

Applications of the TNT®¹ Coupled Transcription/Translation Systems

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¹U.S. Pat. No. 5,324,637 has been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and eukaryotic lysates.

Promega's TNT® Coupled Transcription/Translation Systems are eukaryotic *in vitro* translation systems that enable the synthesis of proteins directly from protein-encoding DNA sequences downstream of T3, T7 or SP6 RNA polymerase promoters. Last year, *Promega Notes* 47 featured an article highlighting new applications of the TNT® Systems (1).

Tables 1-6 contain references using the TNT® Coupled Transcription/Translation Systems. This is not an all-inclusive list, and consists primarily of your submissions to Promega. Each table contains references citing the use of the TNT® System for a particular application area, although in many cases, the TNT® System was used for multiple applications.

Many researchers continue to take advantage of the ease of the coupled system to simply verify their gene constructs (see [Table 1](#)). An identified open reading frame (ORF) can be tested for expression of a protein of expected size by SDS-PAGE. Sometimes, however, proteins do not migrate on SDS-PAGE at the expected size and it can be useful to compare the *in vitro* protein with its *in vivo* counterpart (see [Table 1](#), Krapivinsky *et al.*).

Several research groups have taken advantage of the increased synthesis of the TNT® Systems over standard mRNA-based systems. In one case, a 250kDa protein was synthesized, which was subsequently self-proteolytically cleaved into smaller proteins (see [Table 1](#), Hemmer *et al.*). The TNT® System produced two proteins which were difficult to detect previously, and also produced a much cleaner result. Another group directly compared standard reticulocyte systems using capped or uncapped mRNA to the TNT® Reticulocyte Lysate System. They consistently found better expression in the coupled TNT® System (see [Table 6](#), Herzog, Guilley, and Fritsch).

Table 1. Verification of Cloned Genes.

Description	Reference
Verification that clone expresses a protein of the predicted size	Daigle, I. and Li, C. (1993) <i>apl-1</i> , a <i>Caenorhabditis elegans</i> gene encoding a protein related to the human (beta -amyloid protein precursors. <i>Proc. Natl. Acad. Sci. USA</i> 90 , 12045.
	Mellor, H. <i>et al.</i> (1994) Cloning and characterization of cDNA encoding rat hemin-sensitive initiation factor-2alpha (eIF-2alpha) kinase. <i>J. Biol. Chem.</i> 269 , 10201.
Demonstration that alternative splicing is the molecular basis for isoforms by comparison of radiolabeled <i>in vitro</i> translated proteins expressed from cDNAs to native proteins using immunoprecipitation and Western blots.	Greig, A. <i>et al.</i> (1994) Molecular basis of cardiac troponin T isoform heterogeneity in rabbit heart. <i>Circulation Res.</i> 74 , 41.
Comparison of radiolabeled <i>in vitro</i> translated protein to native protein confirms aberrant migration on SDS-PAGE is due to peculiarities in primary structure.	Krapivinsky, G.B. <i>et al.</i> (1994) Molecular characterization of a swelling-induced chloride conductance regulatory protein, p1Cln <i>Cell</i> 76 , 439.
Demonstration that signal sequence removal has no effect on enzymatic activity by analysis of radiolabeled <i>in vitro</i> translated	Murthy, M.S.R. and Pande, S.V. (1994) A stress-regulated protein, GRP58, a member of thioredoxin superfamily, is a carnitine

proteins expressed in TNT® System with and without the addition of microsomal membranes.	palmitoyltransferase isoenzyme. <i>Biochem. J.</i> 304 , 31.
Increased and cleaner expression of a 250kDa polyprotein and resulting proteolytic proteins in TNT® System over standard mRNA translation system.	Hemmer, O. et al. (1995) Functional characterization of the proteolytic activity of the tomato black ring Nepovirus RNA-1 encoded polyprotein. <i>Virology</i> . 206 , 362.

The fastest growing application of the TNT® Systems is for the identification of molecular interactions. [Table 2](#), contains examples using the TNT® Systems to demonstrate protein:protein, protein:DNA and protein:RNA interactions. Even RNA:RNA interactions are investigated using the TNT® System, presumably because "it is more likely that the mRNA secondary structure would stay in its nascent form", and in this case, make it a better substrate for a hammerhead ribozyme (see [Table 6](#), Karikó *et al.*).

In the field of transcription regulation, many researchers have used radioactively labeled DNA oligo probes together with *in vitro* generated proteins to identify DNA:protein interactions using the Electrophoretic Mobility Shift Assay (EMSA). Protein:protein interactions require a different "handle" for investigation. The most common handle is to engineer a fusion protein (e.g., GST-fusion), which can be purified in large amounts from *E. coli* and then attached to a solid support². Radiolabeled *in vitro* translated proteins are then tested for their ability to bind to the GST-fusion protein. This *in vitro* approach was used by several research groups to verify the protein binding partners identified *in vivo* by the yeast two-hybrid system (see [Table 2](#), Dyck *et al.*, Paroush *et al.*, and Boyd *et al.*). Other "handles" include polyhistidine sequences, biotinylation domains, short epitope sequences or antibodies to one (or more) of the proteins for immunoprecipitations.

²Similar results might be obtained using a "fusionless" approach by incorporating biotinylated lysines into the synthesized polypeptide using Promega's tRNA^{Ascend(TM)} System. After isolation using Promega's SofiLink(TM) Resin, interactions with other "partners" could be detected using the appropriate antibodies. See reference 2 for a description of the tRNA^{Ascend(TM)} System.

Interestingly, two research groups used the ability to co-translate more than one gene product in the same reaction by using multiple DNAs (see [Table 2](#), Sontag *et al.* and Waltzer *et al.*). In one case (Sontag *et al.*), four different DNAs encoding four different gene products were simultaneously co-expressed and then by immunoprecipitation shown to form a complex. Many of the publications also described mutants used to delineate binding domains, often generated by increasing deletions of the carboxyl terminus.

Perhaps the most exciting new applications combine PCR amplification techniques with the TNT® Systems. All of the research groups highlighted in [Table 3](#) use PCR amplified DNA directly in the TNT® Systems. Many genetic diseases and cancers are the result of mutations (stop codon, frameshift or splicing defects) which result in truncated gene products. Normally, DNA based tests are required to identify the mutation, with sequencing being the definitive test. However, it is very labor intensive to screen many samples. In 1993, a group (see [Table 3](#), Roest, P.A. *et al.*) developed a Protein Truncation Test (PTT) to rapidly and easily screen for these truncation mutations. Some groups isolate mRNA and perform reverse transcription (RT)-PCR followed by coupled transcription/translation, while others perform PCR directly from genomic DNA. In either case, a T7 promoter is added upstream of the AUG start site to allow coupled expression in the TNT® Systems.

The PTT system is also useful for researchers studying rapidly mutating genes, such as those found in certain viruses (e.g., HIV, SIV). For example, a group studying the *nef* gene of Simian Immunodeficiency Virus used a simplified protocol that allowed the direct screening of ORFs in cloned PCR products from transformed bacterial colonies. This approach eliminated the need for plasmid preparations to screen for intact *nef* genes, which are required to maintain the high viral loads necessary for the progression to AIDS in SIV-infected macaques (see [Table 3](#), Switzer and Heneine).

Table 2. Protein:Protein, Protein:DNA or Protein:RNA Interactions.

Description	Reference
Determination of binding domain using immobilized GST-fusion deletion mutants and radiolabeled <i>in vitro</i> translated proteins. Also, 2D tryptic peptide mapping to compare <i>in vivo</i> and <i>in vitro</i> proteins. Characterization of antisera.	Kerr, L.D. et al. (1992) The proto-oncogene BCL-3 encodes an IkappaB protein. <i>Genes & Dev.</i> 6 , 2352.
Determination of binding domain using immobilized GST-fusion deletion mutants and radiolabeled <i>in vitro</i> translated proteins.	Sontag, E. et al. (1993) The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation <i>Cell</i> 75 , 887.

Detection of DNA-protein complex formation by EMSA. Co-translation of four proteins to reconstitute a complex detected by immunoprecipitation.	Sif, S. and Gilmore, T.D. (1993) NF-kappaB p100 is one of the high molecular weight proteins complexed with the v-Rel oncoprotein in transformed chicken spleen cells. <i>J. Virol.</i> 67 , 7612.
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Delineation of a transcription activation domain by EMSA of deletion mutants.	Palvimo, J.J. et al. (1993) Dominant negative regulation of trans-activation by the rat androgen receptor: Roles of the N-terminal domain and heterodimer formation. <i>Mol. Endo.</i> 7 , 1399.
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Characterization of antiserum using a GST-fusion protein to specifically block immunoprecipitation of a radiolabeled <i>in vitro</i> translated protein. Partial proteolytic mapping comparing <i>in vitro</i> to <i>in vivo</i> protein.	Gyuris, J. et al. (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. <i>Cell</i> 75 , 791.
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Detection of protein-protein interactions by immuno-precipitation of a purified protein with a radiolabeled <i>in vitro</i> translation protein.	Wu, J.Y. and Maniatis, T. (1993) Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. <i>Cell</i> 75 , 1061.
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Confirmation of ligand-binding region and analysis of mutants using EMSA and partial proteolytic mapping.	Kallio, P.K., Jänne, O.A., and Palvimo, J.J. (1994) Agonists, but not antagonists, alter the conformation of the hormone binding domain of androgen receptor. <i>Endocrinology</i> 134 , 998.
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Cooperative protein-DNA binding and identification of binding domains using truncation mutants with EMSA.	van Dijk, M.A. and Murre, C. (1994) extradenticle raises the DNA binding specificity of homeotic selector gene products. <i>Cell</i> 78 , 1.
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RNA:protein binding demonstrated by immunoprecipitation of wild-type or truncated epitope-tagged <i>in vitro</i> translated proteins mixed with radiolabeled RNA.	Lutz, C.S. and Alwine, J.C. (1994) Direct interaction of the U1 snRNP-A protein with the upstream efficiency element of the SV40 late polyadenylation signal. <i>Gen. Devel.</i> 8 , 576.
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DNA binding demonstrated by binding radiolabeled <i>in vitro</i> translated proteins to ss or dsDNA-cellulose, followed by SDS-PAGE.	Loh, L.C. et al. (1994) Sequence analysis and expression of the murine cytomegalovirus phosphoprotein pp50, a homolog of the human cytomegalovirus UL44 gene product. <i>Virology</i> 200 , 413.
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Mapping of protein binding sites by binding radiolabeled <i>in vitro</i> translated proteins from deletion mutants to immobilized GST-fusion protein.	Patrick, D.R. et al. (1994) Identification of a novel retinoblastoma gene product binding site on human papillomavirus type 16 E7 protein. <i>J. Biol. Chem</i> 269 , 6842.
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Characterization of transcription activation by binding of radiolabeled <i>in vitro</i> translated cellular transcription factors to immobilized GST-fusion protein.	Lukac, D.M., Manuppello, J.R. and Alwine, J.C. (1994) Transcriptional activation by the human cytomegalovirus immediate-early proteins: Requirements for simple promoter structures and interactions with multiple components of the transcription complex. <i>J. Virol.</i> 68 , 5184.
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Determination of protein interactions by binding of radiolabeled <i>in vitro</i> translated proteins to immobilized GST-fusion	Dyck, J.A. et al. (1994) A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. <i>Cell</i> 76 ,

<p>protein. <i>In vitro</i> verification of in vivo results (yeast 2-hybrid and double-labeling immunohistochemistry).</p>	<p>333.</p>

<p>Verification of <i>in vivo</i> results from yeast two-hybrid system by binding of radiolabeled <i>in vitro</i> translated proteins to immobilized GST-fusion protein.</p>	<p>Paroush, Z. et al. (1994) Groucho is required for <i>Drosophila</i> neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. <i>Cell</i> 79, 805.</p>

<p>Identification of the cellular protein binding to a transcriptional activator by cotranslation (2 DNA templates) followed by immunoprecipitation and EMSA.</p>	<p>Boyd, J.M. et al. (1994) Adenovirus E1B 19kDa and Bcl-2 proteins interact with a common set of cellular proteins. <i>Cell</i> 79, 341.</p>

<p>Identification of the cellular protein binding to a transcriptional activator by cotranslation (2 DNA templates) followed by immunoprecipitation and EMSA.</p>	<p>Waltzer, L. et al. (1994) The human Jkappa recombination signal sequence binding protein (RBP-Jkappa) targets the Epstein-Barr virus EBNA2 protein to its DNA responsive elements. <i>EMBO J.</i> 13, 5633.</p>

<p>Identification of a transcription factor binding to a promoter by EMSA and proteolytic clipping bandshift assay.</p>	<p>Laux, G. et al. (1994) The Spi-1/PU.1 and Spi-B ets family transcription factors and the recombination signal binding protein RBP-Jkappa interact with an Epstein-Barr virus nuclear antigen 2 responsive cis-element. <i>EMBO J.</i> 13, 5624.</p>

<p>Identification of DNA binding domains in light responsive gene promoters by EMSA, using PCR DNA templates to synthesize radiolabeled <i>in vitro</i> translation proteins. Demonstration of oligomer formation by chemical crosslinking of radiolabeled <i>in vitro</i> translated proteins.</p>	<p>Lam, E. (1995) Domain analysis of the plant DNA-binding protein GT1a: requirement of four putative alpha-helices for DNA binding and identification of a novel oligomerization region. <i>Mol. Cell Biol.</i> 15, 1014.</p>

<p>Identification of a DNA response element by EMSA and determination of binding constant by Scatchard analysis using radiolabeled <i>in vitro</i> translated repressor.</p>	<p>Lee, H. J. and Chang, C. (1995) Identification of human TR2 orphan receptor response element in the transcriptional initiation site of the Simian Virus 40 major late promoter. <i>J. Biol. Chem.</i> 270, 5434.</p>

<p>DNA binding and bending experiments using <i>in vitro</i> translated isoforms or deletion mutants of the steroid-thyroid-retinoid receptor.</p>	<p>McBroom, L.D.B., Flock, G. and Giguère, V. (1995) The nonconserved hinge region and distinct amino-terminal domains of the RORalpha orphan nuclear receptor isoforms are required for proper DNA bending and RORalpha-DNA interactions. <i>Mol. Cell Biol.</i> 15, 796.</p>

<p>Stoichiometric determination of transcription factors in complex and comparison to <i>in vivo</i> data using coimmunoprecipitation of radiolabeled <i>in vitro</i> translated proteins.</p>	<p>Ernst, M. K., Dunn, L.L. and Rice, N.R. (1995) The PEST-like sequence of IkappaBalph is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers. <i>Mol. Cell Biol.</i> 15, 872.</p>

It is important that expression of eukaryotic genes should be performed in an eukaryotic coupled system to avoid complicating artifacts which can arise if expressed in a prokaryotic coupled system. Many eukaryotic genes contain numerous AGGA (prokaryotic ribosomal binding sites) sequences 5' to internal methionines which result in the production of smaller internal initiation translation products.

Finally, although the current tests are limited to screening for truncated protein, two groups have suggested that all mutations (including

missense) can be detected using PTT in combination with a technique such as isoelectric focusing (see [Table 3](#), Roest *et al.* and Van Der Luut *et al.*). This technology promises to be exciting for the development of future genetic screening tests. An important candidate gene for the PTT screen is BRCA1, which has been shown to produce truncated proteins (see [Table 3](#), Hogervorst *et al.*).

We hope you continue to find new and useful application for the TNT® Systems. Once again, we encourage you to share your application with us so that we can include it in a future issue of *Promega Notes*.

Table 3. Analysis of Truncation Mutations in Cancer and Other Diseases.

Description	Reference
Delineation of APC gene region required for oligomerization of truncation mutants with wild-type APC gene products by <i>in vitro</i> expression and immunoprecipitation.	Su, L.K. et al. (1993) Association between wild type and mutant APC gene products. <i>Cancer Res.</i> 53 , 2728.
Screening for translation-terminating mutations in the dystrophin gene of Duchenne muscular dystrophy patients by RT-PCR followed by coupled transcription/translation.	Roest, P.A. et al. (1993) Protein truncation test (PTT) for rapid detection of translation-terminating mutations. <i>Human Mol. Gen.</i> 2 , 1719.
Screening for translation-termination in the APC gene using the Protein Truncation Test (PTT).	Roest, P.A. et al. (1993) Protein truncation test (PTT) to rapidly screen the DMD gene for translation terminating mutations. <i>Neuromuscular Disord.</i> 3 , 391.
Screening for translation-termination in the APC gene using the Protein Truncation Test (PTT).	Powell, S.M. et al. (1994) APC gene mutation in the utation cluster region are rare in esophageal cancers. <i>Gastroenterology</i> 107 , 1759.
Screening for translation-termination in the APC gene using the Protein Truncation Test (PTT).	Prosser, J. et al. (1994) APC mutation analysis by chemical cleavage of mismatch and a protein truncation assay in familial adenomatous polyposis. <i>Br. J. Cancer</i> 70 , 841.
Screening for translation-termination in the APC gene using the Protein Truncation Test (PTT).	Van Der Luut, R. et al. (1994) Rapid detection of translation-terminating mutations at the adenomatous polyposis coli (APC) gene by direct protein truncation test. <i>Genomics</i> 20 , 1.
Screening for translation-termination in the APC gene using the Protein Truncation Test (PTT).	Tarmin, L. et al. (1995) Adenomatous polyposis coli gene mutations in ulcerative colitis-associated dysplasias and cancers versus sporadic colon neoplasms. <i>Cancer Res.</i> 55 , 2035.
Rapid screening of open reading frames to demonstrate intact SIV nef gene by PCR amplification, cloning PCR product into a T-vector, and then a simplified colony screen using T7-tagged sense promoter primer to produce the correct T7-PCR product for use in the TNT® System.	Switzer, W.M. and Heneine, W. (1995) Rapid screening of open reading frames by protein synthesis with an <i>in vitro</i> transcription and translation assay. <i>BioTechniques</i> 18 , 244.
Screening for translation-terminating BRCA1 mutations found in breast and ovarian cancer by RT-PCR combined with PTT.	Hogervorst, F.B.L. et al. (1995) Rapid detection of BRCA1 mutations by the protein truncation test. <i>Nature Genetics</i> 10 , 208.

Table 4. Mutational Analysis.

Description	Reference
Rapid screening of a library containing all possible amino acids at codon 21 by random PCR-based mutagenesis followed by <i>in vitro</i> coupled transcription/translation. Safety assessment prior to large-scale <i>in vivo</i> culture of toxins.	Johnson, V.G. and Nicholls, P.J. (1994) Histidine 21 does not play a major role in diphtheria toxin catalysis. <i>J. Biol. Chem.</i> 269 , 4349.
Conformation of <i>in vivo</i> results that myristylation is the major determinant for membrane association, using [3H] myristate incorporated into <i>in vitro</i> translated proteins in a TNT® System from PCR-based wild-type and mutant gene constructs.	Busconi, L. and Michel, T. (1994) Endothelial nitric oxide synthase membrane targeting. Evidence against involvement of a specific myristate receptor. <i>J. Biol. Chem.</i> 269 , 25016.

Table 5. In vitro Evolution Studies.

Description	Reference
RNA-based evolution system coupled with a TNT® Translation System. A complex reaction where mRNA is amplified by reverse transcription and T7 RNA polymerase transcription together with translation.	Joyce, G.F. (1993) Evolution of catalytic function. <i>Pure & Appl. Chem.</i> 65 , 1205.

Table 6. Other Applications.

Description	Reference
Demonstration of antiserum specificity to GST-fusion protein.	Gyruis, J. et al. (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. <i>Cell</i> 75 , 791.
Demonstration of nuclear export using radiolabeled <i>in vitro</i> translated proteins after injected into <i>Xenopus</i> oocyte nuclei.	Schmidt-Zachmann, M.S. et al. (1993) Nuclear export of proteins: The role of nuclear retention. <i>Cell</i> 74 , 493.
Screening of epitope library with radioactive <i>in vitro</i> translated polypeptide probes. Also binding and functional effects of point mutant fusion proteins.	Pragnell, M. et al. (1993) Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha1-subunit. <i>Nature</i> 368 , 67.
Inhibition of <i>in vitro</i> synthesis of urokinase plasminogen activator receptor by a hammerhead ribozyme.	Karikó, K. et al. (1994) Lipofectin-aided cell delivery of ribozyme targeted to human urokinase receptor mRNA. <i>FEBS Letters</i> 352 , 41.
Increased synthesis of TNT® System when directly relative to mRNA (+/- cap) system. Analysis of translation initiation.	Herzog, E., Guilley, H. and Fritsch, C. (1995) Translation of the second gene of peanut clump virus RNA 2 occurs by leaky scanning <i>in vitro</i> . <i>Virology</i> 208 , 215.

References

1. Beckler, G.S. (1994) *Promega Notes* **47**, 20.
2. Beckler, G.S. and Hurst, R. (1993) *Promega Notes* **43**, 24.

Ordering Information

Product	Cat. #
TNT® T3 Coupled Reticulocyte Lysate System	L4950
TNT® T7 Coupled Reticulocyte Lysate System	L4610
TNT® SP6 Coupled Reticulocyte Lysate System	L4600
TNT® T7 Coupled Wheat Germ Extract System	L4140
TNT® SP6 Coupled Wheat Germ Extract System	L4130
TNT® T3 Coupled Wheat Germ Extract System	L4120

TNT® Lysates and Extracts are provided in 200µl aliquots and contain endogenous levels of all amino acids. Sufficient reagents are supplied for approximately 40 x 50µl coupled reactions.

Product	Cat. #
TNT® T7/T3 Coupled Reticulocyte Lysate System	L5010
TNT® T7/SP6 Coupled Reticulocyte Lysate System	L5020
TNT® T7/SP6 Coupled Wheat Germ Extract System	L5030
TNT® T7/T3 Coupled Wheat Germ Extract System	L5040

Each system contains sufficient reagents to perform approximately 20 x 50µl translation reactions with each of the polymerases.

Product	Cat. #
TNT® T7 Coupled Transcription/Translation System- Trial Size	L4611

Sufficient coupled reticulocyte lysate reagents included for 8 x 50µl translation reactions or 16 x 25µl reactions.

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