TECHNICAL MANUAL

TnT® T7 Insect Cell Extract Protein Expression System

Instructions for Use of Products
L1101 and L1102

Revised 12/15
TM305

TnT® T7 Insect Cell Extract Protein Expression System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The TnT® T7 Insect Cell Extract Protein Expression System[a,b] is a convenient, quick, single-tube, coupled transcription/translation system for cell-free expression of proteins. It uses the TnT® technology to allow both transcription and translation to occur in a single reaction. The protein synthesis reactions are initiated by adding a DNA template, eliminating the need for the time-consuming process of in vitro RNA synthesis and increasing protein yield.

The extract is made from the commonly used *Spodoptera frugiperda* Sf21 cell line (1). All components necessary for the transcription/translation reaction have been added to the extract and are present in the TnT® T7 ICE Master Mix. To begin protein synthesis, just add the DNA template. This format simplifies and reduces the time required to set up reactions. Reactions are incubated at 28–30°C and are complete within 4 hours.

Protein is expressed from genes cloned downstream of the T7 RNA polymerase promoter. Companion vectors were designed to achieve optimal yield with this system. They contain untranslated region (UTR) sequences 5´ and 3´ of the gene coding region to enhance translation efficiency.

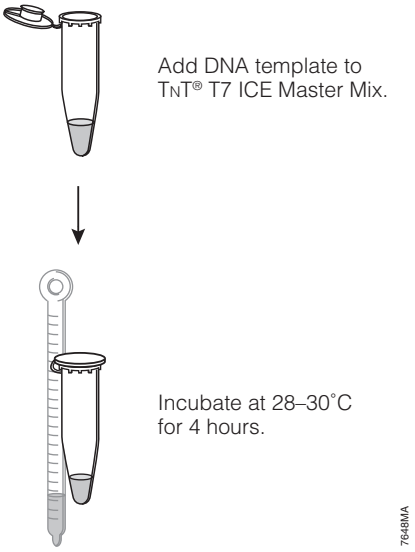

Figure 1. Schematic for the TnT® T7 Insect Cell Extract Protein Expression System.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
TnT® T7 Insect Cell Extract Protein Expression System	**40 × 50µl reactions**	**L1102**

Includes:

- 1.6ml TnT® T7 ICE Master Mix (4 × 400µl)
- 10µg Luciferase ICE T7 Control DNA (1µg/µl)
- 1.25ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
TnT® T7 Insect Cell Extract Protein Expression System	**10 × 50µl reactions**	**L1101**

Includes:

- 400µl TnT® T7 ICE Master Mix
- 10µg Luciferase ICE T7 Control DNA (1µg/µl)
- 1.25ml Nuclease-Free Water

Storage Conditions: Store all components at –70°C. The TnT® T7 ICE Master Mix is sensitive to CO_2 and multiple freeze-thaw cycles, which may have an adverse affect on product activity and performance. The product is shipped in a sealed foil pouch to protect it from CO_2 during shipment on dry ice. Avoid prolonged exposure to CO_2 and dry ice once the foil pouch is opened. **Do not subject the TnT® T7 ICE Master Mix to more than two freeze-thaw cycles.**

3. General Considerations

3.A. Plasmid DNA Template

1. For maximal protein expression with the TnT® T7 Insect Cell Extract Protein Expression System we strongly recommend using the pF25A or pF25K ICE T7 Flexi® Vectors (available separately; Cat.# L1061 and L1081, respectively). The vectors were designed to produce the highest yield with this system and are described in Figure 2.
2. Plasmid DNA can be purified using the PureYield™ Plasmid Miniprep, Midiprep and Maxiprep Systems (Cat.# A1221, A2492 and A2392, respectively), as well as other standard methods. After purification, concentrate the DNA by ethanol precipitation and resuspend in Nuclease-Free Water or TE buffer (10mM Tris, 1mM EDTA) to a concentration of 500ng/µl or greater. Plasmid DNA added to the TnT® T7 Insect Cell Extract Protein Expression System should be of high quality with minimal salt and ribonuclease contamination.
3. Vectors other than the recommended companion vector must be tested for compatibility and expression efficiency. They also must be titrated to determine optimal DNA level to use in a reaction. T7 Control DNA (Cat.# L4821) is not recommended for use with this system.

3.A. Plasmid DNA Template (continued)

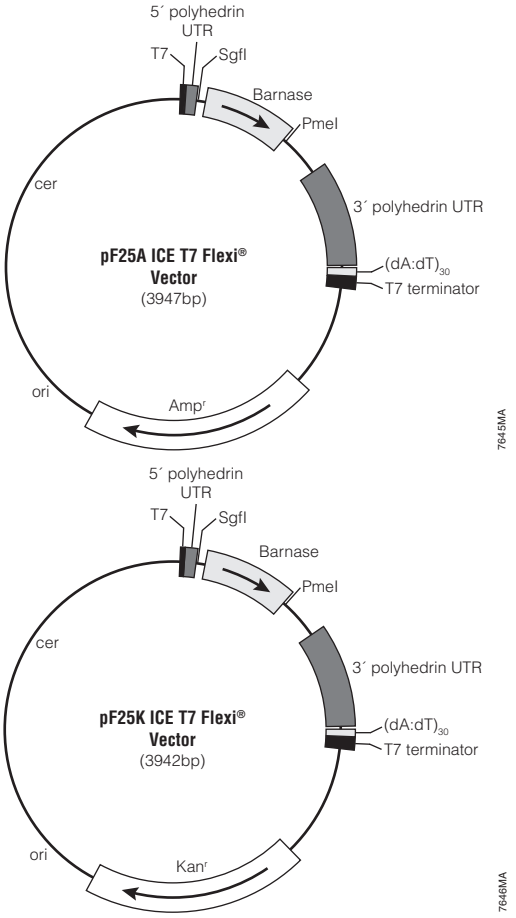

Figure 2. Companion vectors for protein expression. The pF25A and pF25K ICE T7 Flexi® Vectors (Cat.# L1061 and L1081, respectively) and the TnT® T7 Insect Cell Extract Protein Expression System are designed to work together to obtain maximal protein expression. These vectors contain 5´ and 3´ untranslated (UTR) sequences from the baculovirus polyhedrin gene (1,2). These vectors are part of the Flexi® Cloning System. The SgfI and PmeI restriction enzyme sites facilitate the transfer of protein-coding sequences to other Flexi® vectors with different expression options. A lethal barnase gene allows for the positive selection of vectors containing an insert during cloning. The ampicillin (pF25A) and kanamycin (pF25K) resistance genes allow for selection in *E. coli*. Please refer to the *Flexi® Vector Systems Technical Manual* #TM254 for further details on the Flexi® Cloning System technology.

3.B. Creating a Ribonuclease-Free Environment

As RNA is generated during the course of the TnT® reaction, precautions should be taken to reduce the chance of ribonuclease (RNase) contamination. Wear gloves and use RNase-free water, microcentrifuge tubes and pipette tips. RNasin® Plus Ribonuclease Inhibitor is a component of the TnT® T7 ICE Master Mix. There is no need to add additional RNasin® Plus.

3.C. Handling of the Extract

All handling of the TnT® T7 ICE Master Mix should be done at 4°C or on ice. Prepare reactions immediately after the Master Mix has thawed. Any unused Master Mix should be frozen at –70°C as soon as possible after thawing to minimize the loss of translational activity. If possible, quick-freeze the Master Mix with liquid nitrogen before storing it at –70°C. **Do not freeze-thaw the Master Mix more than two times.**

4. TnT® Reaction Protocol

The following is a general protocol for preparing the TnT® reactions.

4.A. General Protocol

1. Immediately before use, remove the needed number of tubes of TnT® T7 ICE Master Mix from storage at –70ºC and transfer them to ice.
2. Thaw the Master Mix on ice, or rapidly thaw it by holding it in your hand (hand warming). Immediately place on ice.
3. After the Master Mix has thawed, mix by gently pipetting, or by gently and briefly vortexing.
4. Immediately put the Master Mix back on ice.
5. Assemble each TnT® reaction by adding the components listed in the table below. The reactions can be set up in 0.5ml or 1.5ml microcentrifuge tubes.

Component	Volume
TnT® T7 ICE Master Mix	40µl
Plasmid DNA Template	4µg
Nuclease-Free Water to a final volume of	50µl

6. After the components have been added, mix the reactions by gently pipetting, or by gently and briefly vortexing.
7. Incubate the TnT® reactions at 28–30°C for 4 hours.
8. Transfer the completed reactions to ice or freeze at –20°C or –70°C.

4.A. General Protocol (continued)

Notes:

1. Return unused Master Mix to –70°C. If possible, quickly freeze the Master Mix in liquid nitrogen to retain maximal activity. The Master Mix can be subjected to two freeze-thaws with minimal loss of activity.
2. Reactions can be scaled by changing the component volumes proportionally. Volumes up to fourfold smaller or larger than 50µl have been tested with no change in performance.
3. A positive control reaction with the Luciferase ICE T7 Control DNA can be used to confirm the activity of the extract.
4. To assemble negative control reactions, do not add Plasmid DNA Template.

4.B. Positive Control Reactions Using Luciferase

The Luciferase ICE T7 Control DNA is provided as a positive control for protein expression. It can be used for the expression of luciferase, a monomeric protein (61kDa) that requires no post-translational processing or modification for enzymatic activity. Only full-length luciferase is active.

To assemble positive control reactions, follow the protocol in Section 4.A and add reagents to the reactions according to the table below.

Component	Volume
TnT® T7 ICE Master Mix	40µl
Luciferase ICE T7 Control DNA (1µg/µl)	4µ1
Nuclease-Free Water to a final volume of	50µl

Notes:

1. Protein production in the luciferase positive control reactions can be analyzed by measuring luciferase enzyme activity. Assay for luciferase activity using the Steady-Glo® Luciferase Assay System (Cat.# E2510) or other Promega Luciferase Assay Systems. These assays require the use of a luminometer. The luciferase assay is sensitive, rapid and easy to perform.

   Luciferase activity can be assayed without purification of the luciferase protein. For example 5µl of the TnT® reaction can be added directly to 100µl of the Steady-Glo® Luciferase Assay System. QuantiLum® Recombinant Luciferase (Cat.# E1701 and E1702) can be used as a positive control in the activity assay.
2. The luciferase positive control reactions also can be performed with the addition of labels as discussed in Section 4.C. The synthesized luciferase then can be detected by gel analysis of the translation products.
3. The luciferase expression product also can be analyzed by Western blot. For detection use Anti-Luciferase pAb (Cat.# G7451).

4.C. Addition of Labels to the Reactions

1. For convenient detection of the synthesized protein products, labels can be added to the reactions and incorporated into the expressed proteins. For example we recommend using the following non-radioactive labeling systems: FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (Cat.# L5001) for fluorescently labeled products or Transcend™ tRNA (Cat.# L5061) for biotinylated products. Both of these systems use modified tRNAs to incorporate label into translated proteins.
2. To label proteins add 1–2µl of the tRNA products to each 50µl reaction. Adjust the water added accordingly such that the final total volume of each reaction is 50µl. See the table below for the setup of a single TnT® reaction.
3. Include negative control reactions in the experiment by omitting the Plasmid DNA Template.
4. For detection of proteins labeled 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 also are available on our Web site at: **www.promega.com/protocols/**
5. For radioactive detection, [^{35}S]methionine also can be used to label proteins. Add 2µl of [^{35}S]methionine (>1,000Ci/mmol; Perkin Elmer) per 50µl reaction. As the Master Mix contains all amino acids, including methionine, the incorporation of radioactivity will depend on the number of methionine residues in the protein and how well the gene is expressed.
6. For more detailed directions for detecting radioactive proteins and measuring the incorporation of radioactivity please refer to the *TnT® Quick Coupled Transcription/Translation Systems Technical Manual* #TM045 or the *TnT® SP6 High-Yield Protein Expression System Technical Manual* #TM282. These Technical Manuals include detailed protocols for the detection of radioactive proteins synthesized in TnT® reactions. The Technical Manuals are available on request or on our Web site at: **www.promega.com/protocols/**

Component	Volume
TnT® T7 ICE Master Mix	40µl
FluoroTect™ Green$_{Lys}$ tRNA, Transcend™ tRNA or [^{35}S]methionine	1–2µ1
Plasmid DNA Template (1µg/µl)	4µg
Nuclease-Free Water to a final volume of	50µl

Note: Include negative control reactions that contain no Plasmid DNA Template.

5. 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	Possible Causes and Comments
Low expression of protein	DNA contains ethanol or salt. Perform an ethanol precipitation to remove contaminants.
	Poor-quality DNA. Check DNA quality by agarose gel electrophoresis and purify a fresh preparation of DNA if necessary.
	The DNA template is not compatible with this system. Use the recommended companion vector.
	Loss of extract activity. The extract should not be used after more than two freeze-thaw cycles. Use the Luciferase ICE T7 Control DNA as a positive control to check protein expression.
Unexpected bands present when labeled expressed protein is analyzed by gel electrophoresis	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.
	Proteolysis of translation product. Add protease inhibitors to the reaction.
Smeared bands or streaking when protein is analyzed by gel electrophoresis	Too much protein loaded on gel. Use 1–5µl of the reaction mixture.
	Protein aggregation. Heat sample in sample buffer for 10 minutes at 70°C before loading onto the gel

6. References

1. Extract provided by Shimadzu Corporation manufactured as in:

   Ezure, T. *et al.* (2006) Cell-free protein synthesis system prepared from insect cells by freeze-thawing. *Biotechnol. Prog.* **22**, 1570–7.

2. Suzuki, T. *et al.* (2006) Performance of expression vector, pTD1, in insect cell-free translation system. *J. Biosci. Bioeng.* **102**, 69–71.

7. Appendix

7.A. Luciferase ICE T7 Control DNA

The Luciferase ICE T7 Control DNA is provided as a positive expression control for the TnT® T7 Insect Cell Extract Protein Expression System. The Control DNA contains the gene for luciferase in the pF25A ICE T7 Flexi® Vector. The map of the Control DNA is in Figure 3. Please note that this vector is intended for use as control expression vector only. It is not intended for use as a cloning vector.

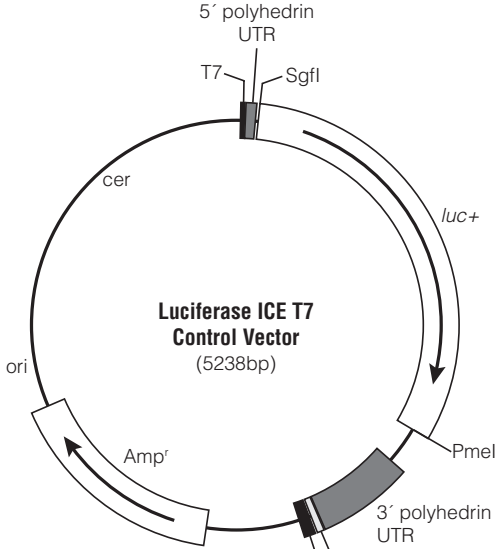

Figure 3. Luciferase ICE T7 Control DNA circle map and sequence reference points (below).

Sequence Reference Points:

T7 RNA polymerase promoter (–17 to +2)	21–39
5´ polyhedrin UTR	40–85
SgfI region	86–93
firefly luciferase gene	95–1750
PmeI region	1745–1752
3´ polyhedrin UTR	1901–2274
synthetic poly(A) region	2279–2308
T7 terminator	2309–2356
β-lactamase (Amp^r) coding region	2737–3597
ColE1-derived plasmid origin of replication	3752–3788
cer site (site for *E. coli* XerCD recombinase)	4459–4744

7.B. Related Products

Companion Vector Products

Product	Size	Cat.#
pF25A ICE T7 Flexi® Vector	20µg	L1061
pF25K ICE T7 Flexi® Vector	20µg	L1081

TnT® Protein Expression Systems

Product	Size	Cat.#
TnT® SP6 High-Yield Wheat Germ Protein Expression System	40 reactions	L3260
	10 reactions	L3261
TnT® T7 Quick Coupled Transcription/Translation System	40 reactions	L1170
	5 reactions	L1171
TnT® SP6 Quick Coupled Transcription/Translation System	40 reactions	L2080
	5 reactions	L2081
TnT® T7 Quick For PCR DNA	40 reactions	L5540

DNA Purification Products

Product	Size	Cat.#
PureYield™ Plasmid Miniprep System	100 preps*	A1223
PureYield™ Plasmid Midiprep System	25 preps*	A2492
PureYield™ Plasmid Maxiprep System	25 preps*	A2393

*Other sizes available.

Protein Labeling Systems

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

Luciferase Assay Systems

Product	Size	Cat. #
Steady-Glo® Luciferase Assay System*	10 ml	E2510
QuantiLum® Recombinant Luciferase*	1mg	E1701
ONE-Glo™ Luciferase Assay System*	10ml	E6110
Bright-Glo™ Luciferase Assay System*	10ml	E2610
Luciferase Assay System*	100 assays	E1500

*Other sizes available.

[a]Use of this vector for applications outside of the TNT® T7 Insect Cell Extract Protein Expression System may require a license from Shimadzu Corporation. For more information contact t-direct@shimadzu-biotech.jp

[b]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.