A Tale of Four Systems

Optimized Gene Expression with the T7 Sample System

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Abstract

The T7 Sample System contains four unique in vitro translation systems for one-step gene expression from circular and linear plasmid DNA templates, as well as nonpurified PCR products. The system contains samples of TᴺT® T7 Quick for PCR DNA, TᴺT® T7 Quick Coupled Transcription/Translation System, TᴺT® T7 Coupled Wheat Germ Extract System and the E. coli *T7 S30 Extract System for Circular DNA. This article discusses the influence of vector and insert sequences on optimal gene expression in both eukaryotic and prokaryotic in vitro translation systems as shown using these four gene expression systems.*

> **The T7 Sample System is designed to allow the quick comparison of four different translation systems in a single experiment.**

Introduction

The use of cell-free systems for in vitro expression of proteins is rapidly expanding with applications in molecular diagnostics, high-throughput target/drug discovery and basic research. In vitro expression systems offer significant time savings over in vivo systems and are easy to use. The use of DNA in a coupled transcription/translation format allows for the development of many in vitro expression applications including protein truncation test (PTT) (1,2), clone verification, functional analysis, mutagenesis and domain mapping, ribosome display (3,4) and in vitro expression cloning (IVEC) (5). The T7 Sample System[a,b,c,d,e] (Cat.# L5900) contains samples of the four in vitro translation systems shown in Table 1. These systems are designed for determining the expression of a particular gene driven by the T7 RNA polymerase promoter. The TᴺT® T7 Quick for PCR DNA System[a,b,c] (Cat.# L5540) and the TᴺT® T7 Quick Coupled Transcription/Translation System[a,b,c,d,e] (Cat.# L1170) are based on Rabbit Reticulocyte Lysate.

The TᴺT® T7 Quick Coupled Transcription/Translation System (eukaryotic) and the S30 Extract System[d,e] (prokaryotic) allow for one-step gene expression from plasmid DNA templates or PCR products. The plasmid or vector backbones that the gene is cloned into can result in significant differences in gene expression in different systems. The standard TᴺT® T7 Quick Coupled Transcription/Translation System is optimized for supercoiled plasmid DNA expression. In comparison, the TᴺT® T7 Coupled Wheat Germ Extract System[a,b,d,e] (Cat.# L4140) is optimized for linearized or circular

Table 1. DNA Templates Recommended for Promega's in vitro Translation Systems.

In vitro Translation System	Circular Plasmid	PCR-Generated DNA	Linear DNA
TᴺT® T7 Quick for PCR DNA System	***	****	****
TᴺT® T7 Quick Coupled Transcription/Translation System	****	**	***
TᴺT® T7 Coupled Wheat Germ Extract System	**	**	****
E. coli T7 S30 Extract System	****	*	*

**** Recommended   *** Satisfactory   ** Possible   * Not Recommended

plasmid DNA containing a T7 transcription terminator. The *E. coli* T7 S30 Extract System for Circular DNA (Cat.# L1020) is optimized for circular plasmid DNA containing a ribosomal binding site (RBS); linear DNA will be degraded and therefore is not recommended.

The TᴺT® T7 Quick for PCR DNA System is optimized for the expression of linear, nonpurified PCR products. PCR-generated DNA is increasingly becoming the template of choice for the TᴺT® Coupled Transcription/Translation Systems, since it is easy to generate and use in comparison to the conventional method of cloning specific targets into plasmid vectors. The required T7 promoter can be amplified from a plasmid containing the gene of interest or can be designed into the forward primer of the PCR product (6). Table 1 summarizes the DNA template recommendations for the systems included in the T7 Sample System.

The T7 Sample System is designed to allow for the quick comparison of translation systems in a single experiment. In this article, we illustrate the influence of vector and insert sequences on expression levels with these different in vitro translation systems.

Template Considerations

Table 1 highlights the recommended template types for each of the translation systems provided in the T7 Sample System. Some systems perform well with many different types of templates.

During eukaryotic translation, the ribosome is thought to scan from the 5' end of the RNA, and translation may begin at the first AUG encountered. Thus any ATGs located in the template DNA upstream of the desired start codon of the insert sequence may cause premature initiation of translation. This premature initiation could then result in a shift of the reading frame and produce a protein larger than expected or result in decreased expression levels.

Vector Backbone	Insert	Expected Protein Size (kDa)	T7 Promoter	Kozak Consensus Sequence	Poly(A) Tail	Ribosomal Binding Site	T7 Terminator Sequence
pRL-null	*Renilla*	36	×	*	×	**	
phRL-null	Synthetic *Renilla*	36	×	*	×	**	
pCite® (1)	Luciferase	61	×	*			
pGEM®2	Luciferase	61	×	*	×	**	
pGEM®2 (linearized)	Luciferase	61	×	*		**	
pGEM®2	GFP	30	×	*	×	**	
pGEM®2 (linearized)	GFP	30	×	*		**	
pIVEX(2)	GFP	30	×	*		***	
TP2	Luciferase	61	×	*		***	×

(1) Novagen, (2) Roche
* Minimal Kozak Consensus Sequence   ** Putative RBS   *** Optimal RBS

Optimal eukaryotic translation initiation will occur if the ATG initiation codon is in a "Kozak consensus sequence" (7) and in the absence of any inhibiting RNA secondary structure. The optimal Kozak consensus sequence is: A/G CC<u>ATG</u>G (the start codon is underlined). Minimal Kozak consensus sequences are A/GXXATGX or XXXATGG.

Enhanced translation of proteins is also observed when using DNA containing a poly(A)+ sequence downstream of the gene of interest. Poly(A)+ sequences have been reported to affect the stability, and therefore the level of translation, of mRNA (8). We have observed that including a poly(A) tail in the template RNA can enhance expression when a perfect Kozak consensus sequence is absent (6). The pRL-null[f], phRL-null[f,g,h,i] and the pGEM®-2 vectors are examples of vectors that contain a minimal Kozak consensus sequence and a poly(A) tail.

There are several things to consider when engineering an insert sequence or vector for expression in an eukaryotic system: (i) the ATG start codon in the sequence should be the first ATG encountered following the transcription start site; (ii) ideally, following the T7 promoter, the ATG is included in a Kozak consensus sequence; (iii) a stop codon should be included at the 3′ terminus of the sequence; and (iv) a synthetic poly(A) tail (30 adenine residues) should be included following the stop codon. Additionally, vectors used in the TNT® T7 Wheat Germ System should contain a T7 terminator sequence. If a T7 terminator sequence is not present, we recommend linearizing the template DNA after the stop codon.

In prokaryotic systems, the selection of a start codon is almost invariably dependent on the presence of a ribosomal binding site (RBS), which contains a signal that marks the start of the reading frame. The part of the site recognized directly by the ribosome is called the Shine-Dalgarno sequence. Therefore, the binding mechanism used by the prokaryotic system does not recognize the ATGs upstream of the ATG start codon unless they happen to contain a properly positioned RBS.

For a prokaryotic system, such as the T7 S30 Extract System, the following should be considered when designing a vector or insert: (i) a T7 promoter should be present; (ii) a prokaryotic RBS should be located approximately 7bp upstream of the ATG start codon; and (iii) a stop codon should be included at the 3′ terminus of the sequence.

Influence of Vector on Expression

It is important to use the vector appropriate for your expression system. To demonstrate the effect of the vector backbone sequence, we cloned the luciferase and GFP genes into four different vectors (Table 2). Figure 1 compares expression levels using the systems included in the T7 Sample System.

Figure 1, Panel A, shows luciferase expression from the pCite®-luc and pGEM®2-luc vectors in the T7 Sample System. The pCite® vector, which contains an internal ribosome entry site (IRES) (9) is designed for enhanced in vitro translation using mammalian extracts, such as rabbit reticulocyte lysate. The luciferase protein expression from the pCite®-luc vector is lowest using the *E. coli* T7 S30 Extract System. This is expected, since the vector is optimized for mammalian expression and lacks a prokaryotic ribosomal binding site. Expression of the pCite®-luc and pGEM®2-luc vectors in the *E. coli* T7 S30 System results in a truncated protein due to translation initiation at an ATG within the coding sequence. Expression of the luciferase protein is highest in the TNT® T7 Quick for PCR lysate when compared to both the TNT® T7 Quick Coupled Transcription/Translation and T7 S30 Systems. This improved expression of the pCite®-luc template in the TNT® T7 Quick for PCR System is due to salt conditions optimal for PCR products.

The pGEM®2-luc vector contains a poly(A) tail as well as a putative RBS. Linearization of the vector and removal of the poly(A) tail by *Eco*ICR I digestion has little effect on the expression of the luciferase gene in the rabbit reticulocyte systems. In contrast, linearization significantly reduces the expression of the luciferase gene (and other eukaryotic genes) in the T7 S30 System (Figure 1,

Panel A). This reduction is a result of exonuclease activity in the T7 S30 Extract System. Thus, the T7 S30 System is recommended for circular templates only.

The presence of an optimal RBS can greatly increase expression in prokaryotic systems. For example, the TP2-luc vector is designed for use with prokaryotic systems and contains an optimal RBS at the correct ATG. As a result, the TP2-luc vector expression level is increased when used in the T7 S30 System (Figure 1, Panel A), unlike the pGEM®2-luc and pCite®-luc vectors, which produce truncated proteins. The production of the correctly sized protein is a result of a reduction of initiation at the internal ATG and increased initiation at the correct ATG using the T7 S30 System. However, TP2-luc expression in eukaryotic systems such as the TNT® T7 Quick Coupled System is reduced when compared to the expression of the pGEM®2-luc vector (Figure 1, Panel A).

In the TNT® T7 Coupled Wheat Germ Extract System, protein production levels will drop if the plasmid does not contain a T7 terminator or if the construct has not been linearized. Figure 1, Panel B, shows that expression levels of GFP increase when the circular pGEM®2-GFP vector is linearized with *Sac* I.

Gene Influence on Expression

To demonstrate the influence of the gene sequence on expression levels, we compared the expression of a synthetic (mammalian optimized) *Renilla* gene (phRL-null) and the unmodified *Renilla* gene (pRL-null) in the
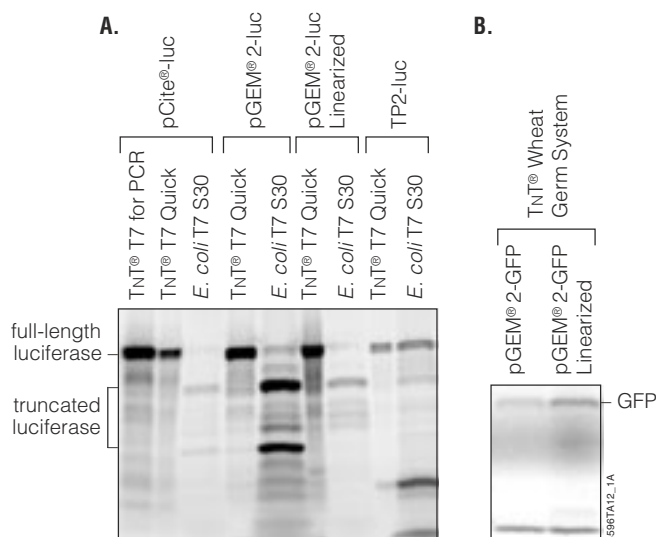
T7 TNT® Coupled Wheat Germ System (Figure 2, Panel A). Expression of the synthetic gene in the TNT® Coupled Wheat Germ System results in decreased expression when compared to the regular *Renilla* gene. However, in the mammalian TNT® T7 Quick Coupled System, the phRL-null plasmid shows better expression. We also compared the expression levels of the pGEM®2-luc and pGEM®2-GFP in the TNT® T7 Quick Coupled System (Figure 2, Panel B). The luciferase gene has a higher expression level than the GFP gene.

Figure 2, Panel B, shows a comparison of the pIVEX-GFP vector and the pGEM®2-GFP vector in the TNT® T7 Quick Coupled System. Because there are multiple ATGs in the GFP sequence upstream of the ATG start codon in the pIVEX plasmid, the GFP expresses as a larger protein in this vector.

Expression of PCR Products

We used four different PCR-generated templates to compare the efficiency of expression of PCR products in the TNT® T7 Quick for PCR DNA versus the TNT® T7 Quick System. The TNT® T7 Quick for PCR DNA expressed protein more efficiently than the TNT® T7 Quick System. PCR products that produce faint undefinable protein bands in the TNT® T7 Quick System are expressed as strong, distinct bands containing few secondary lower molecular weight products in the the TNT® T7 Quick for PCR DNA System (Figure 3). Because the TNT® T7 Quick for PCR DNA is designed specifically for nonpurified, PCR-generated DNA templates, expression levels from plasmid DNA might be reduced (10).
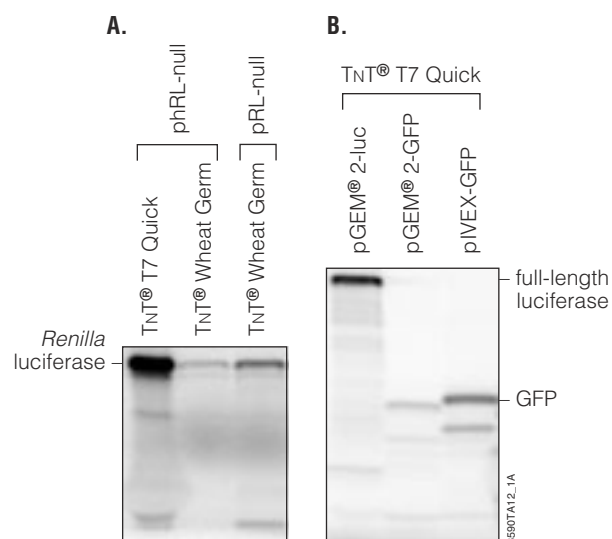

Figure 1. Vector influences on gene expression using the T7 Sample System. The in vitro coupled transcription/translation reactions were performed as described in Technical Bulletin #TB293. Reactions were performed in the presence of 2µl [35S]methionine, and 1µl of each reaction was loaded on a 4–20% Tris-Glycine gel (Invitrogen). The fixed/dried gel was exposed to a PhosphorImager® cassette for 24 hours and visualized using a Molecular Dynamics Storm® 860 PhosphorImager® instrument. **Panel A:** Luciferase expression using the pCite®-luc and TP2-luc vectors compared to the pGEM®2-luc vector in the indicated systems. **Panel B:** GFP expression using circular and linearized pGEM®2-GFP vector in the TNT® T7 Coupled Wheat Germ System.

Figure 2. Gene sequence influences gene expression using the T7 Sample System. The in vitro coupled transcription/translation reactions were performed as described in Figure 1. **Panel A:** Comparison of the expression of the *Renilla* (pRL-null) and synthetic *Renilla* (phRL-null) in the indicated systems. **Panel B:** Comparison of GFP expression from the pGEM®2-GFP and pIVEX-GFP vectors using the TNT® T7 Quick Coupled System. An ATG in the GFP sequence upstream of the ATG start codon in the pIVEX vector results in earlier initiation and thus a larger protein being expressed.
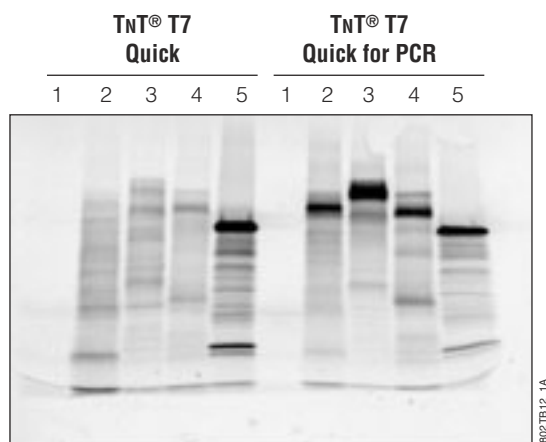
TNT® T7 Quick — Lanes 1 2 3 4 5
TNT® T7 Quick for PCR — Lanes 1 2 3 4 5

Figure 3. Comparison of TNT® T7 Quick Coupled and TNT® T7 Quick for PCR DNA Systems. All transcription/translation reactions were performed as described in Technical Bulletin #TB293. Each reaction (1µl) was analyzed by SDS-PAGE on a 4–20% 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). Lane 1, no DNA controls; lane 2, APC Seg 2 PCR fragment; lane 3, APC Seg 3 PCR fragment; lane 4, BRCA1 Seg 3 PCR fragment; lane 5, Luciferase T7 Control DNA[d]. The chemiluminescence reaction exposure was 10 minutes (a 2-minute exposure was more than adequate for detection of the TNT® T7 Quick for PCR DNA reactions).

Conclusions

The T7 Sample System can be used to determine which translation system will yield the highest level of expression for a particular gene and vector combination. Gene expression can be influenced by both vector and insert sequences. The presence of a Kozak consensus sequence, a poly(A) tail, an ATG start codon not proceeded by previous upstream ATGs and a stop codon at the 3′ terminus of the sequence are all recommended for optimal expression in eukaryotic systems. A T7 terminator should be present or the plasmid should be linearized when the TNT® T7 Wheat Germ System is used. For expression in T7 S30 prokaryotic systems, the presence of a RBS positioned near the initiating ATG and a stop codon at the 3′ terminus of the sequence are recommended.

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Protocols

◆ *T7 Sample System System Technical Bulletin* #TB293, Promega Corporation.
(www.promega.com/tbs/tb293/tb293.html)

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Ordering Information

Product	Size	Cat.#
T7 Sample System	1 each	L5900
TNT® T7 Quick for PCR DNA*	40 reactions	L5540
TNT® T7 Quick Coupled Transcription/Translation System*	40 reactions	L1170
TNT® T7 Coupled Wheat Germ Extract System*	40 reactions	L4140
E. coli T7 S30 Extract System for Circular DNA	30 reactions	L1130
Luciferase T7 Control DNA*	20µg	L4821
pRL-null Vector	20µg	E2271
phRL-null Vector	20µg	E6231

*For Laboratory Use.

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

[b] U.S. Pat. Nos. 5,324,637, 5,492,817, 5,665,563, Australian Pat. No. 660329 and other patents.

[c] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

[d] The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

[e] U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, 5,814,471, Australian Pat. No. 649289 and other patents and patents pending.

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