Technical Manual

TNT® SP6 High-Yield Protein Expression System

INSTRUCTIONS FOR USE OF PRODUCTS L3260 AND L3261.

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TNT® SP6 High-Yield Protein Expression System

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I. Description

The TNT® SP6 High-Yield Protein Expression System[a] is a convenient, quick, single-tube, coupled transcription/translation system designed to express up to 100µg/ml of protein. This cell-free expression system is prepared from an optimized wheat germ extract and contains all the components (tRNA, ribosomes, amino acids, polymerase, and translation initiation, elongation and termination factors) necessary for protein synthesis directly from DNA templates.

The TNT® SP6 High-Yield Protein Expression System expresses genes cloned downstream of an SP6 RNA polymerase promoter. Protein synthesis is initiated by adding the appropriate DNA template and incubating the reaction for 2 hours at 25ºC (Figure 1). The synthesized protein can be analyzed by SDS-PAGE or used directly in numerous applications.

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In general, wheat germ extracts provide some co- and post-translational modifications such as phosphorylation (1), farnesylation (2) and myristoylation (3). Signal sequence recognition and targeting require addition of the signal recognition particle and microsomes to the extract (4–6). Glycosylation can occur with the addition of canine microsomal membranes to the extract but is less efficient than that achieved using reticulocyte lysate systems (7–9). The TnT® SP6 High-Yield Master Mix contains reducing agents and is therefore unable to provide an oxidizing environment that promotes disulfide modifications.

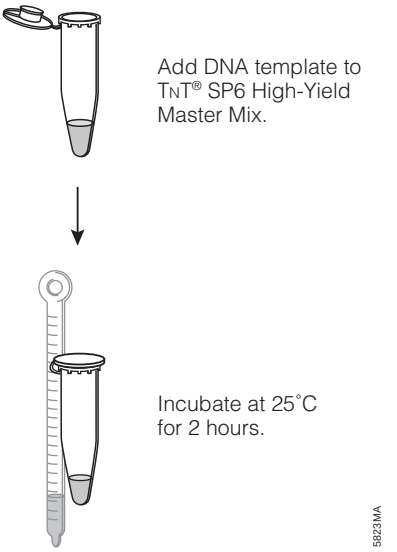

Figure 1. Schematic for the TnT® SP6 High-Yield Protein Expression System.

II. Product Components and Storage Conditions

Product	Size	Cat#
TnT® SP6 High-Yield Protein Expression System	40 × 50µl reactions	L3260

Includes:

- 1.2ml TnT® SP6 High-Yield Master Mix (4 × 300µl)
- 1.25ml Nuclease-Free Water

Product	Size	Cat#
TnT® SP6 High-Yield Protein Expression System	10 × 50µl reactions	L3261

Includes:

- 300µl TnT® SP6 High-Yield Master Mix
- 1.25ml Nuclease-Free Water

Storage Conditions: 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 affect on activity and performance. **Do not subject the components to more than three freeze-thaw cycles.**

III. General Considerations

III.A. Plasmid DNA Template

1. Plasmid DNA can be purified using the PureYield™ Plasmid Midiprep and Maxiprep Systems (Cat.# A2492 and A2392, respectively), as well as other standard methods. Plasmid DNA added to the TnT® SP6 High-Yield Protein Expression System should be of high quality with minimal salt and RNase carryover. To test for the presence of inhibitors, perform control reactions using a template like Luciferase SP6 Control DNA (Cat.# L4741) in the presence and absence of the plasmid DNA.

2. **Optimal results may be dependent on the amount of plasmid DNA added to the reaction.** For pF3 WG (BYDV) Flexi® Vectors (Cat.# L5671 and L5681), which contain barley yellow dwarf virus sequences flanking the protein-coding region (Figure 2), optimal results are obtained with 2–4µg of DNA template per 50µl reaction (10). As a general rule, optimal results for other SP6 promoter-containing vectors (such as pSP64 Poly(A) Vector [Cat.# P1241]) may require up to 10µg of DNA template per 50µl reaction (10).

3. Check the sequence of any non-Flexi® Vector template for the presence of additional upstream start codons. During translation, the ribosome is thought to scan from the 5´ end of the RNA and begin translation at the first AUG encountered. Thus, any AUGs within the untranslated sequence of the insert may cause translation initiation to occur prior to the desired start codon and result in a shift in the reading frame or produce a larger protein than expected.

4. If a non-BYDV plasmid is used, we recommend titrating the DNA (4–10µg per 50µl reaction) to determine the concentration that is optimal for protein synthesis.

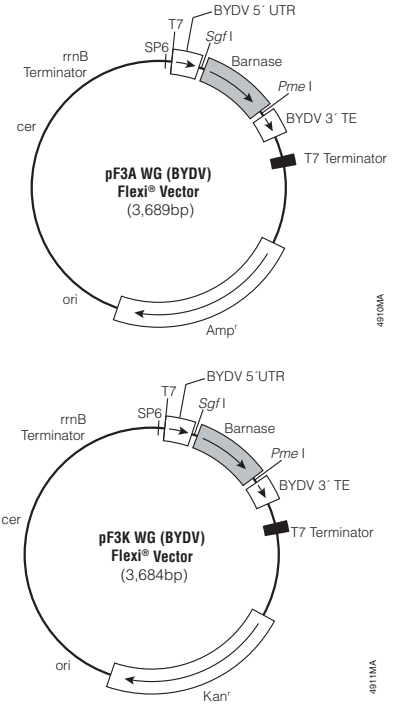

Figure 2. Wheat Germ Flexi® Vectors. The pF3A WG (BYDV) and pF3K WG (BYDV) Flexi® Vectors (Cat.# L5671 and L5681, respectively) are designed for expression of proteins in wheat germ extract. These vectors incorporate sequences from the barley yellow dwarf virus (BYDV) upstream and downstream of the protein 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. Ampicillin- (pF3A WG Vector) and kanamycin- (pF3K WG Vector) resistance genes allow selection in *E. coli.* Please refer to the *Flexi® Vector Systems Technical Manual* #TM254 for further details on the Flexi® Vector technology.

III.B. PCR-Generated Templates

PCR[b]-generated DNA can be used directly from the amplification reaction (e.g., using GoTaq® DNA Polymerase, with either 5X Colorless GoTaq® Reaction Buffer or 5X Green GoTaq® Reaction Buffer or the Access RT-PCR System). When designing PCR primers, you will need to add sequences containing the SP6 Promoter, Kozak consensus sequence and translation initiation sequence (ATG). The design in Figure 3 is recommended for an upstream primer to be used for template generation for expression in the TnT® SP6 High-Yield System. The start codon is underlined; to complete the Kozak consensus sequence the first position of the second codon is typically a G but is sometimes a C.

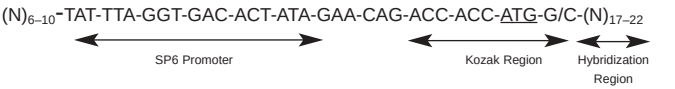

Figure 3. Design of 5´ PCR Primer.

After amplification it is important to analyze the reaction products by agarose gel electrophoresis to ensure that the correct product has been amplified and that no spurious bands are present. We recommend using 5–8µl of the amplification product for coupled transcription/translation.

III.C. Creating a Ribonuclease-Free Environment

RNasin® Plus is included in the TnT® SP6 High-Yield Protein Expression Master Mix; however, precaution should be taken to minimize addition of exogenous RNases to the reaction.

IV. Translation Protocol

The following is a general guideline for setting up a translation reaction. For convenient detection of the synthesized protein we recommend using [^{35}S]methionine, or non-radioactive alternatives such as Transcend™ tRNA (Cat.# L5061) or FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (Cat.# L5001).

Materials to Be Supplied by the User

- [^{35}S]methionine, >1,000Ci/mmol (e.g., Redivue® [^{35}S]methionine, Amersham Biosciences Cat.# AG1094), or Transcend™ tRNA (Cat.# L5061) or FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (Cat.# L5001)

Protocol

1. Remove TNT® SP6 High-Yield Master Mix from storage at –70ºC. Rapidly thaw the Master Mix on ice or by hand warming and immediately place on ice.
2. After the Master Mix has thawed, gently mix several times with a pipette tip or by pipetting.
3. Following the example below, assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube.

Component	Volume
TNT® SP6 High-Yield Master Mix	30µl
DNA template	
pF3 WG (BYDV) Flexi® Vector	2–3µg
or	
Other plasmid DNA	4–10µg
or	
PCR-generated template	5–8µl
[^{35}S]methionine or Transcend™ tRNA or FluoroTect™ Green$_{Lys}$ tRNA (optional)	2–4µl
Nuclease-Free Water to a final volume	50µl

4. Mix gently after all components are added to the reaction tube.
5. Incubate the translation reaction at 25°C for 2 hours.
6. Analyze the results of the translation by SDS-PAGE (See Section V) if the expressed protein has been translated in the presence of radioactive amino acids.

Notes:

1. For plasmid DNA templates other than the pF3 WG (BYDV) Flexi® Vectors, the optimal amount of DNA template per reaction will need to be determined empirically by titration.
2. The TNT® SP6 High-Yield Master Mix contains endogenously biotinylated proteins, which may be detected when translation products are analyzed by SDS-PAGE electroblotting and streptavidin-AP or streptavidin-HRP detection. The 5 major endogenous biotinylated proteins migrate at 200kDa, 80kDa and 32kDa, with a doublet at 17kDa. Comparison of translation products to a control reaction without template will enable distinction between endogenously biotinylated proteins and newly synthesized biotinylated translation product. Thus, it is important to include a negative control reaction containing no DNA. This allows measurement of any background incorporation of labeled amino acids. When using Transcend™ tRNA, the negative control reaction allows identification of endogenous biotinylated proteins.

3. The amount of Transcend™ tRNA or FluoroTect™ Green $_{Lys}$ tRNA that is added to the reaction can be increased up to 4µl to allow more sensitive detection of proteins that contain few lysines or are poorly expressed.

4. Because there is no methionine premix to obtain the desired specific activity of your protein, the amount of [^{35}S]methionine present will depend on how well the gene is expressed and the number of methionines in the protein. For best results we recommend a range of 20–40µCi (2–4µl) of [^{35}S]methionine be added to the TNT® SP6 High-Yield reactions.

5. It is not necessary to add any additional amino acids to express unlabeled proteins.

6. Except for assembly of the reaction and the actual translation incubation, the TNT® SP6 High-Yield Master Mix should be kept at 4°C or on ice. Any unused Master Mix should be refrozen in an ethanol/dry ice bath as soon as possible after thawing to minimize loss of translational activity. Do not expose the Master Mix to more than three freeze-thaw cycles.

7. A positive control reaction with a template such as the Luciferase SP6 Control DNA (Cat.# L4741) can be used to confirm the activity of the extract.

V. Post-Translational Analysis

Materials to Be Supplied by the User

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

- 1M NaOH
- 25% TCA/2% casamino acids (Difco brand, Vitamin Assay Grade)
- 5% TCA
- Whatman® GF/A glass fiber filter (Whatman® Cat.# 1820 021)
- acetone
- Whatman® 3MM filter paper
- 1X SDS sample buffer
- SDS-PAGE running 10X buffer
- SDS polyacrylamide gels*
- Gel fixing solution
- 10% glycerol

*Precast gels are available from a number of manufacturers (e.g., 14% Tris-Glycine and 4–20% Tris-Glycine gradient gels). In addition to convenience and safety, precast gels provide consistent results.

V.A. Determination of Percent Incorporation of Radioactive Label

1. Remove 2µl from the completed translation reaction and add it to 98µl 1M NaOH.
2. Vortex briefly and incubate at 37°C for 10 minutes.
3. At the end of the incubation, add 900µl of ice-cold 25% TCA/2% casamino acids to precipitate the translation product. Incubate on ice for 30 minutes.
4. Wet a Whatman® GF/A glass fiber filter with a small amount of ice-cold 5% TCA. Collect the precipitated translation product by vacuum filtering 250µl of the TCA reaction mix prepared in Step 3. Rinse the filter three times with 1–3ml ice-cold 5% TCA. Rinse once with 1–3ml acetone. Allow the filter to dry at room temperature or under a heat lamp for at least 10 minutes.
5. To determine ^{35}S incorporation, put the filter in the appropriate scintillation cocktail, invert to mix and count in a liquid scintillation counter.
6. To determine total counts, spot a 5µl aliquot of the TCA reaction mix prepared in Step 3 directly onto a filter. Dry the filter for 10 minutes. Count in a liquid scintillation counter as in Step 5.
7. To determine background counts, remove 2µl from a 50µl translation reaction containing no DNA, and proceed as described in Steps 1–5.
8. Determine percent incorporation as follows:

$$\frac{\text{cpm of washed filter (Step 5)}}{\text{cpm of unwashed filter (Step 6)} \times 50} \times 100 = \text{percent incorporation}$$

9. Determine fold stimulation over background as follows:

$$\frac{\text{cpm of washed filter (Step 5)}}{\text{cpm of “no DNA control reaction” filter (Step 7)}} = \text{fold stimulation}$$

V.B. Denaturing Gel Analysis of Radioactive-Labeled Translation Products

A protocol for gel analysis of radiolabeled proteins is given below. For fluorescent detection of proteins using FluoroTect™ Green$_{Lys}$ tRNA, refer to the *FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System Technical Bulletin* #TB285. For colorimetric or chemiluminescent detection using Transcend™ tRNA refer to the *Transcend™ Systems Technical Bulletin* #TB182. These Technical Bulletins are provided with the FluoroTect™ and Transcend™ products, respectively, and are also available on our Web site at: **www.promega.com/tbs/**

1. Once the 50µl translation reaction is complete (or at any desired timepoint), remove a 1µl aliquot and add to 20µl SDS 1X sample buffer. The remainder of the reaction may be stored at –20°C, or at –70°C for long-term storage.
2. Cap the tube and heat at 70°C for 15 minutes to denature the proteins.
3. Load the denatured sample onto an SDS-polyacrylamide gel or store at –20°C. It is not necessary to separate labeled polypeptides from free amino acids by acetone precipitation.
4. Perform electrophoresis according to the gel manufacturer's instructions. Electrophoresis is usually performed until the bromophenol blue dye has run off the bottom of the gel. Disposal of unincorporated label may be easier if the gel is stopped while the dye front remains in the gel, as the dye front also contains the unincorporated labeled amino acids. If transferring the gel to a membrane filter for Western blotting, proceed to Step 7.
5. Fix the gel by soaking in 50% methanol, 7% acetic acid (gel solution 1) for 15 minutes followed by soaking in 7% glycerol, 7% methanol, 7% acetic acid (gel solution 2) for 5–10 minutes.
6. Dry the gel before exposure to film as follows: Soak the gel in 10% glycerol for 5 minutes to prevent cracking during drying. Place the gel on a sheet of Whatman® 3MM filter paper, cover with plastic wrap and dry at 80°C for 30–90 minutes under vacuum using a conventional gel dryer. Dry completely. The gel may also be dried overnight using the Gel Drying Kit (Cat.# V7120). To decrease the likelihood of cracking gradient gels, dry them with the wells pointing down. Expose the gel on Kodak X-OMAT® AR film for 6–15 hours at room temperature.

   Alternatively, the fixed gel can be exposed to a phosphorimaging screen. Phosphorimaging systems provide greater sensitivity, greater speed and the ability to quantitate radioactive bands.
7. For Western blot analysis, transfer (immobilize) the protein from the gel onto a nitrocellulose or PVDF membrane. Detailed procedures for electrophoretic blotting are usually included with commercial devices.

VI. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low translation efficiency	DNA contains ethanol or salt. Perform an ethanol precipitation to remove contaminants.
	Possible cloning error. Verify the sequence of the DNA clone used in the transcription/translation reaction. Include a Kozak sequence.
	Titrate the template DNA to determine the optimal DNA concentration.
	Poor-quality DNA. Perform a control reaction with Luciferase SP6 Control DNA (Cat.# L4741), while titrating the experimental plasmid DNA.
	No PCR DNA product. Check the PCR products on an agarose gel to be sure that the correct PCR product is present. See reference 11 for PCR troubleshooting.
Unexpected bands present on the gel	Possible internal initiation site. Verify by sequencing the cDNA clone. Alter any internal initiation codon by mutagenesis.
	Possible premature termination. Check the template sequence for alternative stop codons.

VII. References

1. Langland, J.O. *et al.* (1996) Phosphorylation of plant eukaryotic initiation factor-2 by the plant-encoded double-stranded RNA-dependent protein kinase, pPKR, and inhibition of protein synthesis in vitro. *J. Biol. Chem.* **271,** 4539–44.
2. Kong, A.M. *et al.* (2000) Cloning and characterization of a 72-kDa inositol-polyphosphate 5-phosphatase localized to the Golgi network. *J. Biol. Chem.* **275**, 24052–64.
3. Martin, K.H. *et al.* (1997) Identification and analysis of three myristoylated vaccinia virus late proteins. *J. Virol.* **71**, 5218–26.
4. Saskena, S. *et al.* (2004) Cotranslational integration and initial sorting at the endoplasmic reticulum translocon of proteins destined for the inner nuclear membrane. *Proc. Natl. Acad. Sci. USA* **101**, 12537–42.
5. Walter, P. and Johnson, A.E. (1994) Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Ann. Rev. Cell Biol.* **10**, 87–119.
6. Walter, P. and Blobel, G. (1983) Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* **96**, 84–93.

7. McGinnes, L.W. *et al.* (2003) Evidence for mixed membrane topology of the newcastle disease virus fusion protein. *J. Virol.* **77**, 1951–63.

8. Rubenstein, J.L.R. and Chappell, T.G. (1983) Construction of a synthetic messenger RNA encoding a membrane protein. *J. Cell Biol.* **96**, 1464–9.

9. Zhan, J-T., Han, E. and Liu, Y. (2000) Role of the ribosome in sequence-specific regulation of membrane targeting and translocation of P-glycoprotein signal-anchor transmembrane segments. *J. Cell Sci.* **113**, 2545–55.

10. Hurst, R. *et al.* (2006) TNT® SP6 High-Yield Protein Expression System: More protein from a coupled transcription/translation system. *Promega Notes* **93**, 15–8.

11. *Protocols and Applications Guide*, Online Edition (2004) Promega Corporation.

VIII. Appendix

VIII.A. Composition of Buffers and Solutions

1X SDS sample buffer

50mM	Tris-HCl (pH 6.8)
2%	SDS
0.1%	bromophenol blue
10%	glycerol
100mM	dithiothreitol

1X SDS sample buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before the buffer is used.

SDS-PAGE polyacrylamide running 10X buffer

30g	Tris base
144g	glycine
100ml	10% SDS

Add water to a final volume of 1L. Store at room temperature.

gel fixing solution

gel solution 1:

50%	methanol
7%	acetic acid

gel solution 2:

7%	glycerol
7%	methanol
7%	acetic acid

VIII.B. Related Products

Product	Size	Cat.#
pF3A WG (BYDV) Flexi® Vector	20µg	L5671
pF3K WG (BYDV) Flexi® Vector	20µg	L5681
pSP64 Poly(A) Vector	20µg	P1241
Wheat Germ Extract Plus	40 × 50µl reactions	L3250
	10 ×50µl reactions	L3251
TNT® T7 Quick Coupled Transcription/Translation System*	40 reactions	L1170
	5 reactions	L1171

* For Laboratory Use.

Product	Size	Cat.#
TNT® SP6 Quick Coupled Transcription/Translation System	40 reactions	L2080
	5 reactions	L2081
TNT® T7 For PCR DNA	40 reactions	L5540
Luciferase SP6 Control DNA	20µg	L4741

For Laboratory Use.

DNA Purification Products

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps*	A2492
PureYield™ Plasmid Maxiprep System	25 preps*	A2393

*Other sizes available

Amplification Products

Product	Concentration	Size	Cat.#
GoTaq® Green Master Mix	2X	100 reactions*	M7122

*Other sizes available.

For Laboratory Use. Catalog numbers may be different in Europe. Premixed solution of GoTaq® DNA Polymerase, GoTaq® Green Reaction Buffer, dNTPs and Mg^{2+}. One reaction refers to a 50µl reaction.

Product	Concentration	Size	Cat.#
GoTaq® DNA Polymerase	5u/µl	100u*	M3001

*Other sizes available.

For Laboratory Use. Catalog numbers may be different in Europe. Includes 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer. Both buffers provide a final concentration of 1.5mM $MgCl_2$.

Product	Size	Cat.#
Access RT-PCR System	100 reactions*	A1250

For Laboratory Use. *Other sizes available.

Protein Labeling Systems

Product	Size	Cat. #
Transcend™ Colorimetric Non-Radioactive Translation Detection System	30 reactions	L5070
Transcend™ Chemiluminescent Non-Radioactive Translation Detection System	30 reactions	L5080
Transcend™ tRNA	30µl	L5061
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System	40 reactions	L5001

For Laboratory Use.

[a]U.S. Pat. Nos. 5,324,637 and 5,492,817, Australian Pat. No. 660329 and Japanese Pat. No. 2904583 have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and eukaryotic lysates.

[b]Patents for the foundational PCR process, European Pat. Nos. 201,184 and 200,362, expired on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.