Technical Manual

Gold TNT® T7/SP6 Express 96 Systems

INSTRUCTIONS FOR USE OF PRODUCTS L5600 AND L5800.

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Gold TNT® T7/SP6 Express 96 Systems

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

I. Description

The Gold TNT® Express 96 Systems[a–d] are formatted to offer the convenience of a 96-well configuration and to enhance experimental expression levels. The dispensed lysate facilitates the use of the TNT® Quick Coupled Transcription/ Translation System for those applications associated with large-scale screening assays such as in vitro expression cloning (IVEC) or high-throughput analysis. Each well contains 20µl of dispensed premium lysate that is evaluated for optimal transcriptional/translational activity, providing reliable and consistent results.

The Gold TNT® Express 96 Systems are for transcription and translation of genes cloned downstream from either the T7 or the SP6 RNA polymerase promoter. To use these systems, plasmid DNA (0.5–1.0µg of pooled cDNA or 0.25–0.5µg individual plasmid DNA) containing the appropriate promoter is added with either unlabeled or ^{35}S-labeled methionine and Nuclease-Free Water to the predispensed 96-well plate and incubated for 60–90 minutes at 30°C. The synthesized proteins may be analyzed by functional testing or by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent detection by fluoroimaging, colorimetric/chemiluminescent methods or autoradiography.

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The Gold TNT® T7 and SP6 Express 96 Systems are ideal for screening large numbers of constructs in a variety of applications. These include:

- Drug screening (affecting translation rates)
- Mutation and detection analysis (i.e., enzyme kinetics)
- Protein-protein interactions (using GST fusion proteins)
- Immunoprecipitation of protein complexes
- Protein dimerization assays
- Ligand-binding region determination/confirmation/ competition assays
- In vitro expression cloning (1) (functional genomics)
- Electrophoretic mobility shift assays (EMSAs) for DNA protein interactions
- DNA footprinting and protein crosslinking studies
- Protein-RNA binding assays
- Post-translational modification tests
- Verification/characterization of cloned genes

For more information and references for these and other applications, see the Protein Expression chapter of the *Protocols and Applications Guide* (2) available at: **www.promega.com/paguide/chap5.htm**

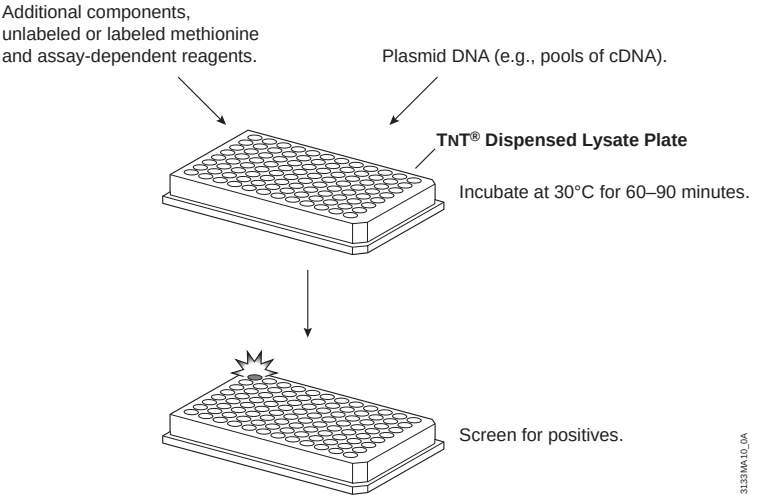

Figure 1. Schematic showing a typical experimental format using the Gold TNT® T7 or SP6 Express 96 Systems.

Selected Citation Using the Gold TNT® T7/SP6 Express 96 Systems

- Pridgeon, J.W., Geetha, T. and Wooten, M.W. (2003) A method to identify p62's UBA domain interacting proteins. *Biol. Proced. Online* 5, 228–37.

  The authors of this paper screened for proteins that bind p62, a novel Src homology domain binding protein. The researchers expressed the Human Adult Brain library of proteins in the presence of [^{35}S]methionine using the Gold TNT® SP6 Express 96 System. The labeled proteins were then added to binding assays in the presence of agarose-immobilized p62 UBA. Eleven proteins were identified and reported in the study.

II. Product Components and Storage Conditions

Product	Cat.#
Gold TNT® T7 Express 96 System	L5600
Gold TNT® SP6 Express 96 System	L5800

Each system contains sufficient reagents to perform 96 × 25µl translation reactions. Includes:

- 1 TNT® T7 or SP6 Dispensed Lysate Plate
- 1 Protocol

Stability/Storage Conditions: Store all components below –65°C. Product is sensitive to CO_2 (avoid prolonged exposure) and multiple freeze-thaw cycles, which may have an adverse effect on activity/performance.

Note that the lysate is sensitive to carbon dioxide released from dry ice. If storing the plate in a freezer containing dry ice, keep the plate sealed until use. **Do not** store the unsealed plate in the presence of dry ice. Prolonged exposure to dry ice causes significant loss of activity. Avoid more than four freeze-thaw cycles or exposure to frequent temperature changes, as these fluctuations can greatly alter product stability.

III. General Considerations

III.A. DNA Template Considerations

DNA Expression Elements

1. While rabbit reticulocyte lysate-based systems are less sensitive to 5´-untranslated region (UTR) secondary structure than other systems, it is still important to avoid strong hairpin secondary structure in the 5´-UTR region, as this can impair translation efficiency (3).

2. We have observed enhanced translation of proteins when using DNA constructs containing a poly(A) sequence downstream of the gene of interest. Poly(A) sequences are important for mRNA stability and can play a role in translation initiation in rabbit reticulocyte lysate (4). For example, we have observed a two- to fivefold increase in the production of luciferase when the gene is cloned into the pSP64 Poly(A) Vector (Cat.# P1241).

Plasmid DNA

1. Residual ethanol should be removed from DNA preparations before they are added to the TnT® T7 or SP6 Dispensed Lysate Plate.

2. For most constructs, optimal results are obtained when 0.5–1.0µg pooled cDNA or 0.25–0.5µg individual plasmid DNA template is used per 20µl of lysate. The use of more than 0.5µg of individual plasmid DNA does not necessarily increase the amount of protein produced.

3. If possible, check the sequence of 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. This may result in a shift in the reading frame, production of a larger protein than expected or decreased expression levels.

III.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 Gold TnT® Express reactions to prevent degradation of RNA, as it is already present in the lysate.

IV. Translation Procedure

The following is a general guideline for setting up a series of transcription/translation reactions.

IV.A. General Protocol for Gold TnT® Express 96 Transcription/Translation Reactions Using Plasmid DNA

Materials to Be Supplied by the User

- Nuclease-Free Water (Cat.# P1193)
- Radiolabeled amino acid (for radioactive detection)
- Methionine, 1mM (for non-radioactive applications)
- Additional substrates or factors as required (e.g., oligonucleotides, peptides, radiolabeled NTPs)
- Plate seals (e.g., AbGene Cat.# AB-0626)

1. Remove the plate from storage and thaw on ice for 10 minutes.

2. Carefully peel away and discard the plate seal (see Note 1 if the entire plate is not to be used).

IV.A. General Protocol for Gold TnT® Express 96 Transcription/Translation Reactions Using Plasmid DNA (continued)

3. For each reaction (i.e., one 20µl predispensed well), add the following components to yield a final reaction volume of 25µl. We recommend including a control reaction containing no DNA to measure any background incorporation of labeled amino acids

Components	Standard Reaction Using [^{35}S]Methionine	Standard Reaction Using Unlabeled Methionine
Methionine, 1mM (mix gently prior to use)	–	0.5µl
[^{35}S]Methionine* (1,000Ci/mmol at 10mCi/ml)	1µl	–
plasmid DNA template (0.5–1.0µg/µl; see Note 5)	1µl	1µl
Nuclease-Free Water to a final volume of	5µl	5µl

*See Note 8 for information on non-radioactive systems.

4. Seal the plate containing the reactions with an adhesive plate seal.
5. Gently vortex the plate to mix.
6. Incubate the reaction at 30°C for 60–90 minutes.
7. Analyze the results of translation.

Notes:

1. If the entire plate is not to be used:
   a. Thaw as in Step 1, then place the plate on ice.
   b. Using a sharp edge, carefully cut through the foil seal, separating the columns or rows you will be using.
   c. Carefully peel away the seal to expose the wells you will be using.
   d. Transfer the contents of each well being used to a fresh 96-well polypropylene plate (e.g., Phenix Cat.# MPX-PCR96-FS) on ice.
   e. Return remaining sealed TnT® Express dispensed lysate to below –65°C storage for later use.

IV.A. General Protocol for Gold TNT® Express 96 Transcription/Translation Reactions Using Plasmid DNA (continued)

2. The TNT® lysate is designed to give high expression for most genetic 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® reaction. If using a construct with a viral leader, we suggest adding additional Mg^{2+} or K^+ to the reaction.

3. We recommend using a grade of [^{35}S]methionine, such as GE Healthcare Bio Sciences Redivue® L-[^{35}S]methionine (GE Healthcare Bio Sciences Cat.# AG1094), which does not cause background labeling of the rabbit reticulocyte lysate 42kDa protein. Background labeling of the 42kDa protein can occur using other grades of label. In addition, a stabilizer has been added to the Redivue® [^{35}S]methionine to increase the stability of this product over conventional radiolabeled amino acids, so that the release of volatile gases is reduced substantially. This [^{35}S]methionine may be stored at 4°C without aliquoting. Other types of ^{35}S-labeled amino acids may be oxidized easily to translation-inhibiting sulfoxides and should be stored in aliquots below –65°C in buffer containing DTT.

4. Except for the actual transcription/translation incubation, **all handling of the Gold TNT® Express 96 System components should be performed at 4°C or on ice**. Optimum results are obtained when any unused wells are quick-frozen below –65°C as soon as possible after thawing to minimize loss of translational activity.

5. For most constructs, optimal results are obtained when 0.5–1.0µg of pooled cDNA template, or 0.25–0.5µg individual plasmid DNA template, is used per 20µl of lysate. The use of more than 0.5µg of individual plasmid DNA does not necessarily increase the amount of protein produced.

   Be sure to use DNA containing the appropriate promoter, T7 or SP6, for the Gold TNT® Express 96 System that you are using.

6. Avoid adding calcium to the transcription/translation reaction. Calcium may re-activate the micrococcal nuclease used to destroy endogenous RNA in the lysate and may result in degradation of DNA or RNA templates.

7. The TNT® lysate contains roughly 100–200mg/ml of endogenous protein.

8. The FluoroTect™ $Green_{Lys}$ and Transcend™ Systems provide convenient methods for non-radioactive detection of translation products. See Section VII for ordering information.

V. Troubleshooting

Symptoms	Causes and Comments
Low translation efficiency	Certain gene constructs may differ in the the Mg^{2+} or K^{+} concentration required for optimal expression in the coupled reaction. Add additional Mg^{2+} or K^{+} to the reaction.
	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 is present in the translation reaction. Residual ethanol should be removed from preparations and labeled amino acids before they are added to the translation reaction.
	Incubation of the reaction at 37°C causes decreased protein synthesis. Incubate the reaction at 30°C.

VI. References

1. Lustig, K.D *et al*. (1997) Small pool expression screening: Identification of genes involved in cell cycle control, apoptosis and early development. *Meth. Enzymol*. **283,** 83–99.
2. *Protocols and Applications Guide*, online edition (2006) Promega Corporation.
3. 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.
4. Jackson, R.J. and Standart, N. (1990) Do the poly(A) tail and 3´ untranslated region control mRNA translation? *Cell* **62**, 15–24.

VII. Related Products

Two basic approaches to in vitro protein synthesis are available: 1) in vitro systems programmed with RNA (translation systems) and 2) those programmed with DNA (coupled transcription/translation systems). Please see our product catalog, available upon request from Promega, or visit our Web site at **www.promega.com** for a complete listing of our in vitro translation systems. A brief listing follows.

VII. Related Products (continued)

TnT® Quick Coupled Transcription/Translation Systems

Product	Size	Cat.#
TnT® T7 Quick Coupled Transcription/Translation System	40 × 50µl reactions	L1170
TnT® SP6 Quick Coupled Transcription/Translation System	40 × 50µl reactions	L2080
TnT® T7 Quick for PCR DNA	40 × 50µl reactions	L5540

For Laboratory Use. Please contact Promega for information on bulk orders.

Canine Pancreatic Microsomal Membranes

Product	Size	Cat.#
Canine Pancreatic Microsomal Membranes	50µl	Y4041

Luciferase Control DNAs

Product	Size	Cat.#
Luciferase SP6 Control DNA	20µg	L4741
Luciferase T7 Control DNA	20µg	L4821
Luciferase T3 Control DNA	20µg	L4941

For Laboratory Use.

Non-Radioactive Detection Systems

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

For Laboratory Use.

DNA Purification

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

[a]U.S. Pat. Nos. 5,324,637 and 5,492,817, European Pat. No. 0 566 714 B1, 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]U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289, European Pat. No. 0 553 234 and Japanese Pat. No. 3171595 have been issued to Promega Corporation for a beetle luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.

[c]U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217, Australian Pat. No. 646803 and Japanese Pat. Nos. 3009458 and 3366596 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor.

[d]U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

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

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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