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

Robin Hurst, M.S., Don Creswell, B.S., Michael Slater, Ph.D., and Elaine Schenborn, Ph.D., Promega Corporation

Abstract

The TNT® SP6 High-Yield Protein Expression System is designed to produce high levels of protein in a single-tube, cell-free format. This system is based on a supplemented wheat germ extract, containing all the components required for coupled SP6 RNA polymerase-dependent transcription and translation. This article compares protein yields from the TNT® SP6 High-Yield System to the conventional wheat germ coupled system. We also demonstrate that the TNT® SP6 High-Yield System performs well in combination with Flexi® Vectors designed for protein expression in wheat germ extracts.

The high levels of cell-free protein expression and the ease of adapting to a high-throughput format will allow the TNT® SP6 High-Yield Protein Expression System to play a significant role in functional cell-free proteomics.

Introduction

In the past, cell-free wheat germ translation systems (1,2) were hampered by low translation efficiency. With the recent advent of improved cell-free wheat germ translation expression systems (3,4), the translation efficiency has substantially increased. However, even these improved cell-free wheat germ translation expression systems are inconvenient to use because they depend on exogenous mRNA, which requires in vitro transcription and purification of the mRNA prior to translation. By comparison, the TNT® SP6 High-Yield Protein Expression System[a,b] (Cat.# L3260) uses a high-activity wheat germ extract supplemented with SP6 RNA polymerase and other components. By coupling transcriptional and translational activities, the TNT® SP6 High-Yield System eliminates the inconvenience of separate in vitro transcription and purification steps for the mRNA while maintaining high levels of protein expression. All that is required is the addition of purified plasmid DNA template containing the SP6 promoter and the protein-coding region for the protein of interest.

The high levels of cell-free protein expression and the ease of adapting to a high-throughput format will allow this system to play a significant role in cell-free functional proteomics. In this introductory article, we describe the new TNT® SP6 High-Yield Protein Expression System, compare its performance to the TNT® SP6 Coupled Wheat Germ System (Cat.# L4130) and demonstrate its compatibility with our Flexi® Vectors.

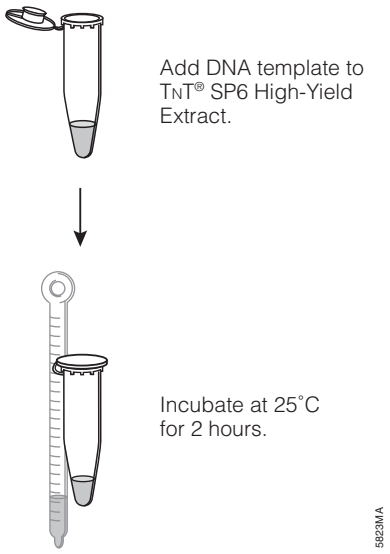

Figure 1. Schematic of the TNT® SP6 High-Yield Protein Expression System.

Single-Tube Format

The TNT® SP6 High-Yield Extract is designed to be easy to use and provide optimal expression. With the exception of the plasmid DNA template and optional protein-labeling reagents, all components for transcription and translation are provided in the extract. The template DNA must contain an SP6 RNA polymerase promoter upstream of the protein-coding region for the protein of interest. Radioactive amino acids and charged tRNAs with modified amino acids, such as Transcend™ and FluoroTect™ tRNAs, can also be used for protein labeling in the TNT® SP6 High-Yield System.

A standard 50µl reaction is comprised of 30µl of the TNT® SP6 High-Yield Extract plus 20µl combined volume of supercoiled plasmid DNA template, optional labeling reagent and Nuclease-Free Water. The reaction is incubated at 25°C, and protein expression is generally complete within 2 hours. A time course of protein synthesis using firefly luciferase, *Renilla* luciferase or the HaloTag™ protein cloned into the pF3K WG (BYDV) Flexi® Vector showed that the maximal yield for all three proteins was obtained between 90 and 180 minutes (data not shown).

The TNT® SP6 High-Yield Extract includes SP6 RNA polymerase and all ribonucleotides required for transcription, as well as amino acids, RNasin® Plus and an energy-regeneration system required for translation. SP6 RNA polymerase transcription and cell-free protein translation have different optimal reaction conditions. The various components in the TNT® SP6 High-Yield Extract are optimized for coupled transcriptional and translational activities. Reaction conditions are balanced for optimal transcription and translation. This optimization maintains high levels of protein expression.

Use High-Quality Plasmid DNA with No Linearization

Supercoiled plasmid DNA template is added directly to the extract with no need to linearize the template, thus making the system easy to use. The plasmid DNA should be of high purity. Purification systems such as the PureYield™ Plasmid Midiprep and Maxiprep Systems (Cat.# A2492 and A2392, respectively) can be used to generate plasmid templates for use in the TNT® SP6 High-Yield System.

Commercially available vectors that are designed for protein expression in wheat germ include the pSP64 Poly(A) Vector (Cat.# P1241), and the pF3A (BYDV) and pF3K (BYDV) Flexi® Vectors (Cat.# L5671 and L5681, respectively) as shown in Figure 2. These two Flexi® Vectors have either an ampicillin- (A) or kanamycin- (K) resistance gene on the vector backbone, which allows appropriate recombinant vector selection (5). Both Flexi® Vectors contain tandem SP6 and T7 RNA polymerase promoters upstream of the *Sgf* I and *Pme* I cloning region. This cloning region is flanked on both ends with sequences from barley yellow dwarf virus (BYDV), which are reported to enhance protein translation in wheat germ extracts in a cap-independent manner (6,7). The pSP64 Poly(A) Vector appends 30 adenosine (A) residues to the 3´ end of a protein-coding RNA, upon transcription, which may provide an advantage for protein translation in cell-free extracts (8).

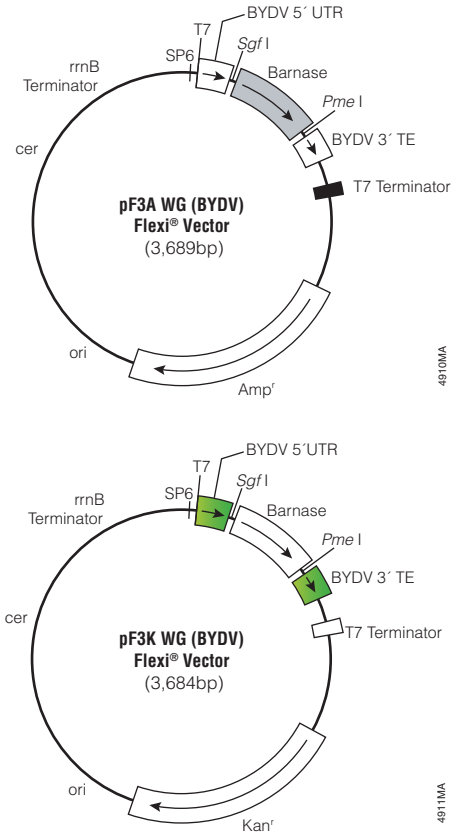

Figure 2. Wheat Germ Flexi® Vectors. The pF3A WG (BYDV) and pF3K WG (BYDV) Flexi® Vectors (Cat.# L5671 and L5681, respectively) are designed for protein expression in wheat germ extract. These vectors incorporate tandem SP6 and T7 RNA polymerase promoters and sequences from barley yellow dwarf virus (BYDV) upstream and downstream of the protein coding region of interest. The vectors contain *Sgf* I and *Pme* I sites to facilitate directional cloning and transfer of protein-coding sequences to other Flexi® Vectors with different expression options. The lethal barnase gene allows positive selection of vectors containing insert, and the ampicillin (A) or kanamycin (K) resistance genes allow selection in *E. coli*.

Produces More Protein than Conventional Wheat Germ Systems

Protein expression capacity of the new TNT® SP6 High-Yield Extract is substantially higher than the corresponding conventional TNT® SP6 Coupled Wheat Germ System (Cat.# L4130) for protein expressed from either of the pF3 WG (BYDV) Flexi® Vectors or the pSP64 Poly(A) Vector. Plasmid DNA concentration profiles that provide the highest levels of protein production, however, differ between the two types of vectors.

Figure 3 depicts results from experiments comparing the effect of plasmid DNA concentrations, as well as the type of vector backbone, on the level of functional protein production. The firefly luciferase protein was expressed from the pF3K WG (BYDV) Flexi® Vector or SP64 Poly(A) Vector (Luciferase SP6 Control DNA; Cat.# L4741) in the TNT® reactions. Protein expression in the TNT® SP6 Coupled Wheat Germ System was optimal at approximately 20ng/µl of input DNA, regardless of the type of vector backbone. In the new TNT® SP6 High-Yield Protein Expression System, maximal protein production with the pF3K WG (BYDV) Flexi® Vector was achieved at ~60ng/µl of input DNA. Even higher protein expression levels were obtained using the TNT® SP6 High-Yield Extract with 240–280ng/µl of Luciferase SP6 Control DNA. This level of luciferase activity corresponds to ~120µg/ml. Overall, functional protein production levels were more than 12-fold higher in the TNT® SP6 High-Yield Extract than in the standard TNT® SP6 Wheat Germ Extract (Table 1).

Direct comparison between the TNT® SP6 High-Yield and TNT® SP6 Coupled Wheat Germ Extracts for functional *Renilla* luciferase protein expressed from a pF3K WG (BYDV) Flexi® Vector template is shown in Figure 4. The highest levels of functional protein were obtained in the TNT® SP6 High-Yield Extract with 40–60ng/µl of input DNA. These levels were more than 50 times greater than those produced in the conventional SP6 coupled extract with an optimum of 20ng/µl input DNA (Table 1). A similar comparison was made for expression of functional HaloTag™ protein from a pF3K WG (BYDV) Flexi® Vector template and is shown in Figure 5. The highest levels of functional protein, assayed by gel separation and binding to the HaloTag™ TMR Ligand (Cat.# G8251), were

Table 1. Fold Increase in Protein Expression in the TNT® SP6 High-Yield Protein Expression System as Compared to the TNT® SP6 Coupled Wheat Germ System.

Vector	Protein	Fold Increase	DNA
pF3K WG (BYDV) Flexi® Vector	Firefly luciferase	72	40–60ng/µl
SP64 Poly(A) Vector	Firefly luciferase	12.2	140–280ng/µl
pF3K WG (BYDV) Flexi® Vector	*Renilla* luciferase	61.5	40–60ng/µl

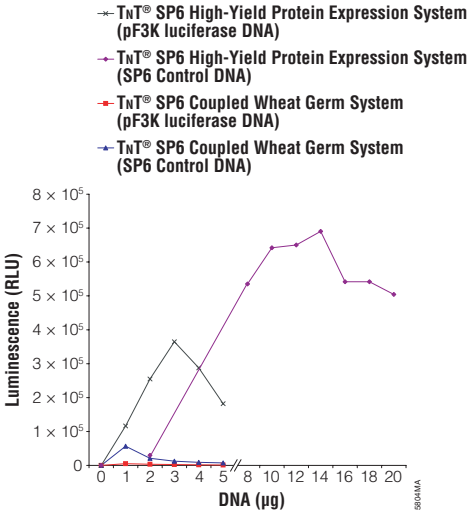

Figure 3. Comparison of firefly luciferase protein expression profiles from different templates. Titrations of circular pF3K firefly luciferase DNA and Luciferase SP6 Control DNA (Cat.# L4741) were compared in the TNT® SP6 High-Yield Protein Expression System (Cat.# L3260) at 25°C and the TNT® SP6 Coupled Wheat Germ Extract System (Cat.# L4130) at 30°C for 3 hours. The coupled transcription/translation reactions were performed according to the Technical Manuals #TM282 and #TB165, respectively. The pF3K firefly luciferase DNA was isolated with the PureYield™ Plasmid Midiprep System (Cat.# A2492) using the combination centrifugation and vacuum protocol described in Technical Manual #TM253. The firefly luciferase activity was measured with the Steady-Glo® Luciferase Assay System (Cat.# E2510) according to Technical Manual #TM051. The reactions were assayed on a TD-20e luminometer (Turner Biosystems).

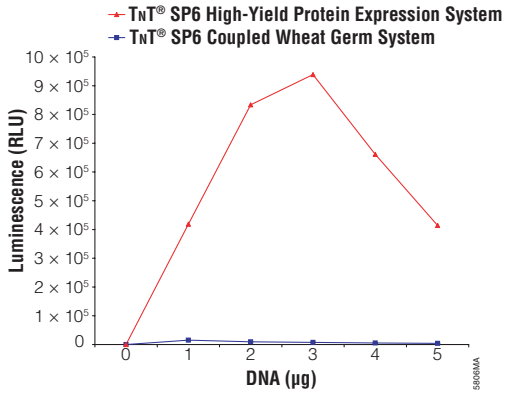

Figure 4. Comparison of *Renilla* luciferase expression. The titrations of circular pF3K h*Renilla* Luciferase DNA (pF3K h*Ren*) were compared in the TNT® SP6 High-Yield Protein Expression System (Cat.# L3260) at 25°C and the TNT® SP6 Coupled Wheat Germ Extract System (Cat.# L4130) at 30°C for 3 hours. The coupled transcription/translation reactions were performed according to Technical Manuals #TM282 and #TB165, respectively. The pF3K h*Ren* Vector was isolated with the PureYield™ Plasmid Midiprep System (Cat.# A2492) using the combination centrifugation and vacuum protocol described in Technical Manual #TM253. The *Renilla* luciferase was assayed using the *Renilla* Luciferase Assay System (Cat.# E2810) with minor alterations. A 2.5µl aliquot of each reaction was diluted 1:1 with a 1X *Renilla* Luciferase Assay Lysis Buffer and incubated at room temperature for 5 minutes. The *Renilla* Luciferase Assay Substrate/Buffer was made according to the Technical Manual #TM055, and 2.5µl of the diluted expression reaction was added to 100µl of the substrate/buffer. The reactions were assayed on a TD-20/20 luminometer (Turner Biosystems).

obtained with 40ng/µl input DNA in the TNT® SP6 High-Yield Extract. Expression of HaloTag™ protein was significantly higher in the TNT® SP6 High-Yield Extract and estimated at ~110µg/ml when compared to titrations of a purified HaloTag™ protein using fluorimaging analysis.

When using BYDV-containing vectors, such as the pF3K WG (BYDV) Flexi® Vector and the pF3A WG (BYDV) Flexi® Vector, 40–60ng/µl of template DNA is optimal, while non-BYDV-containing vectors require more DNA template for maximal protein expression. Because other non-BYDV SP6 expression vectors will have different optimum template DNA values than the Luciferase SP6 Control DNA (data not shown), we recommend titrating the template DNA to determine the correct amount of template DNA for maximal expression. Higher levels of protein production can be obtained using pSP64 Poly(A)-type vector constructs in the TNT® SP6 High-Yield System compared to the levels obtained in the TNT® SP6 Coupled Wheat Germ System; however, higher levels of template DNA are required (Table 1).

Performance characteristics of the TNT® SP6 High-Yield Protein Expression System are compatible with higher-throughput applications. These features include the combination of higher levels of expressed protein, minimal reagent additions and short incubation times performed at room temperature.

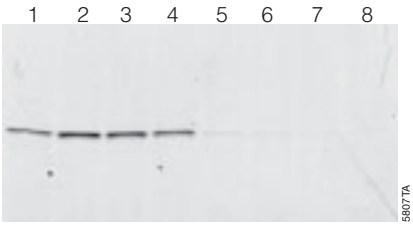

Figure 5. Comparison of HaloTag™ protein expression. Various amounts of circular pF3K HaloTag™ Vector were transcribed and translated using the TNT® SP6 High-Yield Protein Expression System (Cat.# L3260) at 25°C for 3 hours (lanes 1–4) or the TNT® SP6 Coupled Wheat Germ Extract System (Cat.# L4130) at 30°C for 3 hours (lanes 5–8). Lanes 1 and 5 contain reactions performed with 1µg of DNA; lanes 2 and 6, 2µg of DNA; lanes 3 and 7, 3µg of DNA; lanes 4 and 8, 4µg of DNA. The coupled transcription/translation reactions were performed according to the Technical Manuals #TM282 and #TB165. The pF3K HaloTag™ DNA was isolated with the PureYield™ Plasmid Midiprep System (Cat.# A2492) using the combination centrifugation and vacuum protocol described in Technical Manual #TM253. The HaloTag™ Protein was quantitated by incubating 1µl of the coupled transcription/translation reactions in 10µl of PBS and 8µM of the HaloTag™ TMR Ligand (Cat.# G8251) for 1 hour at room temperature. After the incubation, 10µl of 2X SDS sample buffer was added, and the reactions were incubated at 95°C for 2 minutes. The samples were cooled and then loaded onto a 4–20% tris-glycine gel (Invitrogen Cat.# EC6028). The gel was imaged on the Typhoon® 9410 in the Fluorescence mode (600V, 526 SP/Green [532nm]).

Summary

We demonstrated that the new TNT® SP6 High-Yield Protein Expression System produced substantially more protein than the conventional TNT® SP6 Coupled Wheat Germ System while maintaining the ease of use that cell-free and coupled transcription and translation conditions afford. Whereas protein production in the conventional TNT® Wheat Germ Extract generally peaked at 20ng/µl of input DNA, the TNT® SP6 High-Yield Extract protein production capacity was greater and accommodated a higher amount of template DNA. Moreover, when the protein-coding region was flanked by BYDV translation-enhancing sequences, 40–60ng/µl of template plasmid consistently provided high yields of protein with the TNT® SP6 High-Yield System. These features will allow the new TNT® SP6 High-Yield Protein Expression System to play a significant role in functional cell-free proteomics.

References

1. Roberts, B.E. and Paterson, B.M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2330–4.
2. Erickson, A.H. and Blobel, G. (1983) *Meth. of Enzymol.* **96**, 38–50.
3. Madin, K. *et al.* (2000) *Proc. Natl. Acad. Sci. USA* **97**, 559–64.
4. Hurst, R. *et al.* (2005) *Promega Notes* **90**, 19–21.
5. Guo, L., Allen, E.M. and Miller, W.A. (2001) *Mol. Cell* **7**, 1103–9.
6. Gallie, D.R. and Tanguay, R. (1994) *J. Biol. Chem.* **269**, 17166–73.
7. Guo, L., Allen, E.M. and Miller, W.A. (2000) *RNA* **6**, 1808–20.
8. *Flexi® Vector Systems Technical Manual*, #TM254, Promega Corporation.

Protocols

- *TNT® SP6 Coupled Wheat Germ Extract System Technical Manual*, #TB165, Promega Corporation. www.promega.com/tbs/tb165/tb165.html
- *Flexi® Vector Systems Technical Manual*, #TM254, Promega Corporation. www.promega.com/tbs/tm254/tm254.html

Ordering Information

Product	Size	Cat.#
TNT® SP6 High-Yield Protein Expression System	10 reactions	L3261
	40 reactions	L3260

[a] U.S. Pat. Nos. 5,324,637 and 5,492,817, Australian Pat. No. 660329 and other patents.

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

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.