

A New TNT® System for Enhanced Expression of PCR DNA



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TNT® T7 Quick for PCR DNA^(a,b) is a rapid and convenient coupled transcription/translation system designed for expression from PCR-generated templates. The system does not require any post-amplification purification of the template and can produce up to 5 times more protein than other commercially available kits.

INTRODUCTION

With multiple genomes now completely sequenced and the human genome sequence to be completed within 3 years, an increasing amount of sequence information is being rapidly scrutinized to identify genes and their corresponding functions. Much of this research is moving towards understanding gene function in the cellular, tissue and natural context. This has led to rapid development of “knock-out/knock-in” systems (e.g., mice) and a growing number of cell-based assays. However, due to the limitations of each method (e.g., lethal knock-outs), multiple approaches are usually required to detect the gene product function. These can include rapid cell-free extract expression and assay methodologies. These functional strategies are usually intertwined with complementary nucleic acid sequence approaches such as DNA/RNA microarrays (chips) and bioinformatics. As more individual Single Nucleotide Polymorphisms (SNPs) are identified and we move into the age of personalized medicine, the biological significance of these genetic variations or polymorphisms can be studied. These studies can be performed using protein biochemical and in vitro expression methodologies for functional analysis.

ORIGINAL TNT® SYSTEMS

Promega revolutionized in vitro expression of cDNAs in 1992 with the introduction of the DNA-programmed coupled eukaryotic TNT® Expression Systems^(a,b,c,d) (2,3). Coupled one-step TNT® Systems offered advantages of speed, simplicity and increased efficiency over the existing two-step mRNA-based expression systems (4,5). The three original TNT® Systems (T7, T3 and SP6 Rabbit Reticulocyte Lysate) were optimized for expression of plasmid DNA. The benefits of using DNA in a coupled format promoted the development of a wide range of in vitro expression applications.

These applications include (see also references 1 and 6):

- gene construct verification
- determination of protein function
- detection of molecular interactions or environments (protein:protein, protein:DNA/RNA, ligand binding)
- detection of post-translational modifications
- detection of disease-causing mutations (PTT or IVSP)
- development of eukaryotic ribosomal display systems for cell-free protein evolution
- identify novel genes using In Vitro Expression Cloning (IVEC)

TNT® QUICK SYSTEMS

Although extremely successful, we realized that the original TNT® Systems could be further optimized for fidelity and expression of plasmid genetic constructs as well as improved in terms of speed and convenience. In 1996 and 1998, the TNT® T7 and SP6 Quick Coupled Transcription/Translation Systems^(a,b,c,d) were introduced (7). These systems combined the lysate extract, polymerase and re-optimized buffers into a simple “Master Mix”. The reduced number of pipetting steps and efficiency of the TNT® Quick Systems promoted rapid acceptance and have proven to be useful for a number of applications (8).

NEW SYSTEM: TNT® T7 QUICK FOR PCR DNA EXPRESSION

Increasingly, PCR-generated DNA has become the template of choice in TNT® System reactions. The ability to analyze PCR products in the TNT® System without additional purification would be highly advantageous. Therefore, we developed a new TNT® system optimized for transcription/translation expression from PCR products directly from the amplification reaction. The coupled reaction conditions for PCR-generated DNA are different than those for the optimal expression of plasmid DNA. Because this product is specifically designed for PCR product expression, transcription levels from plasmid DNA are reduced. Therefore, we recommend the use of the TNT® T7 Quick System for plasmid DNA expression.

PCR PRIMER DESIGN

We have successfully used several software programs: OLIGO® Primer Analysis software, PrimerSelect™ Expert Sequence Analysis software and Primer3 (available to the public at www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to assist in choosing primers for amplification. General recommendations for PCR conditions can be found in many sources including *PCR Protocols* (9) and the *Promega PCR Protocols and Reference Guide* (10). For assistance with optimization and troubleshooting amplification reactions, please refer to *Amplification Assistant*SM on our web site at: www.promega.com/amplification/.

EXPRESSION SEQUENCES

5' Primer: A T7 phage polymerase promoter is required for transcription initiation from the DNA template. If a T7 promoter is already present in a genetic construct 5' to the gene to be amplified (in the plasmid vector), primers can be designed to anneal to the vector upstream from the T7 promoter. Alternatively, a T7 promoter can be added directly to the amplification product through incorporation of the promoter sequence into the 5' oligonucleotide sequence. After choosing annealing sites for the complementary oligos, translation may require additional sequences on the 5' oligonucleotide. To ensure efficient translation initiation, a Kozak consen-

Table 1. Design of 5' Primers for PCR DNA Expression.

Restriction Site Sequence	Bacteriophage Promoter T7 Sequence	Spacer	Kozak Translation Initiation Sequence	Gene Target Sequence (match ~20)
GGATCC	TAATACGACTCACTATAGGG	AG	CCACCATG	N ₂₀
GGATCC	TAATACGACTCACTATAGGG	AG	CCACCATGG	N ₂₀
GGATCC	TAATACGACTCACTATAGG	AACAG	CCACCATG	N ₂₀
NNN	TAATACGACTCACTATAGG	AACAG	CCACCATGG	N ₂₀

This table includes examples of 5' primers incorporating a T7 promoter that have been successfully used for initiating efficient transcription/translation expression from PCR DNA. See Hogervorst *et al.* (15) for references of the primer sequences.

sequence should also be present. Typically, when amplifying from the 5' UTR of a target cDNA, the native Kozak sequence is present (11). However, when amplifying from an internal AUG, a Kozak consensus sequence must be added (see Table 1). Recent literature suggests that there is polymorphism within the Kozak sequence and that certain sequences show increased translational efficiency *in vitro* and *in vivo* (12). Additional sequence (6–10 nucleotides) added upstream of the T7 consensus sequence ensures efficient RNA polymerase binding and RNA production. See Table 1 for 5' primer sequence considerations.

3' Primer: The 3' primer typically matches the carboxy terminus of the gene or some position downstream from the termination codon and is generally 22–26 nucleotides in length. Some researchers have engineered an in-frame termination codon (e.g., TAA) into the 3' primer sequence if the native termination codon is not present. The added termination codon may be useful to achieve multiple rounds of translation by allowing the release of the ribosome from the peptidyl-tRNA.

DIRECT PCR DNA ADDITION

We have tested a number of PCR systems including: Promega's Access RT-PCR System[®] (Cat.# A1250), Roche Diagnostics' High Fidelity and Expand[™] Long Template PCR Systems, Gibco BRL's Platinum *Taq*, Stratagene's *Taq* Plus and PanVera's *Z-Taq*. We have determined that the DNA products from all these systems, as well as other amplification protocols, may be added directly to the TnT[®] T7 Quick for PCR DNA reaction without purification (data not shown). This direct addition is highly advantageous over competitor's two-step transcription/translation systems that require a chloroform extraction of PCR DNA prior to expression.

BENCHMARKING RESULTS

To demonstrate its increased efficiency for expressing PCR DNA, the new TnT[®] T7 Quick for PCR DNA system was compared to the original TnT[®] T7 Quick System and to two other *in vitro* expression systems. In contrast to the TnT[®] Systems, the *in vitro* systems supplied by other vendors are designed to be two-step reactions, with separate transcription and translation reactions.

Four different DNA templates were used in this study. Three different primer sets were used to amplify regions of the *APC* (*Adenomatous polyposis coli*) gene, involved in hereditary colon cancer (exon 15, Seg 2 and Seg 3) and the *BRCA1* gene, involved in hereditary breast cancer (exon 11, Seg 3), from genomic DNA (Cat.# G3041). These primer sets were derived from: "The Protein Truncation Test, A Manual of Methods and Primers" produced by Ed Edkins *et al.* (13). The Luciferase T7 Control DNA (Cat.# L4821) was used to test expression from plasmid DNA.

Significant improvements in expression levels and fidelity are observed in the TnT[®] T7 Quick for PCR DNA reactions (Figures 1 and 2, and Table 2). The new TnT[®] Quick system is up to 10 times more efficient than the original TnT[®] T7 Quick System for expression from PCR DNA. Faint undefinable protein bands from PCR products in the original TnT[®] system are now clear, strong, distinct bands with few secondary lower molecular weight products. Note the approximately 50% reduction in expression from plasmid DNA in the new TnT[®] T7 Quick system. Figure 1, Panels B and C, compares the new TnT[®] T7 Quick for PCR DNA to two different two-step transcription/translation expression systems. Vendor B reactions programmed with PCR DNA result in very weak protein bands with many secondary lower molecular weight products. The vendor A system produced stronger, more distinct protein bands than that of vendor B, yet these bands have only 6–25% the intensity of those generated from the new Promega system.

The *in vitro*-expressed proteins may alternatively be visualized through the incorporation of biotinylated lysine (14). Figure 2 is similar to the radioactive comparisons (Figure 1) but uses colorimetric or chemiluminescent detection of the biotinylated proteins. In the TnT[®] T7 Quick for PCR DNA reactions, Promega's Transcend[™] tRNA was used. Vendor A's analogous system was used in their two-step transcription/translation reactions. The TnT[®] T7 Quick for PCR DNA system produced substantially improved results over the original TnT[®] T7 Quick System as well as the other vendors' systems. Also, all the Transcend[™] System programmed reactions demonstrated more sensitive detection than vendor A's system (Figure 2, Panel B).

PCR-SPECIFIC APPLICATIONS

A number of TnT[®] System applications can be enhanced by, or require, amplified DNA. Some important examples include:

PTT: The Protein Truncation Test (PTT) is a rapid method for diagnostic detection of genetic diseases in a number of medically important genes, such as *BRCA1* (15), *APC* gene (16) and the large Duchenne Muscular Dystrophy gene (17). Blood is usually used as the source of either genomic DNA or mRNA for PCR or RT-PCR. TnT[®] analysis is used to identify mutations that result in truncated gene products. The main advantages of PTT include the ability to scan large (2–3kb) DNA/RNA segments quickly and that the method only detects truncation mutations, avoiding the fruitless

Table 2. Quantitation of Protein Bands by [³⁵S]Methionine Detection With a Molecular Dynamics PhosphorImager[®] Instrument.

Gene Product	TnT [®] T7 Quick	TnT [®] T7 Quick for PCR DNA	Vendor A	Vendor B
Seg 2 <i>APC</i>	7.0%	100%	25.0%	4.0%
Seg 3 <i>APC</i>	3.3%	100%	13.0%	2.2%
Seg 3 <i>BRCA1</i>	20.9%	100%	6.0%	7.8%
Control Plasmid	204.0%	100%	16.3%	6.1%

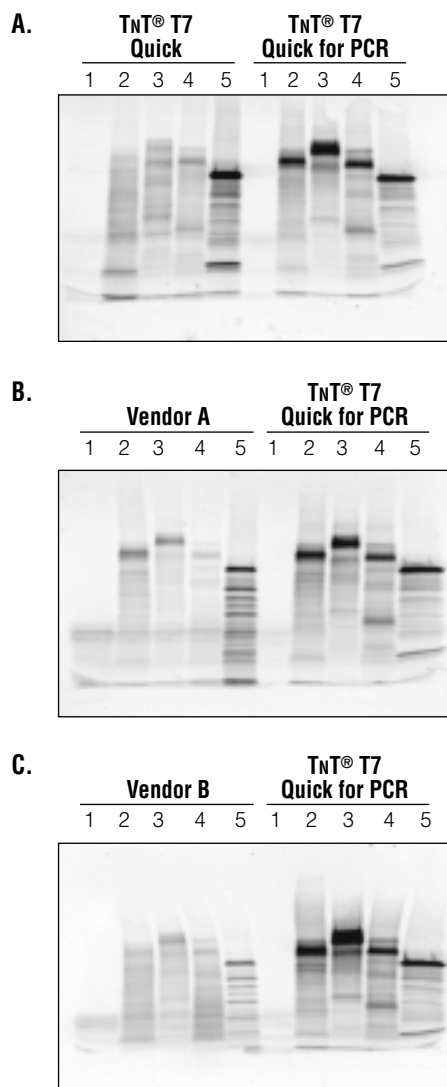


Figure 1. Radioactive [³⁵S]methionine comparison of the new TnT® T7 Quick for PCR DNA to the original TnT® T7 Quick System and Vendors A and B systems. PCR and plasmid DNA-programmed reactions were also compared. All linked transcription/translation reactions were performed using a single batch according to the vendors' instructions. Promega protocols (TM045 and TM235) were followed for the appropriate TnT® System reactions. Each reaction (1µl) was analyzed by SDS-PAGE on a 4–20% Novex™ gel. The separated proteins were transferred to a sheet of PVDF (Bio-Rad, Sequi-Blot) and then simultaneously exposed to a single PhosphorImager® cassette plate for 12 hours (a 2-hour exposure was adequate to visualize the products from the TnT® T7 Quick for PCR DNA reaction). Lanes 1 contain no DNA controls; lanes 2, *APC* Seg 2 PCR DNA; lanes 3, *APC* Seg 3 PCR DNA; lanes 4, *BRCA1* Seg 3 PCR DNA; lanes 5, the Luciferase T7 Control DNA.

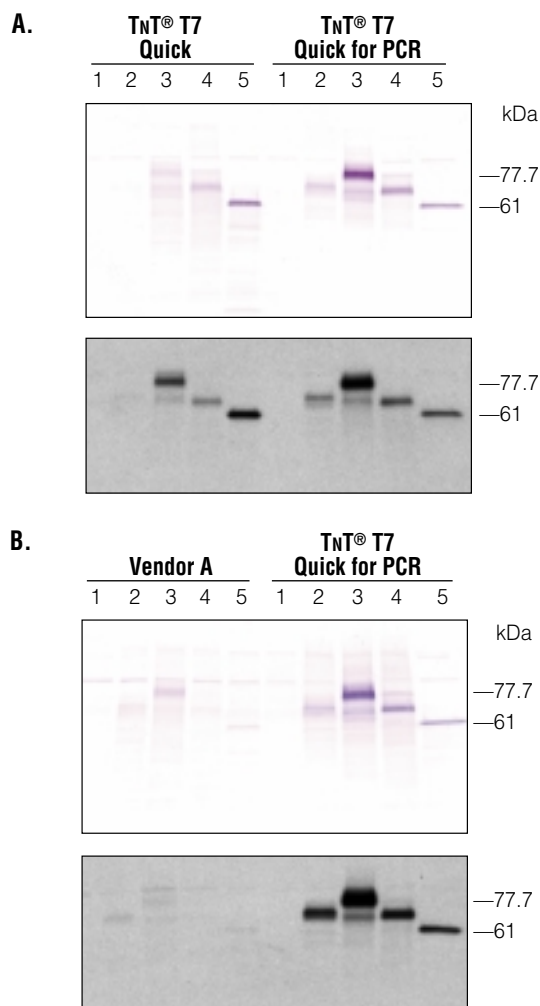


Figure 2. Non-Isotopic comparison of the new TnT® T7 Quick for PCR DNA expression to the original TnT® T7 Quick System (Panel A) and Vendor A (Panel B) using colorimetric (top) and chemiluminescence (bottom) detection. All linked transcription/translation reactions were performed according to the vendor's instructions. Each reaction contained 1µl of vendor-specific biotinylated-lysine-tRNA (Transcend™ tRNA for Promega's system). Gel analysis and Western blots were performed as described in the legend to Figure 1. The PVDF membranes were incubated with Western Blue® Stabilized Substrate (Cat.# S3841; colorimetric detection) and Transcend™ Chemiluminescent Substrates A and B (chemiluminescent detection). The chemiluminescence reaction exposure was for 10 minutes (a 2-minute exposure was more than adequate for detection of the TnT® T7 Quick for PCR DNA reactions). Lanes 1 contain the no DNA controls; lanes 2, *APC* Seg 2 PCR DNA; lanes 3, *APC* Seg 3 PCR DNA; lanes 4, *BRCA1* Seg 3 PCR DNA; lanes 5, the Luciferase T7 Control DNA.



evaluation of polymorphisms. Each gene requires a particular combination of PCR and RT-PCR with overlapping PCR expression products for optimal mutation detection (see Table 1 and reference 18 for primer considerations). For a comprehensive list of PTT publications, see reference 6.

IVEC: In vitro expression cloning (IVEC) is a systematic and broadly applicable method for the genetic characterization and biochemical screening of genes and cognate proteins (19). The original IVEC protocol first expresses small pools (50–100 clones) of cDNAs cloned as plasmids in TnT® System reactions (20). However, PCR can be used successfully to amplify gene pools from cDNA libraries by utilizing flanking vector sequences for primers (data not shown).

Ribosome Display: Ribosome display utilizes cell-free systems to transcribe a DNA library, translate the mRNA pools and, using a variety of techniques, retain the proteins and their encoding mRNAs attached to the ribosomes. As described, the protein-mRNA-ribosome complexes are screened for binding to a target, and the retained mRNA is amplified using RT-PCR. The resulting DNA is then used for further rounds of selection (1). The first eukaryotic-based system centered around a coupled transcription/translation rabbit reticulocyte system used antibody-ribosome-mRNA (ARM) complexes for rapid selection to monitor the evolution of antibody-combining sites (21). ARMs carrying single-chain (VH/K) binding fragments specific for progesterone have been selected using antigen-coupled magnetic beads. Selection simultaneously captured the genetic information as mRNA, making it possible to generate and amplify cDNA by single-step RT-PCR on the ribosome-bound mRNA for further manipulation. Using mutant libraries, antigen-binding ARMs were enriched by a factor of 10⁴–10⁵-fold in a single cycle (21), with further enrichment in repeated cycles. Such an approach has obvious potential for the selection of receptors or peptides from libraries.

CONCLUSION

The new TnT® T7 Quick for PCR DNA system represents the next generation of one-step, one-tube systems for in vitro expression of PCR-generated DNA. This system simplifies PCR-based functional gene analysis or identification of truncation mutations using PTT and can be used effectively in a non-isotopic format.

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Ordering Information

Product	Size	Cat.#
TnT® T7 Quick for PCR DNA	40 reactions	L5540

Related Products

Product	Size	Cat.#
Access RT-PCR System	20 reactions	A1260
	100 reactions	A1250
	500 reactions	A1280

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