

# Characterization of TNT® T7 Quick for PCR DNA



By Natalie Betz, Ph.D.  
Promega Corporation

## ABSTRACT

*TNT® T7 Quick for PCR DNA is a rapid and convenient coupled transcription/translation system designed for expression of PCR-generated DNA templates. The system is quite robust and able to express a variety of proteins ranging in size from 10–150kDa. This article discusses the effects on expression of PCR product size and purity, AUG start codon context and the presence of a poly(A) tail.*

## INTRODUCTION

The importance of matching a genetic sequence with a particular protein function is becoming increasingly important as the amount of genome sequence information increases and becomes readily available to researchers. Multiple approaches are usually required to determine the function of any particular gene. Many of these studies can be performed using protein biochemistry and in vitro expression methodologies, such as in vitro translation.

The three original TNT® Systems<sup>(a,b,c,d)</sup> (T7, T3 and SP6 Rabbit Reticulocyte Lysate) were optimized for the expression of plasmid DNA. Using DNA in a coupled transcription/translation format allowed the development of many in vitro expression applications, including gene construct verification, determination of protein function, detection of molecular interactions, detection of post-translational modifications, detection of disease-causing mutations by protein truncation test (PTT) or in vitro synthesized protein assay (IVSP), in vitro expression cloning<sup>(e)</sup> (IVEC) and ribosome display systems for cell-free protein evolution (1).

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PCR<sup>(f)</sup>-generated DNA has increasingly become the template of choice for TNT® coupled transcription/translation reactions due to the ease of generating and using PCR products directly versus cloning specific targets by conventional means into plasmid vectors that contain genetic expression elements. TNT® T7 Quick for PCR DNA<sup>(a,b,g)</sup> was optimized for the expression of linear, unpurified PCR products. In comparison, the standard TNT® T7 Quick System<sup>(a,b,c,d,g)</sup> is optimized for plasmid DNA expression.

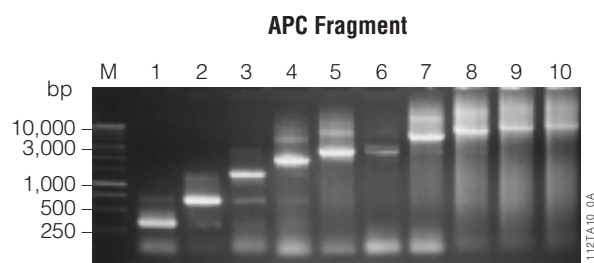
A T7 phage RNA polymerase promoter is required for transcription initiation from the PCR product DNA template. The T7 promoter may either be amplified from the plasmid vector containing the gene of interest, or the T7 promoter can be designed into the PCR product by addition to the forward or 5' amplification primer. To ensure efficient translation initiation, a Kozak consensus sequence should also be present. The reverse or 3' primer typically matches the carboxy terminus of the gene of interest and includes a stop codon (TAA, TGA or TAG). Reference 2 discusses effective primer design.

Earlier work using TNT® T7 Quick for PCR DNA demonstrated that the system does not require any post-amplification purification of the template DNA and can produce up to five times more protein than other commercially available kits (Technical Manual #TM235). The purpose of this set of experiments was to further characterize TNT® T7 Quick for PCR DNA. Reaction parameters investigated include effect of template size, PCR product purification, context of the ATG start codon and presence of a poly(A) tail.

## EFFECT OF PCR PRODUCT SIZE

The 8.7kb *APC* (adenomatous polyposis coli) locus (3,4) was amplified by RT-PCR from HeLa cell total RNA and cloned into the pSP64 Poly(A) Vector (Cat.# P1241). Restriction enzyme digestion and partial sequencing verified that the clone carried the *APC* cDNA.

To investigate the effect of PCR product size on expression in this system, increasingly larger portions of the *APC* cDNA were amplified (Figure 1) using a forward primer containing a T7 RNA polymerase promoter with an ATG start codon and a reverse primer containing a stop codon (Figure 3, primers A and D). The PCR products ranged in size from 377–8,786bp, which should express proteins between 10–280kDa (Table 1).



**Figure 1. Agarose gel of PCR products amplified from an 8.7kb *APC* cDNA template.** DNA fragment sizes are listed in Table 1. Lane M, 1kb DNA Ladder (Cat.# G5711).

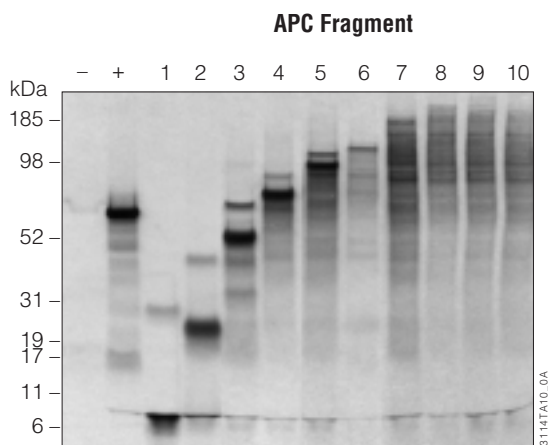
**Table 1. Expected Protein Size for Tested APC PCR Fragments.**

APC PCR Fragment #	PCR Product Size (bp)	Expected Protein Size (kDa)
1	377	10
2	707	20
3	1,376	40
4	2,039	60
5	2,669	80
6	3,335	100
7	5,000	150
8	6,668	200
9	8,000	240
10	8,786	280

Each APC PCR product was translated in vitro using TNT® T7 Quick for PCR DNA (5µl unpurified PCR product per reaction). The results are shown in Figure 2. TNT® T7 Quick for PCR DNA is able to translate proteins between 10–150kDa (lanes 1–7), although other smaller bands are visible in lane 6 (100kDa) and lane 7 (150kDa). No obvious bands at 200, 240 or 280kDa were detectable in lanes 8–10, but optimization of the translation reaction might allow expression of such large proteins (such as optimizing magnesium and potassium levels, lowering translation temperature, adding a poly(A) tail or increasing lysate concentration).

**EFFECT OF ATG START CODON CONTEXT AND PRESENCE OF POLY(A) TAIL**

Translation initiation occurs at an ATG (AUG) start codon present in the RNA template. For optimal translation initiation, the ATG codon is present in a Kozak consensus sequence. The presence of a poly(A) tail on the 3' end of a mRNA template also appears to enhance translation (see reference 5 for a review on translation initiation). To investigate the importance of these features when



**Figure 2. In vitro translation of APC PCR products using TNT® T7 Quick for PCR DNA.** The in vitro coupled transcription/translation reactions were performed as described in Technical Manual #TM235. Reactions were performed in the presence of 4µl [<sup>35</sup>S]methionine, and 2µl of each reaction were resolved per lane. All translation reactions were resolved on 4–12% Novex NuPAGE™ Tris-Bis gels in MES running buffer (Invitrogen). Negative control (-), no DNA template. Positive control (+), 100ng Luciferase T7 Control DNA<sup>(d)</sup> (Cat.# L4821).

expressing DNA templates in TNT® T7 Quick for PCR DNA, two different APC products were amplified (encoding the 20kDa or the 60kDa protein) using forward primers that contained either a perfect Kozak consensus sequence (Figure 3, primer A), the endogenous APC ATG start codon, which is not a perfect Kozak consensus sequence (primer B), or a minimal T7 promoter and ATG start codon (primer C). In addition, the 60kDa APC template amplification either used a reverse primer that ended in a stop codon (primer D) or a stop codon followed by a poly(T)<sub>30</sub> tail, which introduces a poly(A)<sub>30</sub> tail into the RNA transcript (primer E).

**Forward Primers**

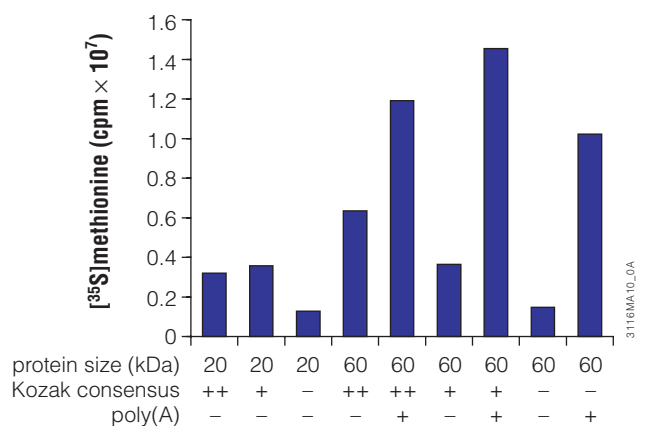
Spacer	T7 Promoter	Spacer	Start Codon	APC Sequence
A) 5'-GGATCC	TAATACGACTCACTATAGGG	AACAG	CCACCATGG	CTGCAGCTTATATGATC
B) 5'-GGATCC	TAATACGACTCACTATAGGG	GTAGC	CAAGGATGG	
C)	TAATACGACTCACTATAGGG		ATGG	

**Reverse Primers**

Poly(A) <sub>30</sub> Tail	Stop Codon	APC Sequence (specific for each PCR Product)
D) 5'-	TAA	APC Sequence (specific for each PCR Product)
E) 5'- T <sub>30</sub>	TAA	APC Sequence

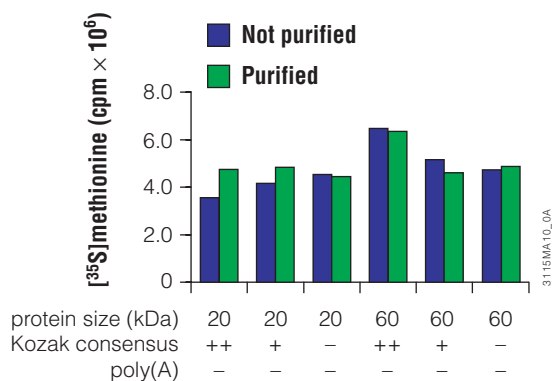
**Figure 3. Forward and reverse primers used for amplification of the APC PCR products.**

3113MA10\_GA



**Figure 4. Effect of ATG start codon context and presence of poly(A) tail on expression of APC PCR products with TnT® T7 Quick for PCR DNA.** The PCR products used as templates for coupled transcription/translation reactions varied by the size of the protein produced (20kDa or 60kDa); the Kozak context of the start codon (perfect Kozak sequence [++], imperfect Kozak sequence [+], or no Kozak sequence [-]); and presence (+) or absence (-) of a poly(A) tail. An equal number of template molecules ( $1.8 \times 10^{11}$ ) were used in each reaction.

An equal number of template molecules ( $1.8 \times 10^{11}$ ) were used in each transcription/translation reaction. The results are shown in Figure 4. The 20kDa APC product showed reduced expression when only a minimal T7 promoter plus ATG start codon were incorporated into the PCR product. Expression of the 60kDa product was reduced when the endogenous APC start codon was used instead of a perfect Kozak consensus sequence. This level of expression was further reduced when only a minimal T7 promoter and ATG start codon were used. The reduced level of expression exhibited when a perfect Kozak consensus sequence was absent could be compensated for by including a poly(A)<sub>30</sub> tail into the template RNA. The presence of a Kozak consensus sequence produces optimal expression, but a nonperfect start codon context can be compensated for by the addition of a poly(T)<sub>30</sub> tail into the PCR product template DNA.



**Figure 5. Effect of PCR product purification on expression in TnT® T7 Quick for PCR DNA.** PCR products (50μl) were used directly in the expression reaction or were purified using the Wizard® PCR Preps DNA Purification System (Cat.# A7170). The templates used were as described in Figure 4.

### EFFECT OF PCR PRODUCT PURIFICATION

Aliquots (50μl) of each APC PCR were purified using the Wizard® PCR Preps DNA Purification System<sup>(h)</sup> (Cat.# A7170 and A2180) and eluted in 50μl Nuclease-Free Water. Assuming 96–99% yield (Technical Bulletin #TB118), the PCR product concentration should be approximately equal before and after purification. Aliquots of either unpurified or purified APC PCR products (5μl) were then expressed in vitro using TnT® T7 Quick for PCR DNA. The amount of radioactivity in each band was quantitated by phosphorimaging. Figure 5 shows that, for the PCR products tested, purification is not necessary.

### CONCLUSIONS

The new TnT® T7 Quick for PCR DNA allows for the convenient expression of PCR products ranging from 350–5,000bp. Larger products can potentially be used with optimization of the translation reaction. In most cases, purification of the PCR products are not necessary. An ATG start codon within a Kozak consensus sequence or the presence of a poly(A) tail promote higher levels of expression.

## REFERENCES

1. *Bibliography of References Using the TNT® Coupled Transcription/Translation Systems* #BL001, Promega Corporation. ([www.promega.com/techserv/tntbib.html](http://www.promega.com/techserv/tntbib.html))
2. Beckler, G. *et al.* (2000) *Promega Notes* **74**, 10–13.
3. Kinzler, K.W. *et al.* (1991) *Science* **253**, 661–665.
4. Nishishio, I. *et al.* (1991) *Science* **253**, 665–669.
5. Sachs, A.B. and Varani, G. (2000) *Nat. Struct. Biol.* **7**, 356–361.

## PROTOCOLS

- ▶ *TNT® T7 Quick for PCR DNA Technical Manual* #TM235, Promega Corporation. ([www.promega.com/tbs/tm235/tm235.html](http://www.promega.com/tbs/tm235/tm235.html))
- ▶ *Wizard® PCR Preps DNA Purification System Technical Bulletin* #TB118, Promega Corporation. ([www.promega.com/tbs/tb118/tb118.html](http://www.promega.com/tbs/tb118/tb118.html))



NATALIE BETZ



## Ordering Information

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

## Related Products

Product	Size	Cat.#
Wizard® PCR Preps DNA Purification System	50 preps	A7170
	250 preps	A2180

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## PRODUCT BIBLIOGRAPHY

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608-274-4330  
1-800-356-9526  
techserv@promega.com