}$ tRNA (Cat.# L5001). After the translations were completed, 5 µl of each TNT® reaction was incubated with 1 µl of RNase ONE™ Ribonuclease (Cat.# M4261) and 4 µl of water for 15 minutes at room temperature to remove unincorporated FluoroTect™ tRNA. Four microliters of the RNase-ONE™-Ribonuclease treated sample was incubated for 10 minutes at 70°C in sample buffer before resolving on a 4–12% NuPAGE® Bis-Tris SDS-polyacrylamide gel. Proteins were detected by fluorescent scanning on a Typhoon® 9410 using the recommended filters for fluorescein. Lane M, fluorescent marker (Invitrogen); lane 1, no-template negative control reaction; lanes 2–11, proteins 1–10 as listed in Table 1; lane 12, luciferase control reaction.

TNT® reactions were performed as described in the manuals for the TNT® Quick Coupled and T7 ICE Systems (3,4). Both systems use a master mix format containing all the components necessary for transcription and translation except plasmid template. Both systems also allow the option of adding a labeled amino acid, which allows the newly synthesized protein to be easily detected. The proteins in these experiments were fluorescently labeled at lysine residues by including 1 µl of FluoroTect™ $Green_{Lys}$ tRNA in the reactions. The proteins were analyzed by gel electrophoresis followed by imaging. To determine if the expressed proteins were soluble, one aliquot from each reaction was centrifuged for 15 minutes at 14,000 rpm to pellet insoluble material, while another aliquot was used to detect total translated protein. We observed no difference between soluble and total protein for any of the target proteins tested (data not shown). This indicates that all proteins were completely soluble in the TNT® reactions.

Expression was detected for all ten proteins, although the detection of the expressed proteins is both protein and lysate-dependent (Figure 1). Band intensity reflects both the amount of protein synthesized and the number of labeled lysine residues in the protein (Table 1). SDS-PAGE analysis of small proteins is distorted with RRL Systems because the RRL contains hemoglobin. The hemoglobin band extends from 11 to 19 kDa on the gel and shows endogenous fluorescence. In addition, the red color of the heme can increase background in some absorbance-based activity assays. In contrast, the ICE lysate is clear and the endogenous proteins give only faint bands with fluorescent detection. As a result, we were able to detect a 13.2 kDa protein, MAR 1, when it was expressed with the ICE system. The MAR 1 protein only contains 6 lysines for labeling, the fewest lysines per protein in the panel studied. Overall, ICE reactions tend to give higher protein yield than RRL reactions (5). This is not reflected in the signal intensity because FluoroTect™ $Green_{Lys}$ tRNA has a lower labeling efficiency in the ICE lysate. However, which lysate gives the best yield of active protein varies by protein.

EXPRESS ACTIVE PROTEINS

We can often express proteins with detectable enzymatic activity directly from a TNT® reaction without purifying the protein. For example, we expressed procaspase 6 in both the ICE and RRL systems and measured enzyme activity with the Caspase-Glo® 6 Assay (Figure 2). Samples from the ICE reactions gave much greater activity than samples from RRL reactions, even though RRL gave very good full-length protein expression (see lane 6 in Figure 1). Neither sample was treated to activate the caspase 6 enzyme. It could be that ICE produced more proteins and/or contains endogenous caspase-activating proteins that are absent in RRL, leading to proteolytic activation of procaspase 6.

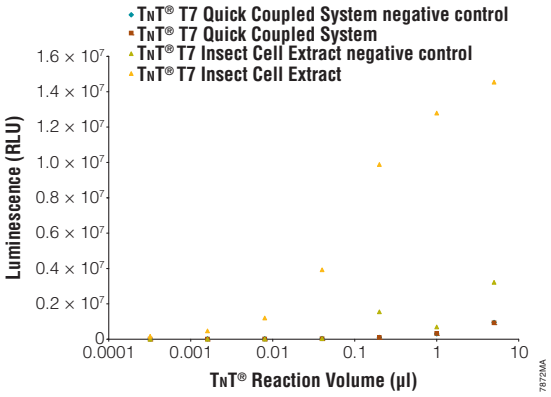

Figure 2. Caspase-Glo® 6 activity. Tenfold serial dilutions of caspase 6 TNT® reactions were made in PBS. Each sample contained 100 µl of the diluted TNT® reaction and 100 µl Caspase-Glo® 6 reagent. Samples were incubated for 30 minutes on a plate shaker at room temperature, then luminescence was read in a GloMax® 96 luminometer.

SUMMARY

Eukaryotic cell-free TNT® systems provide a rapid method for protein expression. These systems save time and avoid the solubility problems associated with bacterial expression. Soluble, functional protein can be produced from plasmid DNA in one to four hours. The TNT® systems allow you to incorporate labeled amino acids into the synthesized protein. This makes it easier to track the newly synthesized protein by SDS-PAGE as well as in downstream applications.

REFERENCES

1. Slater, M. *et al. (2005) Promega Notes* **91,** 21–5.
2. Slater, M. *(2006) Promega Notes* **93,** 8–10.
3. *TNT® T7 Insect Cell Extract Protein Expression Technical Manual* #TM305, Promega Corporation.
4. *TNT® T7 Quick Coupled Transcription/Translation System Technical Manual* #TM045, Promega Corporation.
5. Leippe, D. *et al.* (2008) *Promega Notes* **100**, 11–2.

PROTOCOLS

- *TNT® T7 Insect Cell Extract Protein Expression Technical Manual* #TM305, Promega Corporation
  www.promega.com/tbs/tm305/tm305.html
- *TNT® T7 Quick Coupled Transcription/Translation System Technical Manual* #TM045, Promega Corporation
  www.promega.com/tbs/tm045/tm045.html

ORDERING INFORMATION

Product	Size	Cat.#
TNT® T7 Insect Cell Extract Protein Expression System	40 reactions	L1102
	10 reactions	L1101
TNT® T7 Quick Coupled Transcription/Translation System †	40 reactions	L1170
	5 reactions	L1171
pF25A ICE T7 Flexi® Vector	20 µg	L1061
pF25K ICE T7 Flexi® Vector	20 µg	L1081
Flexi® System, Entry/Transfer	5 entry/20 transfer	C8640
Flexi® System, Transfer	100 transfer	C8820
PureYield™ Plasmid Miniprep System*	50 preps	A1221
PureYield™ Plasmid Midiprep System*	25 preps	A2492
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System†	40 reactions	L5001
Caspase-Glo® 6 Assay* †	10 ml	G0970
RNase ONE™ Ribonuclease* †	1,000 units	M4261
GloMax® 96 Microplate Luminometer		E6501

*Additional sizes available. †For Laboratory Use.

[a]Ezure, T., Suzuki, T., Higashide, S., Shintani, E., Endo, K., Kobayashi, S., Shikata, M., Ito, M., Tanimizu, K., Nishimura, O. (2006) Cell-free protein synthesis system prepared from insect cells by freeze-thawing. ***Biotechnol. Prog.*** **22**, 1570–7.

[b]For Laboratory Use. Any use of the product for diagnostics requiring clearance or approval by the FDA may require a license under Mayo Clinic U.S. Pat. Nos. 6,027,913 and 6,361,949.

[c]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.