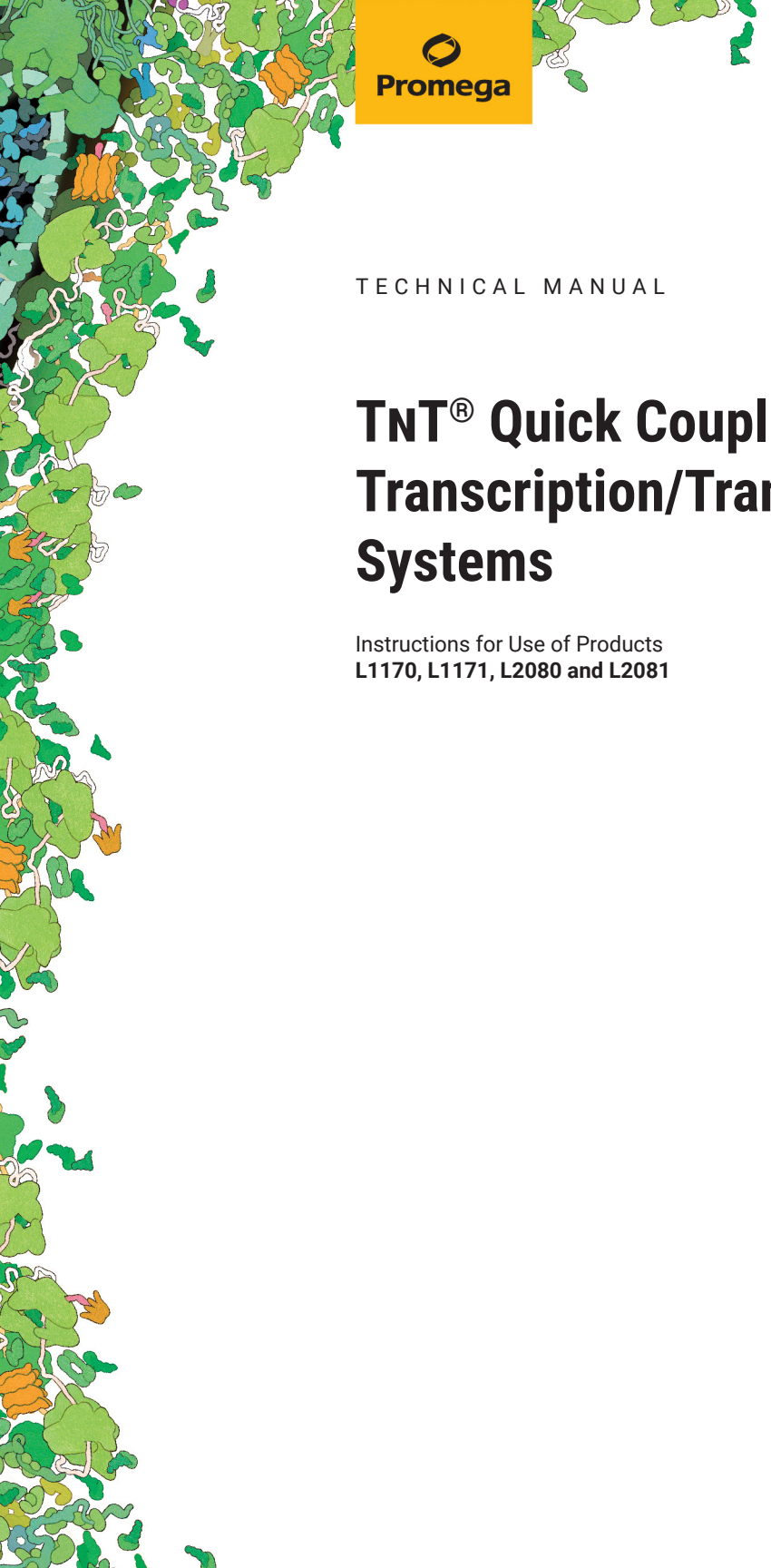

TECHNICAL MANUAL

TnT® Quick Coupled Transcription/Translation Systems

Instructions for Use of Products
L1170, L1171, L2080 and L2081

Revised 8/22
TM045

Promega

TNT® Quick Coupled Transcription/ Translation Systems

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® Quick Coupled Transcription/Translation System is a mammalian cell-free protein synthesis system. It simplifies the transcription and translation of DNA sequences cloned in plasmid vectors containing a T7 or SP6 promoter by providing a rabbit reticulocyte extract that contains the T7 or SP6 RNA polymerase for transcription along with all necessary components for translation (1). This system can produce moderate amounts of recombinant proteins (up to 5 micrograms of protein per milliliter of reaction) within an hour. The protein yields are lower when compared to *E. coli* systems; however, this system is advantageous for the production of larger, more complex proteins, and can achieve post-translational modifications that are not found in prokaryotic expression systems.

To use these systems, 0.2–2.0µg of circular plasmid DNA containing a T7 or SP6 promoter is added to an aliquot of the TnT® Quick Master Mix and incubated in a 50µl reaction volume for 60–90 minutes at 30°C (Figure 1). The TnT® Quick Coupled System can be used with PCR templates containing T7 promoters. The synthesized proteins can be labeled (using Transcend™ Non-Radioactive or FluoroTect™ Green Labeling System) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and appropriate detection system. Included with the TnT® Quick System is a luciferase-encoding control plasmid and Luciferase Assay Reagent, which can be used for detection of functionally active luciferase protein. Refer to Section 6 for details.

Synthesizing a protein of the correct size is a useful way to verify the gene product of a particular DNA sequence. The reaction can be performed with smaller volumes (as low as 5µl) for high-throughput screening in a 96-well plate format. The amount of protein synthesized increases proportionally with reaction volume. Small amounts of target protein can be purified using affinity tags (e.g., HaloTag® or metal-affinity tag) for downstream analysis. Proteins expressed in the cell-free system also can be used for a variety of functional studies, such enzymatic analysis, protein interactions and post-translational modification.

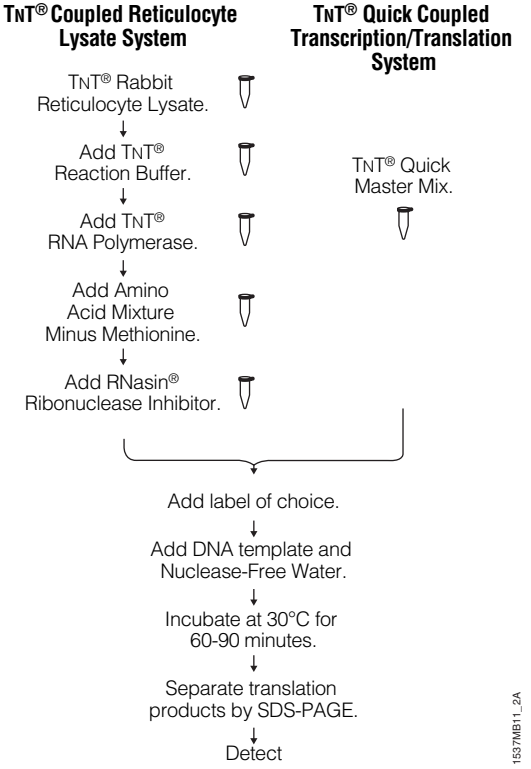

Figure 1. Comparison of the TnT® Coupled Reticulocyte Lysate System and the TnT® Quick Coupled Transcription/Translation System protocols.

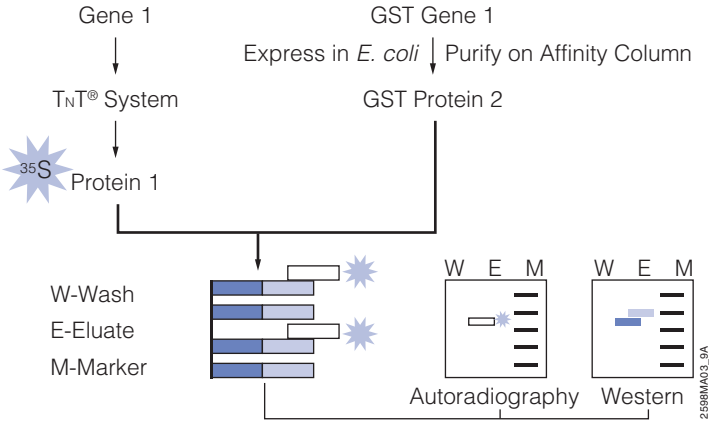

Figure 2. Studying protein:protein interactions using the TNT® Systems. This schematic shows translation of one protein with radioactive [^{35}S]methionine in a TNT® System reaction. Large amounts of the suspected partner protein are expressed and purified. A fusion tag (most commonly GST) is incorporated into this second protein to facilitate purification and subsequent capture steps. After the GST fusion protein is immobilized on sepharose (GST pulldowns), it is mixed with the protein produced in the TNT® reaction. The sepharose is washed to remove unbound protein, and the remaining bound proteins are eluted and analyzed on a gel. This technique allows measurement of the protein:protein interactions for both proteins and is often used to verify the in vivo results obtained from yeast two-hybrid experiments. The MagneGST™ Pull-Down System (Cat.# V8870) can be used for GST pull-down experiments.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
TnT® T7 Quick Coupled Transcription/Translation System	**40 reactions**	**L1170**
TnT® SP6 Quick Coupled Transcription/Translation System	**40 reactions**	**L2080**

Each system contains sufficient reagents to perform approximately 40 × 50µl translation reactions. Includes:

- 1.6ml TnT® Quick Master Mix (8 × 200µl)
- 5µg SP6 or T7 Luciferase Control DNA (0.5µg/µl)
- 100µl T7 TnT® PCR Enhancer (L1170 only)
- 50µl Methionine, 1mM
- 250µl Luciferase Assay Reagent
- 1.25ml Nuclease-Free Water

PRODUCT	SIZE	CAT.#
TnT® T7 Quick Coupled Transcription/Translation System, Trial Size	**5 reactions**	**L1171**
TnT® SP6 Quick Coupled Transcription/Translation System, Trial Size	**5 reactions**	**L2081**

Each system contains sufficient reagents to perform approximately 5 × 50µl translation reactions. Includes:

- 200µl TnT® Quick Master Mix
- 5µg SP6 or T7 Luciferase Control DNA (0.5µg/µl)
- 100µl T7 TnT® PCR Enhancer (L1171 only)
- 50µl Methionine, 1mM

Storage and Stability: Store all components at below –65°C. Product components are sensitive to CO_2 (avoid prolonged exposure), frequent temperature fluctuations and multiple freeze-thaw cycles, which can adversely affect stability, activity and performance. Luciferase Assay Reagent (LAR) is stable for at least 12 months if stored and handled properly.

Note that the systems are shipped in foil packaging because the system is sensitive to carbon dioxide released from dry ice. If storing the system in a freezer containing dry ice, keep system components sealed in foil packaging for best results. **Do not** store the unfoiled lysate in the presence of dry ice. Prolonged exposure to dry ice causes significant loss of activity. The expiration date for the TnT® Quick Master Mix is listed on the product vial. **Do not** freeze-thaw the Master Mix more than two times.

3. General Considerations

3.A. Transcription/Translation Considerations

DNA Template Considerations

Start Codon. Check the sequence on the DNA 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 transcribed portion of the vector or 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 production of a larger protein than expected.

5´ UTR. While Rabbit Reticulocyte Lysate-based systems are less sensitive to 5´-untranslated region (UTR) secondary structure than other systems, strong hairpin secondary structure in the 5´-UTR region can impair translation efficiency (2).

Kozak Sequence. Optimal translation will occur if the AUG initiation codon is in a "Kozak consensus" context (A/GCCAUGG).

Stop Codon. For truncated templates, adding a stop codon (usually UAA) will prevent ribosomes from stalling at the ends of RNAs.

PolyA. DNA constructs containing a poly(A) sequence downstream of the gene of interest generate more protein. Poly(A) sequences are important for mRNA stability and can play a role in translation initiation in Rabbit Reticulocyte Lysate (3).

Plasmid Considerations

- The TnT® Quick Coupled Systems are optimized for use with circular plasmid templates, such as pTnT™ (Cat.# L5610), pSP64 Poly(A) Vector (Cat.# P1241) or pCMVTnT™ (Cat.# L5620).
- For most constructs, optimal results are obtained when 1µg of plasmid DNA template is used. However, we have used 0.2–2.0µg of DNA template and obtained satisfactory levels of translation. Using more than 1µg of plasmid does not necessarily increase the amount of protein produced.
- Linearized plasmid templates should only be used with the TnT® T7 Quick Coupled System. We do not recommend using linear DNA with the SP6 System due to reduced transcription efficiencies.
- If linearizing plasmid DNA, avoid restriction enzymes that yield 3´-overhangs (PstI, KpnI, SacI, SacII, BstXI, NsiI, ApaI and AatII), as aberrant transcription products can be produced (4). If no alternative enzyme is available, the 3´-overhang can be removed by adding T4 DNA polymerase.
- For linearized DNA, include 1µl of the T7 TnT® PCR Enhancer in each 50µl reaction.

3.A. Transcription/Translation Considerations (continued)

PCR Considerations

- PCR templates can be used with the TnT® T7 Quick Coupled System. We do not recommend using PCR templates with the SP6 System due to reduced transcription efficiencies.
  Note: For coupled transcription/translation from PCR-generated templates, Promega offers TnT® T7 Quick for PCR DNA (Cat.# L5540).
- Because PCR DNA templates are usually much smaller than plasmid templates, the amount of DNA necessary for optimal expression is often less than for inserts cloned into plasmid vectors (e.g., for a 500bp PCR product, use 100–800ng for each 50µl TnT® Quick reaction).
- PCR products (5–7µl) can be used directly from the amplification reaction.
- For PCR products, include 1µl of the T7 TnT® PCR Enhancer in each 50µl reaction.

Primer Design Considerations for Incorporating a T7 Promoter into a PCR Product for in vitro Transcription/Translation.

Primer	Required	Desired
Forward Primer: 5´-($N_{6–10}$) TAA TAC GAC TCA CTA TAG GG ($N_{3–6}$) CCA CCA TGG ($N_{17–22}$)-3´	T7 promoter sequence (5´-TAA TAC GAC TCA CTA TAG GG-3´)	Kozak consensus sequence (5´-CCACCATGG-3´)
	ATG start codon (5´-ATG-3´)	6–10 bases upstream of promoter. Improves efficiency of promoter.
	Gene-specific sequence ($N_{17–22}$)	3- to 6-base spacer between promoter sequence and Kozak sequence. Ensures transcription starts a few bases upstream of the Kozak sequence and allows better ribosome binding to RNA.
Reverse Primer: 5´-T30 stop anticodon ($N_{17–22}$)-3´	Gene-specific sequence. Needed to allow priming of the target gene.	Reverse complement of stop codon (TTA, CTA or TCA). Terminates translation.
		Reverse complement of poly(A) tail T30.

3.B. Creating a Ribonuclease-Free Environment

To reduce the chance of RNase contamination, gloves should be worn when setting up experiments, and microcentrifuge tubes and pipette tips should be RNase-free. It is not necessary to add Recombinant RNasin® Ribonuclease Inhibitor to the TnT® Quick reactions to prevent degradation of RNA, because it is already present in the TnT® Quick Master Mix.

3.C. Handling of TnT® Quick Master Mix

Except for the actual transcription/translation incubation, all handling of the TnT® Quick Master Mix should be done at 4°C. Any unused Master Mix should be refrozen as soon as possible after thawing to minimize loss of translational activity. Optimum results are obtained when any unused Master Mix is quick-frozen with liquid nitrogen as soon as possible after thawing to minimize loss of translational activity. Do not freeze-thaw the Master Mix more than two times.

4. Translation Procedure

The following is a general guideline for setting up a transcription/translation reaction. Also provided are examples of standard reactions using [^{35}S]methionine (radioactive), Transcend™ Non-Radioactive Detection System (colorimetric or chemiluminescent) or FluoroTect™ Green$_{Lys}$ Systems (fluorescent). Using the Transcend™ Systems, biotinylated lysine residues are incorporated into nascent proteins during translation. This biotinylated lysine is added to the transcription/translation reaction as a precharged ε-labeled, biotinylated lysine-tRNA complex (Transcend™ tRNA) rather than a free amino acid. For more information on the Transcend™ Systems, see Technical Bulletin #TB182. The FluoroTect™ System uses a charged lysine tRNA labeled with the fluorophore, BODIPY®-FL, to incorporate fluorescently labeled lysine residues into the in vitro translation product. For more information on the FluoroTect™ System, see Technical Bulletin #TB285.

Note: Technical Manuals and Bulletins are available online at: **www.promega.com/protocols/**

4.A. General Protocol for TnT® Quick Coupled Transcription/Translation Reactions Using Plasmid DNA

Materials to Be Supplied by the User

- Nuclease-Free Water (Cat.# P1193)
- **optional:** Transcend™ tRNA (Cat.# L5061) or Transcend™ Colorimetric (Cat.# L5070) **or**
- **optional:** Chemiluminescent (Cat.# L5080) Translation Detection System **or**
- **optional:** FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (for fluorescent detection; (Cat.# L5001) **or**
- **optional:** Radiolabeled amino acid (for radioactive detection; see Section 5). We recommend using a grade of [^{35}S] methionine, PerkinElmer EasyTag™ L-[^{35}S]methionine (PerkinElmer Cat.# NEG709A), which does not cause the background labeling of the rabbit reticulocyte lysate 42kDa protein. Background labeling of the 42kDa protein can occur using other grades of label.

  Between 10–40µCi (1–4µl) of [^{35}S]methionine (1,000Ci/mmol at 10mCi/ml) can be added to the TnT® Quick reactions, depending upon the balance between labeling efficiency and cost. For gene constructs that express well and contain several methionines, the 10µCi level (1µl) is sufficient for adequate detection.

1. Remove the reagents from storage at –65°C. Rapidly thaw the TnT® Quick Master Mix by hand-warming and place on ice. The other components can be thawed at room temperature and then stored on ice.
2. Following the examples in Table 1 below, assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube. After adding all of the components, gently mix by pipetting. If necessary, centrifuge briefly to return the reaction to the bottom of the tube.
3. We recommend including a negative control reaction containing no added DNA. This reaction allows measurement of any background incorporation of labeled amino acids.
4. Incubate the reaction at 30°C for 60–90 minutes.
5. Analyze the results of translation. See Section 5.A.

4.B. Positive Control Reactions using Firefly Luciferase

The assay for firefly luciferase is extremely sensitive, rapid and easy to perform. It is a good control for in vitro translations because only full-length firefly luciferase is active. Additionally, luciferase is a monomeric protein (61kD) that does not require post-translational processing or modification for enzymatic activity. The control reaction can be performed with or without the addition of radiolabeled amino acids, Transcend™ tRNA or FluoroTect™ $Green_{Lys}$ tRNA.

See Section 6 for measurement of luciferase activity.

Table 1. Examples of TnT® Quick Reactions.

		Label			
Components	**Unlabeled**	**Positive Control**	**[^{35}S]Methionine**	**Transcend™ tRNA**	**FluoroTect™ $Green_{Lys}$ tRNA**
TnT® Quick Master Mix[a]	40µl	40µl	40µl	40µl	40µl
DNA template (0.2–2.0µg)[b,c]		2µl			
Methionine, 1mM	1µl	1µl	–	1µl	1µl
[^{35}S]methionine	–	–	2µl	–	–
Transcend™ Biotin-Lysyl-tRNA[d]	–		–	1–2µl	–
FluoroTect™ $Green_{Lys}$ tRNA[d]	–		–	–	1–2µl
Nuclease-Free Water to a final volume of[e]	**50µl**	**50µl**	**50µl**	**50µl**	**50µl**

[a]The TnT® Quick Master Mix is designed to give the highest expression for most expression constructs. However, we have observed that certain gene constructs may differ in the Mg^{2+} and K^{+} concentrations required for optimal expression in the coupled reaction. For example, some viral leaders will increase translation efficiency and fidelity if additional magnesium acetate and potassium chloride are added to the TnT® Quick reaction. If using a construct with a viral leader, we suggest adding 1–2µl of the T7 TnT® PCR Enhancer.

[b]Avoid adding calcium to the transcription/translation reaction. Calcium can reactivate the micrococcal nuclease used to destroy endogenous RNA in the Master Mix and result in degradation of DNA or RNA templates.

[c]We recommend using 1µl of the T7 TnT® PCR Enhancer in a 50µl reaction to increase transcription/translation when using PCR-generated DNA, linear plasmid or viral-enhanced plasmids.

[d]The level of added Transcend™ tRNA and FluoroTect™ $Green_{Lys}$ tRNA can be increased (1–4µl) to allow more sensitive detection of proteins that contain few lysines or are poorly expressed.

[e]Small-scale reactions may be performed by reducing the recommended volumes proportionally.

5. Post-Translational Analysis

Several methods can be used for detection of newly synthesized protein, depending on the labeling method chosen.

- Unlabeled proteins can be detected on Western blots. The TnT® Quick Master Mix contains roughly 100–200mg/ml of endogenous protein. The newly translated protein band will not be visible on an SDS-PAGE gel stained with Coomassie® Blue because of the high background of endogenous proteins.
- Transcend™ biotinylated lysine-labeled proteins can be detected with streptavidin reagents on a Western blot. See the *Transcend™ Non-Radioactive Translation Detection System Technical Bulletin*, #TB182, for details.
- Fluorescently labeled proteins with FluoroTect™ tRNA can be detected on SDS-PAGE as directed in the *FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling Systems Technical Bulletin*, #TB285.
- Radioactive labeled proteins can be detected by scintillation counting or on SDS-PAGE with imaging or radiography

5.A. SDS-Polyacrylamide Gel Analysis of Translation Products

The most widely applicable and versatile method for analysis of in vitro translation products is polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) and a discontinuous buffer system. Precast gels are available from a number of manufacturers. In addition to convenience and safety, precast gels provide consistent results.

Materials to Be Supplied by the User

- precast SDS polyacrylamide gels (e.g., NuPAGE® Bis-Tris 4–12% acrylamide gel)
- 4X SDS-polyacrylamide sample buffer
- RNase ONE™ Ribonuclease (Cat.# M4261, M4265) or RNase A Solution (Cat.# A7973)
- 1X TBS

1. Dilute RNase ONE™ Ribonuclease or RNase A solution by mixing 1µl with 9µl of 1X TBS.
2. Once the transcription/translation reaction is complete (or at any desired time point), remove a 5µl aliquot and add to it to a microcentrifuge tube containing 14µl of 1X TBS and 1µl of diluted RNase solution. Incubate at room temperature for 5 minutes. Store unused portion of the reaction at –20°C.

   Note: Ribonuclease treatment of the sample can reduce the background generated by unincorporated fluorescent or biotinylated tRNAs when used in the reaction. This step is optional.
3. Add 5µl of 4X SDS-polyacrylamide sample buffer and tap to mix.
4. Close the tube securely. Heat at 70ºC for 2–10 minutes.

   Note: The FluoroTect™ tRNA is sensitive to extreme heating. Do not exceed 70°C for more than 2–3 minutes. Heating the sample at 100°C can cause protein aggregation, so denaturation may need to be performed at lower temperatures (e.g., 20 minutes at 60°C or 3–4 minutes at 80–85°C).

See “Helpful hints for analyzing in vitro transcription/translation reactions on polyacrylamide gels” at: **www.promega.com/resources/pubhub/enotes/trouble-free-sdspage-analysis-of-proteins-synthesized-in-tnt-cell-free-expression-systems/** .

5. Load 10µl of the prepared sample on an SDS-PAGE gel. (This is equivalent to 2µl of the original reaction). Loading too much of the reaction per lane can cause protein aggregation and poor separation of proteins.
6. Perform electrophoresis using standard conditions for your apparatus. Typically, electrophoresis is carried out at a constant current of 20mA until the bromophenol blue dye has run to the bottom of the gel.
7. Detect target protein expression by one of the following methods:
   a. Western blot (see Section 5.B)
   b. Fluorescent detection when labeling proteins with FluoroTect™ tRNA as directed in the *FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling Systems Technical Bulletin* #TB285.
   c. Detection of biotinylated protein when labeling proteins with Transcend™ tRNA as directed in the *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin* #TB182.

5.B. Western Blot Analysis

Materials to Be Supplied by the User

- PVDF membrane
- Western blot apparatus
- blot-qualified BSA (Cat.# W3841)
- TBST (1X TBS + 0.1% Tween® 20)
- primary antibody for your protein of interest
- secondary antibody (against species of primary Ab)
- detection reagents (for label on secondary Ab)
- **optional:** Anti-Luciferase pAb (Cat.# G7451) for positive control analysis

1. Run an aliquot of the reaction on SDS-PAGE as described in Section 5.A.
2. Following electrophoresis, remove the gel and place it in water.
3. Transfer the proteins to a PVDF membrane using a Western blotting system.
4. Block the membrane using 15ml of 5% blot-qualified BSA in TBST (1X TBS + 0.1% Tween® 20). Incubate for 1 hour with gentle shaking.
5. Dilute your primary antibody in 1X TBST.

   Note: We recommend that you titrate your primary antibody dilutions to determine what dilution produces the best results for your protein.
6. Following incubation, remove the blocking solution from the membrane and add 15ml of diluted primary antibody.
7. Incubate the membrane with the primary antibody at room temperature for 1 hour with gentle shaking.
8. Remove the primary antibody solution, then wash the membrane with 15ml of 1X TBST for 5 minutes with gentle shaking.
9. Repeat the wash 5 more times for a total of six washes.

10. Dilute your secondary antibody in 1X TBST as recommended by the supplier.
11. Following that last wash, remove buffer from the membrane and add 15ml of diluted secondary antibody.
12. Incubate the membrane with the secondary antibody for 1 hour with gentle shaking.
13. Following the incubation, remove the secondary antibody solution, and wash the membrane with 15ml of 1X TBST for five minutes. Repeat for a total of six washes.
14. Proceed to the detection method appropriate for your secondary antibody.

6. Positive Control Luciferase Assays

Materials to Be Supplied by the User

- luminometer
- white 96-well plate or tube to fit the luminometer
- **optional:** 1X Glo Lysis Buffer (Cat.# E2661; Section 6.B)
- **optional:** Bright-Glo™ Luciferase Assay System (Cat.# E2610) **or** ONE-Glo™ Luciferase Assay System (Cat.# E6110; Section 6.B)

6.A. Assays Using Luciferase Assay Reagent

1. Dispense 50µl of the Luciferase Assay Reagent into luminometer tubes or microplate.
2. Program the luminometer to perform a 2-second measurement delay followed by a 10-second measurement read for luciferase activity.
3. Add 2.5µl of cell lysate to luminometer tube or microplate containing the Luciferase Assay Reagent. Mix by pipetting 2–3 times or vortex briefly.
4. Place the tube or microplate into the luminometer and initiate reading.

6.B. Assays Using Bright-Glo™ or ONE-Glo™ Luciferase Assay

Alternatively, prepare Bright-Glo™ or ONE-Glo™ Luciferase Assay reagent according to the instructions in the technical manual. The reagent should be equilibrated to room temperature and mixed thoroughly by vortexing before beginning measurements. Dilute the samples to be assayed in 1X Glo Lysis Buffer.

1. Add 2µl of the translation reaction to 98µl of 1X Glo Lysis Buffer and add to a luminometer tube or microplate. Mix by pipetting 2–3 times or vortex briefly.
2. Add 100µl of Bright-Glo™ or ONE-Glo™ Reagent. Mix on a plate shaker or vortex.
3. Put the tube or plate in the luminometer and read the signal for 1 second per sample.

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7. 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
The control reaction produces no luciferase	Loss of reaction component(s) activity. The lysate should not be used after more than two freeze-thaw cycles. Do not use reagents after the expiration date. Prolonged exposure of unfoiled lysate to dry ice can cause loss of activity.
	Ethanol or salt present in the reaction may inhibit translation.
Low translation efficiency	Certain gene constructs may require different Mg^{2+} and K^{+} concentrations for optimal expression. Add 1–3µl of the T7 TNT® PCR Enhancer.
	Calcium is present in the translation reaction. Avoid adding calcium to the translation reaction. Calcium may reactivate the micrococcal nuclease used to destroy endogenous mRNA in the lysate and result in degradation of the DNA or mRNA template.
	Ethanol present in the translation reaction. Residual ethanol should be removed from template DNA preparations and amino acids before they are added to the translation reaction.
	Poly(A) tail. Addition of poly(A) tail to the template may increase translation efficiency (see Section 3.A).
	Incubation of the reaction at 37°C causes decreased protein synthesis. Incubate the reaction at 30°C, the optimal temperature.
Unexpected bands present at higher molecular weights or bands stuck in stacking gel	Denaturing temperature too high. Denature sample at a lower temperature (e.g., 60–80°C) for 10–15 minutes.
Unexpected bands present on the gel	Proteolysis of translation product. Add protease inhibitors, such as α2-macroglobulin, leupeptin or chymostatin (0.5–1µg/ml).
	More than one peptide is translated from the template. Leaky scanning for translation initiation can result in translation initiating at internal methionines. Optimizing the Mg^{2+} or K^{+} concentration can increase fidelity.
	[^{35}S]methionine used is not translation grade or is beyond its expiration date. There are reports of a 42kDa band with some grades of [^{35}S]methionine. We recommend Perkin-Elmer EasyTag™ L-[^{35}S]methionine (Perkin-Elmer Cat.# NEG709A) to avoid this 42kDa band.

Symptoms	Causes and Comments
Unexpected bands present on the gel (continued)	Globin may appear on the autoradiogram or stained gel. It appears as a broad band migrating at 10–15kDa.
	Aminoacyl tRNAs may produce background bands (~25kDa). Add RNase A to the lysate reaction (after completion) to a final concentration of 0.2mg/ml. Incubate for 5 minutes at 30°C.
	Oxidized β-mercaptoethanol is present or not enough SDS in the loading buffer. Use a loading buffer that contains 2% SDS and 100mM DTT.
Unexpected bands present when isolating polyhistidine-tagged protein	A nickel-based resin is used to purify polyhistidine-tagged proteins. Hemoglobin present in the rabbit reticulocyte lysate will bind to the nickel and co-elute with the polyhistidine-tagged protein. Use an alternate purification tag to isolate the protein from the TNT® lysate and avoid this problem.
Smearing on the gel	Too much protein loaded on the gel. Check the amount of sample loaded on the gel and the amount of loading buffer. Too much protein loaded can cause smearing.
	Acrylamide concentration in the gel is too low. Acrylamide concentration can be increased to 12%.
High background levels when performing Western blots	Primary antibody concentration too high. Increase the dilution of the primary antibody.

8. References

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2. Frances, V., Morle, F. and Godet, J. (1992) Identification of two critical base pairings in 5´ untranslated regions affecting translation efficiency of synthetic uncapped globin mRNAs. *Biochim. Biophys. Acta.* **1130**, 29–37.
3. Jackson, R.J. and Standart, N. (1990) Do the poly(A) tail and 3´ untranslated region control mRNA translation? *Cell* **62**, 15–24.
4. Schenborn, E.T. and Mierendorf, R.C. (1985) A novel transcription property of SP6 and T7 RNA polymerases: Dependence on template structure. *Nucl. Acids Res.* **13**, 6223–36.

9. Appendix

9.A. Composition of Buffers and Solutions

T7 TnT® PCR Enhancer

0.5M	KCl
12.5mM	$Mg(OAc)_2$

In nanopure water.

TBST

50mM	Tris HCl (pH7.4)
150mM	NaCl
0.1%	Tween®-20

9.B. Luciferase SP6/T7 Control DNAs

The Luciferase SP6/T7 Control DNAs are used as functional controls in the TnT® Quick Coupled Transcription/Translation System. The Control DNAs contain the gene for luciferase under transcriptional control of a phage RNA polymerase promoter. The constructs carry a 30bp poly[d(A)/d(T)] tail following the luciferase gene. The maps of the Luciferase SP6 Control DNA and T7 Control DNA are shown in Figures 3 and 4, respectively. Please note that these vectors are intended for use as control luciferase expression vectors only. They are not intended for use as cloning vectors.

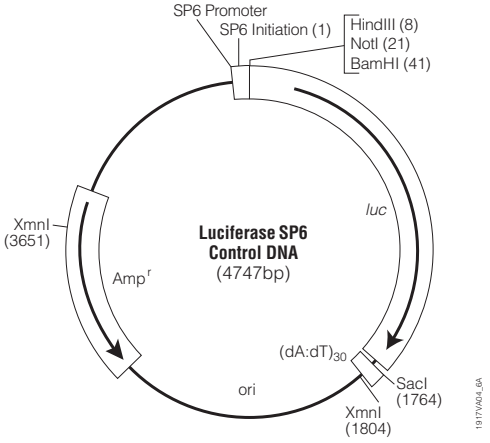

Figure 3. Luciferase SP6 Control DNA circle map and sequence reference points. Additional description: Amp^r, β-lactamase gene (resistant to ampicillin); ori, origin of plasmid replication.

Luciferase SP6 Control DNA Sequence Reference Points:

SP6 RNA polymerase initiation	1
GLPrimer2	49–71
Luciferase gene	48–1700
Poly(A) $(dA)_{30}$	1767–1796
pUC/M13 reverse primer (17mer)	1833–1817
pUC/M13 reverse primer (22mer)	1838–1817
β-lactamase gene (Amp^r)	3838–2978
SP6 RNA polymerase promoter primer	4731–1
SP6 RNA polymerase promoter	4731–3

9.B. Luciferase SP6/T7 Control DNAs (continued)

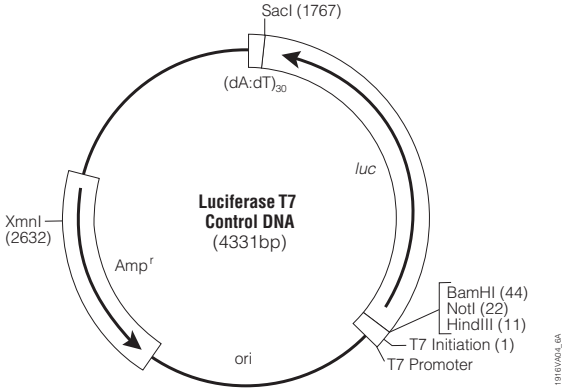

Figure 4. Luciferase T7 Control DNA circle map and sequence reference points. Additional description: Amp^r, β-lactamase gene (resistant to ampicillin); ori, origin of plasmid replication.

Luciferase T7 Control DNA Sequence Reference Points:

T7 RNA polymerase initiation	1
GLPrimer2	52–74
Luciferase gene	51–1700
Poly(A) $(dA)_{30}$	1770–1799
β-lactamase gene (Amp^r)	2444–3304
T7 RNA polymerase promoter	4315–3
T7 RNA polymerase promoter primer	4315–3

9.C. Determining Percent Incorporation of Radioactive Label

Materials to Be Supplied by the User

- 1M NaOH/2% H_2O_2
- 25% TCA/2% casamino acids
- 5% TCA
- Whatman GF/A glass fiber filter
- acetone
- scintillation fluid
- scintillation counter

1. After the translation reaction is complete, remove 2µl from the reaction, and add it to 98µl of 1M NaOH/ 2% H_2O_2.
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.
5. Rinse the filter 3 times with 1–3ml of ice-cold 5% TCA.
6. Rinse once with 1–3ml of acetone. Allow the filter to dry at room temperature or under a heat lamp for at least 10 minutes.
7. For determination of ^{35}S incorporation, put the filter in the appropriate scintillation cocktail, invert to mix and count in a liquid scintillation counter.
8. To determine total counts present in the reaction, spot a 5µl aliquot of the TCA reaction mix directly onto a filter. Dry the filter for 10 minutes. Count in a liquid scintillation counter as in Step 7.
9. To determine background counts, remove 2µl from a negative control translation reaction containing no DNA and proceed as described in Steps 1–7.
10. Perform the following calculation to determine percent incorporation:

$$\frac{\text{cpm of washed filter (Step 7)}}{\text{cpm of unwashed filter (Step 8)} \times 50} \times 100 = \text{percent incorporation}$$

11. Perform the following calculation to determine the fold stimulation over background:

$$\frac{\text{cpm of washed filter (Step 7)}}{\text{cpm of “no DNA control reaction” filter (Step 9)}} = \text{fold stimulation}$$

Promega

9.D. Related Products

TnT® Coupled Reticulocyte Lysate Systems

Product	Size	Cat.#
TnT® T7 Insect Cell Extract Protein Expression System	40 reactions	L1102
S30 T7 High-Yield Protein Expression System	24 reactions	L1110
TnT® SP6 High-Yield Wheat Germ Protein Expression System	40 reactions	L3260
TnT® SP6 Coupled Reticulocyte Lysate System	40 reactions	L4600
TnT® T7 Coupled Reticulocyte Lysate System	40 reactions	L4610
TnT® T3 Coupled Reticulocyte Lysate System	40 reactions	L4950
TnT® T7 Quick for PCR DNA	40 reactions	L5540

Translation Detection

Product	Size	Cat.#
Transcend™ Colorimetric Translation Detection System	30 reactions	L5070

Translation Labeling

Product	Size	Cat.#
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System	40 reactions	L5001

Vectors

Product	Size	Cat.#
pTnT® Vector	20µg	L5610
pCMVTnT® Vector	20µg	L5620
pSP64 Poly(A) Vector	20µg	P1241

10. Summary of Changes

The following changes were made to the 8/22 revision of this document:

1. Section 1 was updated.
2. Section 3 was modified to provide cloning hints and additional details on plasmid use.
3. Sections 4.A–C and 5 were combined into Section 4.A. Subsequent sections were renumbered.
4. Section 7.B, Determining Percent Incorporation of Radioactive Label, is now Section 9.C.
5. The cover image and font were updated.