

Applications of the TNT[®] T7 Quick Coupled Transcription/Translation System



Gregory S. Beckler
Promega Corporation

Use of the TNT[®] T7 Quick System for the Detection of Translation-Terminating Mutations in a Protein Truncation Test

Matthew Cayouette
LabCorp of America
1912 Alexander Drive
Research Triangle Park, NC 27709

Analysis of Interactions between Proteinases and Serpins Expressed *in vitro* Using the TNT[®] T7 Quick System

Peter C. Turner
Department of Molecular Genetics and Microbiology
University of Florida
Gainesville, FL 32610

Applications of the TNT[®] T7 Quick Coupled Transcription/Translation System^(a)



By Gregory S. Beckler
Promega Corporation

The TNT[®] T7 Quick System^(b) offers researchers a one-tube, coupled transcription/translation method for eukaryotic *in vitro* protein expression (1). Coupled transcription/translation systems, first introduced by Promega in 1992, eliminate the laborious task of synthesizing large amounts of potentially labile mRNA (2,3) by allowing direct addition of DNA to the translation reaction. The TNT[®] T7 Quick System further simplifies the process by combining the T7 RNA Polymerase and other components in solution to form a single TNT[®] T7 Quick Master Mix. This Master Mix has been carefully adjusted to maximize both expression and fidelity for most gene constructs. The inclusion of Recombinant RNasin[®] Ribonuclease Inhibitor^(c) directly in the TNT[®] T7 Quick Master Mix protects against problems caused by the introduction of RNases carried over in the DNA solutions prepared using some miniprep protocols. In addition to circular plasmid DNA, linear DNA templates -- such as those generated by PCR^(d) amplification -- can also be used in the TNT[®] Systems (4).

The following articles highlight some of the advantages of the new TNT[®] T7 Quick System. The first article describes how the system simplifies and optimizes the ability to screen for truncation mutations in the protein truncation test (PTT; 5). Certain genetic diseases and cancers arise from mutations in particular genes. In some of these diseases, such as in familial adenomatous polyposis (FAP), the disease state is primarily due to the introduction of a stop codon into the reading frame, through either a point mutation, a frameshift mutation or a mutation in the mRNA splice sites. To detect the corresponding truncated gene product, either genomic DNA or mRNA can be amplified, with the 5' (sense) primer designed to introduce a T7 promoter upstream of the coding sequence. The resulting amplified DNA is added to TNT[®] T7 Quick reactions and the products are analyzed by SDS-polyacrylamide gel electrophoresis for the appearance of truncated proteins. The TNT[®] T7 Quick System makes it easier to screen many samples through reduced pipetting steps, simplifying many research applications.

The second article demonstrates the ability to further optimize the TNT[®] T7 Quick System through the addition of salts, such as magnesium acetate, to the standard reaction. While most genetic constructs tested so far in the TNT[®] T7 Quick System are efficiently expressed without further optimization, certain genetic constructs either benefit by, or actually require, additional magnesium or potassium for optimal expression. After successfully expressing several types of serpins (a class of proteinase inhibitors) the researchers were able to demonstrate the functionality of the expressed proteins. Radioactively labeled, *in vitro*-generated serpins were shown to be

functional inhibitors of cysteine and serine proteinases as demonstrated through efficient binding and subsequent cleavage.

References

1. Hurst, R. *et al.* (1996) *Promega Notes* **58**, 8.
2. Pelham, H.R.B. and Jackson, R.J. (1976) *Eur. J. Biochem.* **67**, 247.
3. Krieg, P. and Melton, D. (1984) *Nucl. Acids Res.* **12**, 7057.
4. *Bibliography of References using the TNT[®] Coupled Transcription/Translation Systems* #BL001, Promega Corporation.
5. Roest, P.A. *et al.* *Human Mol. Genetics* **2**, 1719.

^(a)For research use only; not for use in diagnostic procedures.

^(b)U.S. Pat. Nos. 5,324,637 and 5,492,817 have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and eukaryotic lysates.

^(c)U.S. Pat. No. 5,552,302 has been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor (PRI). Inhibitors of Angiogenin, which comprises a segment of human PRI, is the subject of U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687 assigned to the President and Fellows of Harvard College and exclusively licensed to Promega Corporation.

^(d)PCR is a patented process. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

Use of the TNT[®] T7 Quick System for the Detection of Translation-Terminating Mutations in a Protein Truncation Test

By Matthew Cayouette*

*LabCorp of America, 1912 Alexander Drive, Research Triangle Park, NC 27709, Telephone: 800-533-0567, Fax: 919-361-7797

Protein truncation tests are powerful research methods for detecting frameshift and nonsense mutations in many genes. In this update, we describe the use of the TNT[®] T7 Quick System^(a) to characterize proteins produced by normal and mutant forms of the APC gene, which is responsible for familial adenomatous polyposis (FAP).

^(a)U.S. Pat. Nos. 5,324,637 and 5,492,817 have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and eukaryotic lysates.

Introduction

Cancer is now recognized as part of a complex group of diseases which results from changes at the genetic level. Presently, most cancer-causing genes can be classified as either oncogenes or tumor suppressor genes. Simplistically, expression of the oncogene or inactivation of the tumor suppressor gene plays a key role in cancer development. Most cancers arise from the accumulation of a number of different genetic mutations, often in several genes. Interestingly, in some cancer genes, many of the inherited or acquired mutations are either nonsense or frameshift mutations resulting in the expression of a truncated protein which leads to development of the disease.

One such gene is the *APC* tumor suppressor gene which plays a key role in familial adenomatous polyposis (FAP), a syndrome characterized by the presence of multiple colorectal polyps. FAP affects about 1 in 5,000 individuals in the United States, and is a dominantly inherited disorder, with affected individuals inheriting a mutation in one copy of the *APC* gene located on chromosome 5q21 (1-3). The *APC* gene is 8.5kb, distributed over 15 exons and encodes a 2,843 amino acid protein (4). Exon 15 is quite large (6kb) and contains approximately 70% of the known mutations (5). Analysis of the *APC* gene has shown that over 80% of the mutations identified result in a truncated protein, making the protein truncation test (PTT) an effective research method for screening mutations.

A schematic diagram of the PTT is presented in [Figure 1](#) (6). A gene or gene segment is amplified from either genomic DNA via PCR^(b), or mRNA via RT-PCR^(b), with the sense primer containing signals for transcription (T7 promoter) and translation (optimal eukaryotic translation initiation sequence). *In vitro* transcription and translation of the PCR product in a TNT[®] reaction followed by SDS-polyacrylamide gel analysis identifies truncated proteins in the form of novel, faster-migrating bands. Modification of the procedure using the TNT[®] T7 Quick System allows for a quick and easy method to detect mutations by PTT. The TNT[®] T7 Quick System was developed for maximal full-length expression of most gene constructs while still retaining the option for further salt optimization for particular gene constructs (such as those containing viral 5' leader translation-enhancing sequences). In this report, we

describe the use of the TNT[®] T7 Quick System to detect proteins produced by normal and mutant forms of the *APC* gene.

^(b) PCR is a patented process. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

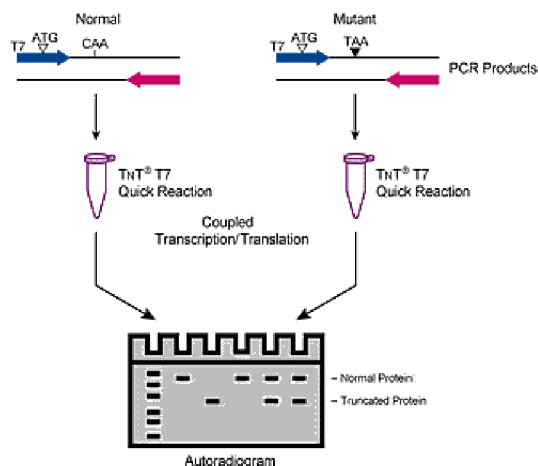


Figure 1. Schematic diagram of the Protein Truncation Test. This example illustrates normal and mutant forms of a gene segment amplified by PCR^(b). The mutation of a glutamine codon (CAA) to a stop codon (TAA) is shown.

Overview of the FAP Test Protocol

Lymphocytes were isolated from whole blood samples for RNA isolation. Total cellular RNA was isolated using a standard guanidinium isothiocyanate procedure (7) and DNA was isolated from whole blood (8). cDNA was prepared using Superscript[®] II Reverse Transcriptase (Life Technologies) in the presence of dNTPs and random hexamers. PCR was carried out using overlapping primer sets (for 5 segments) with the sense primer of each set containing signals for *in vitro* initiation of transcription and subsequent eukaryotic translation (6). The PCR products were analyzed by agarose gel electrophoresis (Figure 2).

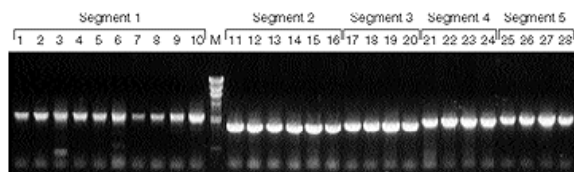


Figure 2. Analysis of PCR and RT-PCR products by agarose gel electrophoresis. The *APC* gene was amplified in five overlapping segments with sense primers containing sequences needed for *in vitro* transcription and eukaryotic translation. Aliquots of each PCR reaction were examined by electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide. Lanes 1-10: RT-PCR product of segment 1; Lanes 11-16: PCR product of segment 2; Lanes 17-20: PCR product of segment 3; Lanes 21-24: PCR product of segment 4; Lanes 25-28: PCR product of segment 5. Segments 2-5 were amplified from genomic DNA.

The amplified products were then subjected to *in vitro* transcription and translation. Five microliters of PCR product was added to 43 μ l of TNT[®] T7 Quick Lysate and 2 μ l of [³⁵S]methionine, and the reaction was incubated at 30°C for 90 minutes (9). A 3 μ l aliquot of this reaction was added to 20 μ l of reducing buffer and the sample was then mixed, boiled for 5 minutes, and loaded onto a 12.5% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed with acetic acid:methanol:water (10:30:60), soaked in an intensifying solution, dried and exposed to X-ray film.

Figure 3 presents the results of the PTT corresponding to segments 1-3 of the *APC* gene. Each specimen produces a germline band corresponding to the full length normal protein. A specimen heterozygous for a truncating mutation will also contain a second, novel band of similar intensity to the full length product. We observed that the segment 1 and segment 3 products produced only germline and background bands (Figure 3, Lanes 1-7 and Lanes 14-17, respectively). In contrast, analysis of the segment 2 products showed that truncated proteins were expressed in two related individuals (Figure 3, Lanes 9-10) and in two unrelated individuals (Lanes 11-12).

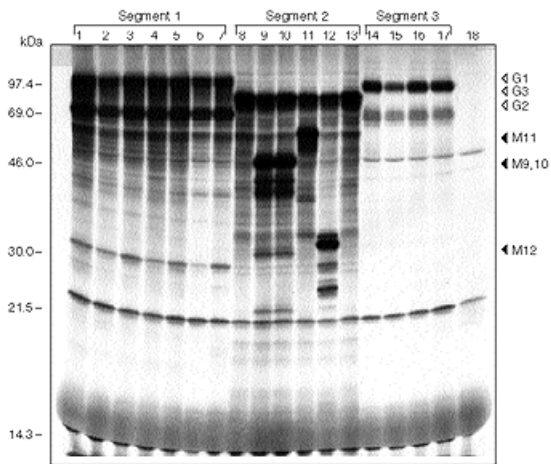


Figure 3. Protein truncation test results for *APC* gene segments 1-3. PCR products corresponding to segments 1-3 of the *APC* gene were used in separate TNT[®] T7 Quick reactions. The reaction products were separated on a 12.5% SDS-polyacrylamide gel, following which the gel was fixed, enhanced and dried. Data from an overnight exposure to X-ray film is shown. Lanes 1-7: segment 1 product; Lanes 8-13: segment 2 product; Lanes 14-17: segment 3 product. Lane 18 is a negative control TNT[®] T7 Quick reaction with no added template. The mobilities of germline and mutant products are indicated as follows: G1, G2 and G3 -- germline products for segments 1, 2 and 3, respectively; M9,10, M11 and M12 -- mutant products for segment 2 in Lanes 9, 10, 11 and 12, respectively.

Summary

The TNT[®] T7 Quick System facilitates the rapid and easy analysis of genes which have a high proportion of truncating mutations. Its ease of use makes the TNT[®] T7 Quick System an ideal reagent for performing protein truncation tests in genetic and epidemiological research.

References

1. Herrera, L. *et al.* (1986) *Am. J. Med. Genet.* **25**, 473.
2. Bodmer, W.F. *et al.* (1987) *Nature* **328**, 614.
3. Leppert, M. *et al.* (1987) *Science* **238**, 1411.
4. Van der Luijt, R. *et al.* (1996) *Genomics* **20**, 1.
5. Miyoshi *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4452.
6. Powell, S. *et al.* (1993) *N. Engl. J. Med.* **329**, 1982.
7. Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156.
8. Miller, S.A. *et al.* (1988) *Nucl. Acids Research* **16**, 1215.
9. TNT[®] T7 Quick Coupled Transcription/Translation Technical Manual #TM045, Promega Corporation.

Analysis of Interactions between Proteinases and Serpins Expressed *in vitro* Using the TNT[®] T7 Quick System

By Peter C. Turner*

*Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL 32610, Telephone: 352-392-9784, Fax: 352-392-3133, E-mail: turner@icbr.ifas.ufl.edu

The interactions between proteinases and natural inhibitory proteins (serpins) can be conveniently studied using radiolabeled serpins expressed in vitro with the TNT[®] T7 Quick Coupled Transcription/Translation System^(a). Most serpins can be efficiently expressed using the standard TNT[®] T7 Quick System protocol. However, some serpins, and presumably other proteins, are not expressed efficiently unless additional magnesium is included in the coupled transcription/translation reaction. We also demonstrate that serpins synthesized using the TNT[®] T7 Quick System are functionally active in protein-protein interactions.

^(a)U.S. Pat. Nos. 5,324,637 and 5,492,817 have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and

Introduction

Serpins (*serine proteinase inhibitors*) constitute a protein superfamily that includes inhibitors of serine proteinases involved in inflammation, blood coagulation and fibrinolysis. Poxviruses are the only known viruses that encode and express serpins. The cowpox virus *crmA* (cytokine response modifier A) protein is the prototypic poxvirus serpin (1) and has both anti-inflammatory and anti-apoptosis activity by virtue of its ability to inhibit the cysteine proteinase ICE (interleukin-1 beta converting enzyme) (1) and the serine proteinase granzyme B (2). Serpins expressed *in vitro* by coupled transcription/translation have been used to study protein conformational stability and complex formation between serpins and proteinases (3). Our laboratory has characterized the interactions between poxvirus serpin proteins (expressed using the TNT[®] T7 Quick System) and purified proteinases. Both complex formation and cleavage can be assessed by this method.

Expression of functionally active serpins

The serpins SPI-1 (4) and *crmA* (also known as SPI-2) (1) from the orthopoxviruses rabbitpox and cowpox virus, respectively, were efficiently expressed using the TNT[®] T7 Quick System without titrating Mg²⁺ (Figure 1A). However, we found that the swinepox virus SPI-7 (5) and the myxoma virus SERP2 (6) serpins were only synthesized *in vitro* when the magnesium level was increased by approximately 0.5mM (Figure 1B and 1C). SPI-7 expression was strongly dependent on the Mg²⁺ concentration, with optimum expression at 0.5mM additional magnesium ion (Figure 1B). The quantity of SERP2 protein synthesized was greatest at additional Mg²⁺ concentrations between 0.5mM and 1mM (Figure 1C). We obtained successful expression of serpins coupled to the T7 promoter using the plasmids pGEM^{®(b)}-5Zf(+), pALTER^{®(b)}-Ex1, pET-16b and derivatives of pTM1 (7).

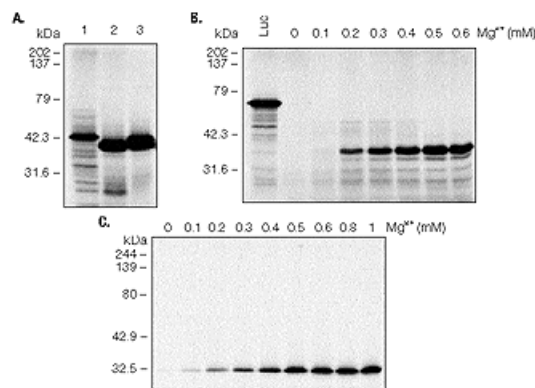


Figure 1. Magnesium-dependence of poxvirus serpin transcription/translation *in vitro*. Plasmid DNAs containing poxvirus serpin genes coupled to the T7 promoter were transcribed and translated using the TNT[®] T7 Quick System (9) with 20 μ Ci of [³⁵S]methionine per 20 μ l reaction. A 2 μ l aliquot of each TNT[®] reaction was analyzed by electrophoresis on 10% SDS-polyacrylamide gels followed by fluorography. **Panel A:** Expression of rabbitpox virus SPI-1 (Lane 1) and cowpox virus *crmA* (untagged and histidine-tagged; Lanes 2 and 3) was efficient using no additional magnesium. **Panel B:** The first lane shows expression of the luciferase control (Luc) provided with the TNT[®] System. The following lanes show expression of histidine-tagged swinepox SPI-7 protein from the vector pET-16b in the presence of additional Mg²⁺ (amounts added over the basal level are indicated above each lane). **Panel C:** Expression of the myxoma virus serpin SERP2 from the pGEM[®]-5Zf(+) Vector with varying levels of added Mg²⁺ (amounts added over the basal level are indicated above each lane).

^(b)U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

We used radiolabeled *crmA*, SPI-7 and SERP2 proteins synthesized using the TNT[®] T7 Quick System to test their interaction with cysteine and serine proteinases. [³⁵S]methionine-labeled *crmA* protein synthesized *in vitro* formed a stable 1:1 complex with ICE that could be detected by electrophoresis on native, non-denaturing protein gels followed by autoradiography (Figure 2A). The complex was not seen on denaturing SDS-polyacrylamide gels following treatment with reducing agents (Figure 2B). In addition, we observed that cleavage of *crmA* by ICE could be clearly detected in an SDS-polyacrylamide gel, and the amount of cleavage increased with increasing amounts of ICE (Figure 2B). Thus, the *crmA* protein expressed by the TNT[®] T7 Quick System interacts with ICE in the same way as does purified *crmA* protein from conventional expression systems (8). Folding of serpins produced in the TNT[®] T7 Quick System appears to be accurate as judged by migration on native gels and by the ability of the serpins to complex with or be cleaved by appropriate proteinases

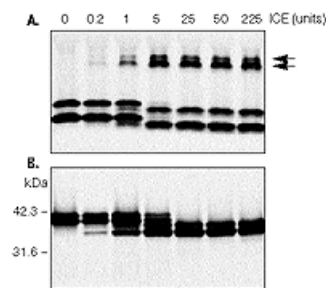


Figure 2. Radiolabeled *crmA* protein complexes with ICE and is cleaved by ICE. Histidine-tagged *crmA* protein was synthesized by *in vitro* transcription/translation using the TNT[®] T7 Quick System (9). The protein migrated as a doublet, presumably due to some internal initiation at the methionine codon following the His₁₀ tag at the N-terminus. Equal aliquots of [³⁵S]methionine-labeled, histidine-tagged *crmA* protein were incubated with varying amounts of ICE for 15 minutes at 37°C. The units of ICE are indicated above each lane. The samples were analyzed by electrophoresis on native acrylamide (**Panel A**) and SDS-polyacrylamide (**Panel B**) gels. Stable complexes between ICE and *crmA*, and between ICE and His-*crmA*, are indicated in the native gel (**Panel A**) by arrows.

Summary

The TNT[®] T7 Quick System provides efficient synthesis of proteins for use in studying protein-protein interactions. The system also allows optimization of salt conditions for constructs that do not express well under standard conditions. The one-tube reaction is convenient in that the proteins to be expressed do not need to be "tagged" and many site-directed mutant or chimeric serpins can be studied without requiring laborious purification.

References

1. Ray, C.A. *et al.* (1992) *Cell* **69**, 597.
2. Quan, L.T. *et al.* (1995) *J. Biol. Chem.* **270**, 10377.
3. Komiyama, T. *et al.* (1994) In: *Techniques in Protein Chemistry V*, Crabb, J.W., ed., San Diego Academic Press.
4. Ali, A.N. *et al.* (1994) *Virology* **202**, 305.
5. Massung, R.F., Jayarama V. and Moyer, R.W. (1993) *Virology* **197**, 511.
6. Petit, F. *et al.* (1996) *J. Virol.* **70**, 5860.
7. Moss, B. *et al.* (1990) *Nature* **348**, 91.
8. Komiyama T. *et al.* (1994) *J. Biol. Chem.* **269**, 19331.
9. TNT[®] T7 Quick Coupled Transcription/Translation Technical Manual #TM045, Promega Corporation.

Ordering Information

Product	Size	Cat.#
TNT [®] T7 Quick Coupled Transcription/Translation System		L1170
TNT [®] T7 Quick Coupled Transcription/Translation System, Trial Size		L1171
Potassium Chloride, 2.5M	200µl	L4591
Magnesium Acetate, 25mM	100µl	L4581

© 1996 Promega Corporation. All Rights Reserved.

pALTER, pGEM, RNasin and TNT are trademarks of Promega Corporation and are registered with the U.S. Patent and Trademark Office.

Superscript is a registered trademark of Life Technologies, Inc.