



Promega

Technical Bulletin

TnT[®] T7 Quick for PCR DNA

INSTRUCTIONS FOR USE OF PRODUCT L5540.



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TnT[®] T7 Quick for PCR DNA

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1. Description

TnT[®] T7 Quick for PCR DNA^(a,b,c) is a rapid, convenient, coupled transcription/translation system designed for optimum expression of PCR templates. For most PCR templates, the TnT[®] T7 Quick for PCR DNA reactions produce up to 5 times more protein than other commercially available kits. The PCR-generated DNA can be used directly from the amplification reaction (1). PCR products from amplification protocols and commercially available systems have been successfully tested (e.g., Access RT-PCR System [Cat.# A1250], GoTaq[®] DNA Polymerase [Cat.# M3001], Roche Diagnostics High Fidelity and Expand[™] Long Template PCR Systems, and Invitrogen Platinum Taq). This system will work with any other comparable PCR amplification scheme. The PCR products may be added directly to the TnT[®] T7 Quick for PCR DNA reaction without purification.

To use TnT[®] T7 Quick for PCR DNA, a PCR fragment containing a T7 promoter is added to the TnT[®] T7 PCR Quick Master Mix and incubated for 60–90 minutes at 30°C. The synthesized proteins then can be analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Section 6.B) followed by autoradiography or phosphorimaging.

For peer-reviewed articles citing use of this product, please visit:
www.promega.com/citations/

2. PCR DNA Template Considerations

The ability to directly analyze PCR products with the TnT[®] T7 Quick for PCR DNA system is highly advantageous. The quality of the results is dependent on the ability to obtain discrete, specific PCR products. The selection of primers is an important first step in this process. Many researchers now use computer programs to assist in choosing primers for amplification. Programs successfully used at Promega include OLIGO[®] and Primer Select expert sequence analysis software. It is recommended that each PCR reaction be optimized, as different thermostable DNA polymerases may require different reaction conditions. However, standard amplification protocols are often satisfactory. Commonly encountered problems include low or no yield of product, nonspecific amplification products and “primer-dimers,” which compete with the template for primer hybridization.

After choosing the complementary oligonucleotides, additional sequences may be needed in the 5' oligonucleotide. These include a phage polymerase promoter for transcription of the DNA template, as well as the Kozak consensus sequence (if not already present) to enable efficient translation of the RNA. Typically, when amplifying from the 5' UTR of a target cDNA, the native Kozak sequence is present (2). However, if translation will be initialized from an internal AUG, a Kozak consensus sequence should be added for optimal protein expression (Figure 1). Recent literature suggests that there is polymorphism within the Kozak sequence, and certain sequences show increased translation efficiency in vitro and in vivo (3). Finally, it is recommended that some additional nucleotides be added upstream of the T7 consensus sequence to ensure efficient RNA production. General recommendations for PCR conditions can be found in many sources including *PCR Protocols* (4). After amplification, it is important to analyze the samples by agarose gel electrophoresis to ensure that the correct product has been amplified and that no spurious bands are present. PCR-generated DNA fragments may be used directly in the TNT® T7 Quick for PCR DNA reaction without purification.

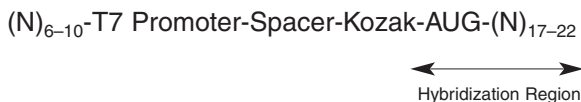


Figure 1. Design of 5' PCR Primer.

3. Product Components and Storage Conditions

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

Each system contains sufficient reagents to perform approximately 40 × 50µl translation reactions. Includes:

- 1.6ml TNT® T7 PCR Quick Master Mix (8 × 200µl)
- 50µl Methionine, 1mM
- 1.25ml Nuclease-Free Water

Storage and Stability: Store all components at -70°C. Product is sensitive to CO₂ (avoid prolonged exposure) and multiple freeze-thaw cycles, which may have an adverse effect on activity/performance.

4. General Considerations

4.A. Creating a Ribonuclease-Free Environment

To reduce the chance of RNase contamination, gloves should be worn when setting up experiments, and microcentrifuge tubes and pipette tips should be RNase-free. It is not necessary to add Recombinant RNasin® Ribonuclease Inhibitor to the TNT® T7 PCR Quick reactions to prevent degradation of RNA, as it is already present in the TNT® T7 PCR Quick Master Mix.

4.B. Handling of Lysate

Except for the actual transcription/translation incubation, all handling of the TNT® T7 PCR Quick Master Mix should be done at 4°C. Any unused Master Mix should be refrozen as soon as possible after thawing to minimize loss of translational activity (see Section 5.A, Note 2). Do not freeze-thaw the Master Mix more than two times.

5. Transcription/Translation Procedure

The following is a general guideline for setting up a transcription/translation reaction. Also provided are examples of standard reactions using [³⁵S]methionine (radioactive), Transcend™ Non-Radioactive Detection Systems or FluoroTect™ Green_{Lys} in vitro Translation Labeling System. Using the Transcend™ Systems, biotinylated lysine residues are incorporated into nascent proteins during translation. This biotinylated lysine is added to the transcription/translation reaction as a precharged ε-labeled, biotinylated lysine-tRNA complex (Transcend™ tRNA) rather than a free amino acid. Using the FluoroTect™ System, in vitro translation products are fluorescently labeled through the use of a modified charged lysine transfer RNA labeled with the fluorophore BODIPY®-FL. The fluorescent lysine is added to the translation reaction as a charged epsilon-labeled fluorescent lysine-tRNA complex (FluoroTect™ Green_{Lys} tRNA) rather than a free amino acid .

For more information on the Transcend™ Systems, request Technical Bulletin #TB182. For more information about the FluoroTect™ System, request Technical Bulletin #TB285. These documents may be requested from Promega Corporation and are also available on the Internet at: www.promega.com/tbs/

5.A. General Protocol for TNT® T7 Quick for PCR DNA Reactions

Materials to Be Supplied by the User

- radiolabeled amino acid (for radioactive detection), Transcend™ tRNA (Cat.# L5061) and Transcend™ Colorimetric (Cat.# L5070) or Chemiluminescent (Cat.# L5080) Translation Detection Systems (for non-radioactive detection), or FluoroTect™ GreenLys tRNA with the FluoroTect™ Green_{Lys} in vitro Translation Labeling System (L5001).
- Remove the reagents from -70°C. Rapidly thaw the TNT® T7 PCR Quick Master Mix by hand-warming and place on ice. The other components can be thawed at room temperature and then stored on ice.
 - Following the example below, assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube. After addition of all the components, gently mix by pipetting. If necessary, centrifuge briefly to return the reaction to the bottom of the tube. For additional information on performing a TNT® T7 PCR Quick reaction, see Notes 1–4 at the end of this section.
 - We recommend including a control reaction containing no added DNA. This reaction allows measurement of any background incorporation of labeled amino acids.

Note: The level of added Transcend™ tRNA can be increased (up to 4μl) to allow more sensitive detection of proteins that contain few lysines or are poorly expressed.

Components	Standard Reaction Using [³⁵ S]methionine	Standard Reaction Using Transcend™ tRNA	Standard Reaction Using FluoroTect™ Green _{Lys} tRNA
TNT® T7 PCR Quick Master Mix (see Note 3, below)	40μl	40μl	40μl
Methionine, 1mM (mix gently prior to use)	-	1μl	1μl
[³⁵ S]methionine (1,000Ci/mmol at 10mCi/ml) (see Note 1, below)	1–4μl	-	-
PCR-generated DNA template(s) (see Note 4, below)	2.5–5μl	2.5–5μl	2.5–5μl
Transcend™ Biotin-Lysyl-tRNA	-	1–2μl	-
FluoroTect™ Green _{Lys} tRNA	-	-	1–2μl
Nuclease-Free Water to a final volume of	50μl	50μl	50μl

- Incubate the reaction at 30°C for 60–90 minutes.
- Analyze the results of translation. Procedures for determination of radiolabel incorporation (Section 6.A) and SDS-PAGE analysis of translation products (Section 6.B) are provided.

5.A. General Protocol for TNT[®] T7 Quick for PCR DNA Reactions (continued)

Notes:

1. We recommend using a grade of [³⁵S]methionine, such as PerkinElmer EasyTag[™] L-[³⁵S]methionine (PerkinElmer Cat.# NEG709A), which does not cause the background labeling of the rabbit reticulocyte lysate 42kDa protein. Background labeling of the 42kDa protein can occur using other grades of label (5). Between 10–40μCi (1–4μl) of [³⁵S]methionine can be added to the TNT[®] Quick reactions, depending upon the balance between labeling efficiency and cost. For gene constructs that express well and contain several methionines, the 10μCi level (1μl) is sufficient for adequate detection.
2. Except for the transcription/translation incubation, all handling of the TNT[®] Quick System components should be done at 4°C or on ice. Optimum results are obtained when any unused Master Mix is quick-frozen with liquid nitrogen as soon as possible after thawing to minimize loss of translational activity.
3. Avoid adding calcium to the transcription/translation reaction. Calcium may reactivate the micrococcal nuclease used to destroy endogenous RNA in the Master Mix and result in degradation of DNA or RNA templates.
4. PCR-generated templates can be used directly from the amplification reaction. We recommend using 2.5–5μl from the amplification reaction, but up to 7μl can be used in a 50μl reaction. For PCR-generated DNA that has been purified following amplification, we recommend using 100–800ng of the purified product for each reaction.

6. Post-Translational Analysis

Materials to Be Supplied by the User

(Solution compositions are provided in Section 9.A.)

- 1M NaOH
- 25% TCA/2% casamino acids (Difco brand, Vitamin Assay Grade)
- 5% TCA
- Whatman GF/A glass fiber filter (Whatman Cat.# 1820 021)
- acetone
- Whatman 3MM filter paper
- 30% acrylamide solution
- separating gel 4X buffer
- stacking gel 4X buffer
- SDS sample buffer
- SDS polyacrylamide gels
- optional: precast polyacrylamide gels

6.A. Determination of Percent Incorporation of Radioactive Label

1. After the 50 μ l translation reaction is completed, remove 2 μ l from the reaction and add it to 98 μ l of 1M NaOH/2% H₂O₂.
2. Vortex briefly and incubate at 37°C for 10 minutes.
3. At the end of the incubation, add 900 μ l of ice-cold 25% TCA/2% casamino acids to precipitate the translation product. Incubate on ice for 30 minutes.
4. Wet a Whatman GF/A glass fiber filter with a small amount of ice-cold 5% TCA. Collect the precipitated translation product by vacuum filtering 250 μ l of the TCA reaction mix. Rinse the filter 3 times with 1–3ml of ice-cold 5% TCA. Rinse once with 1–3ml of acetone. Allow the filter to dry at room temperature or under a heat lamp for at least 10 minutes.
5. For determination of ³⁵S incorporation, put the filter in the appropriate scintillation mixture, invert to mix and count in a liquid scintillation counter.
6. To determine total counts present in the reaction, spot a 5 μ l aliquot of the TCA reaction mix directly onto a filter. Dry the filter for 10 minutes. Count in a liquid scintillation counter as in Step 5.
7. To determine background counts, remove 2 μ l from a 50 μ l translation reaction containing no DNA and proceed as described in Steps 1–5.
8. Perform the following calculation to determine percent incorporation:

$$\frac{\text{cpm of washed filter (Step 5)}}{\text{cpm of unwashed filter (Step 6)} \times 50} \times 100 = \text{percent incorporation}$$

9. Perform the following calculation to determine the fold stimulation over background:

$$\frac{\text{cpm of washed filter (Step 5)}}{\text{cpm of "no DNA control reaction" filter (Step 7)}} = \text{fold stimulation}$$

6.B. Denaturing Gel Analysis of Translation Products

For information on preparation of SDS-polyacrylamide gels and separation of proteins by electrophoresis, please see reference 1.

Alternatively, precast polyacrylamide gels are available from a number of manufacturers. For protein analysis, Invitrogen Corporation and Bio-Rad Laboratories, Inc., offer a variety of precast mini-gels, which are compatible with their vertical electrophoresis and blotter systems. These companies offer Tris-Glycine, Tricine and Bis-Tris gels for resolution of proteins under different conditions and over a broad spectrum of protein sizes. The Invitrogen Novex® 4–20% Tris-Glycine gradient gels (Cat.# EC6025 or EC60355) and the Bio-Rad Ready Gel 4–20% Tris-Glycine Gel, 10-well (Cat.# 161-0903) are convenient for resolving proteins over a wide range of molecular weights. In addition to convenience and safety, precast gels provide consistent results.

1. Once the 50 μ l translation reaction is complete (or at any desired timepoint), remove a 1–2 μ l aliquot and add it to 20 μ l of SDS sample buffer. The remainder of the reaction may be stored at –20°C.
2. Cap the tube and heat at 100°C for 2 minutes to denature the proteins. This may cause protein aggregation. Incubation at a lower temperature (e.g., 20 minutes at 60°C, 10 minutes at 70°C or 3–4 minutes at 80–85°C) may be more appropriate.
3. The cooled denatured sample then can be loaded onto an SDS-polyacrylamide gel. It is not necessary to separate labeled polypeptides from free amino acids by acetone precipitation.
4. Typically, electrophoresis is performed at a constant current of 15mA in the stacking gel and 30mA in the separating gel (or 30mA for a gradient gel). Electrophoresis is usually performed until the bromophenol blue dye has run off the bottom of the gel. Disposal of unincorporated label may be easier if the gel is stopped while the dye front remains in the gel, as the dye front also contains unincorporated labeled amino acids. If transferring the gel to a membrane filter for Western blotting or using phosphorimaging for visualization, proceed to Step 7.

5. Place the polyacrylamide gel in a plastic box and cover the gel with fixing solution (as prepared in Section 9.A) for 30 minutes. Agitate slowly on an orbital shaker. Pour off the fixing solution. Proceed to Step 6 (gel drying prior to film exposure).

Optional: Labeled protein bands in gels may be visualized by autoradiography or fluorography. Fluorography dramatically increases the sensitivity of detection of ^{35}S -, ^{14}C - and ^3H -labeled proteins and is recommended for analysis of in vitro translation products. The increased detection sensitivity of fluorography is obtained by infusing an organic scintillant into the gel. The scintillant converts the emitted energy of the isotope to visible light and increases the proportion of energy that may be detected by X-ray film. Commercial reagents, such as Amplify[®] Reagent (GE Healthcare Bio-sciences), can be used for fluorographic enhancement of signal. Alternatively, the fixed gel can be exposed to a phosphorimaging screen. These systems provide greater sensitivity, greater speed and the ability to quantitate the radioactive bands.

6. Dry the gel before exposure to film as follows: Soak the gel in 7% acetic acid, 7% methanol, 10% glycerol for 5 minutes to prevent the gel from cracking during drying. Place the gel on a sheet of Whatman 3MM filter paper, cover with plastic wrap and dry at 80°C for 30–90 minutes under a vacuum using a conventional gel dryer; dry completely. The gel also may be dried overnight using the Gel Drying Kit (Cat.# V7120). To decrease the likelihood of cracking gradient gels, dry them with the wells pointing down. Expose the gel on Kodak X-OMAT[®] AR film for 1–6 hours at -70°C (with fluorography) or 6–15 hours at room temperature (with autoradiography).
7. For analysis of proteins by Western blot or phosphorimaging, transfer (immobilize) the protein from the gel onto nitrocellulose or PVDF membrane (6,7). Detailed procedures for electrophoretic blotting usually are included with commercial devices and can be found in references 6, 8, 9 and 10. A general discussion of Western blotting with PVDF membranes is found in reference 11. PVDF membranes must be pre-wet in methanol or ethanol before equilibrating in transfer buffer. The blot then may be subjected to immunodetection analysis or phosphorimaging. For more information, refer to the *Protocols and Applications Guide*, Online Edition (12).

Note: When detecting proteins by phosphorimaging, transferring the proteins to a membrane sharpens the bands.

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
Low or inefficient protein synthesis	Calcium is present in the translation reaction. Avoid adding calcium to the translation reaction. Calcium may reactivate the micrococcal nuclease used to destroy endogenous mRNA in the lysate and result in degradation of the DNA or mRNA template.
	Ethanol is present in the reaction. Residual ethanol should be removed from preparations of PCR-generated DNA.
	Incubation of the reaction at 37°C causes decreased protein synthesis. Incubate the reaction at 30°C.
	No PCR DNA product. Check the PCR products on an agarose gel to be sure that the correct PCR product is present. If the correct product is not present, see Section 2.
Unexpected bands are present at higher molecular weights	Denaturing temperature is too high. Denature sample at a lower temperature (e.g., 60–80°C) for 10–15 minutes.
Unexpected bands are present on the gel	Proteolysis of translation product. Add protease inhibitors, such as α 2-macroglobulin, leupeptin or chymostatin (0.5–1 μ g/ml).
	More than one peptide is translated from the template. Leaky scanning for translation initiation can result in translation initiating at internal methionines. Fidelity may be increased by optimizing the Mg ²⁺ or K ⁺ concentration (13).
	The [³⁵ S]methionine used is not of translational grade or is beyond its expiration date. We recommend PerkinElmer EasyTag™ L-[³⁵ S]-methionine (PerkinElmer Cat.# NEG709A) to avoid this 42kDa band.
	Globin may appear on the autoradiogram or stained gel. Globin may show on a stained gel and occasionally as a faint image on the autoradiogram. It appears as a broad band migrating at 10–15kDa.

Symptoms	Causes and Comments
Unexpected bands are present on the gel (continued)	Aminoacyl tRNAs may produce background bands (~25kDa). Add RNase A to the lysate reaction (after completion) to a final concentration of 0.2mg/ml. Incubate for 5 minutes at 30°C.
	Oxidized β -mercaptoethanol is present or not enough SDS in the loading buffer. Use a loading buffer that contains 2% SDS and 100mM DTT.
There is smearing on the gel	Gel not clean. Gel must be washed before placing on film. Once electrophoresis is complete, soak the gel in either a standard Coomassie® destaining solution (50% methanol, 7.5% glacial acetic acid) or in water for 15-30 minutes prior to drying.
	Too much protein loaded on the gel. Check the amount of samples loaded on the gel and the amount of loading buffer. Too much protein loaded on the gel can cause smearing.
	Acrylamide concentration in the gel is too low. Acrylamide concentration can be increased to 12%.

8. References

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9. Appendix

9.A. Composition of Buffers and Solutions

acrylamide solution, 30%

30g acrylamide
0.8g bisacrylamide

Add water to a final volume of 100ml. Store at 4°C.

fixing solution

50% methanol
10% glacial acetic acid
40% water

1X SDS gel-loading buffer

50mM Tris-HCl (pH 6.8)
2% SDS
0.1% bromophenol blue
10% glycerol
100mM dithiothreitol

1X SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before the buffer is used.

SDS polyacrylamide running 10X buffer

30g Tris base
144g glycine
100ml 10% SDS

Add deionized water to a final volume of 1 liter. Store at room temperature.

separating gel 4X buffer

18.17g Tris base
4ml 10% SDS

Bring the volume to approximately 80ml with deionized water. Adjust to pH 8.8 with 12N HCl and add deionized water to a final volume of 100ml. Store at room temperature.

stacking gel 4X buffer

6.06g Tris base
4ml 10% SDS

Bring the volume to approximately 80ml with deionized water. Adjust to pH 6.8 with 12N HCl and add deionized water to a final volume of 100ml. Store at room temperature.

9.B. Related Products

TNT® Quick Coupled Transcription/Translation Systems

Product	Size	Cat.#
TNT® T7 Quick Coupled Transcription/Translation System	40 × 50µl reactions	L1170
TNT® T7 Quick Coupled Transcription/Translation System, Trial Size	5 × 50µl reactions	L1171
TNT® SP6 Quick Coupled Transcription/Translation System	40 × 50µl reactions	L2080
TNT® SP6 Quick Coupled Transcription/Translation System, Trial Size	5 × 50µl reactions	L2081

TNT® Coupled Reticulocyte Lysate Systems

Product	Size	Cat.#
TNT® SP6 Coupled Reticulocyte Lysate System	40 × 50µl reactions	L4600
TNT® SP6 Coupled Reticulocyte Lysate System, Trial Size	8 × 50µl reactions	L4601
TNT® T7 Coupled Reticulocyte Lysate System	40 × 50µl reactions	L4610
TNT® T7 Coupled Reticulocyte Lysate System, Trial Size	8 × 50µl reactions	L4611
TNT® T3 Coupled Reticulocyte Lysate System	40 × 50µl reactions	L4950
TNT® T7/T3 Coupled Reticulocyte Lysate System	40 × 50µl reactions	L5010
TNT® T7/SP6 Coupled Reticulocyte Lysate System	40 × 50µl reactions	L5020

Rabbit Reticulocyte Lysate

Product	Size	Cat.#
Rabbit Reticulocyte Lysate System, Nuclease-Treated*	5 × 200µl	L4960
Rabbit Reticulocyte Lysate System, Untreated	1ml	L4151

Bulk Rabbit Reticulocyte Lysate is available from Promega.

Flexi® Rabbit Reticulocyte Lysate System

Product	Size	Cat.#
Flexi® Rabbit Reticulocyte Lysate System	5 × 200µl	L4540

Bulk Flexi® Rabbit Reticulocyte Lysate is available from Promega.

Amino Acid Mixtures

Product	Size	Cat.#
Amino Acid Mixture Minus Leucine	175µl	L9951
Amino Acid Mixture Minus Methionine	175µl	L9961
Amino Acid Mixture Minus Cysteine	175µl	L4471
Amino Acid Mixture, Complete	175µl	L4461
Amino Acid Mixture Minus Methionine and Cysteine	175µl	L5511

Reverse Transcription

Product	Size	Cat.#
Improm-II™ Reverse Transcription System	100 reactions	A3800
Access RT-PCR System	100 reactions	A1250
	500 reactions	A1280
Access RT-PCR Introductory System	20 reactions	A1260

Transcend™ Non-Radioactive Translation Detection Systems

Product	Cat.#
Transcend™ Colorimetric Translation Detection System	L5070

Each system contains sufficient reagents to label 30 × 50µl translation reactions and perform colorimetric detection of biotinylated proteins on 6 blots (5.5 × 7cm) using Streptavidin-AP Conjugate and Western Blue® Substrate.

Product	Cat.#
Transcend™ Chemiluminescent Translation Detection System	L5080

Each system contains sufficient reagents to label 30 × 50µl translation reactions and perform chemiluminescent detection of biotinylated proteins on 6 blots (5.5 × 7cm) using Streptavidin-AP Conjugate and Transcend™ Chemiluminescent Substrate.

Product	Size	Cat.#
Transcend™ Biotinylated tRNA	30µl	L5061

Thirty microliters of Transcend™ Biotinylated tRNA is sufficient for 30 × 50µl translation reactions.

FluoroTect™ Green_{Lys} in vitro Translation Labeling System

Product	Size	Cat.#
FluoroTect™ Green _{Lys} in vitro Translation Labeling System	40 reactions	L5001

^(a)U.S. Pat. Nos. 5,324,637 and 5,492,817, Australian Pat. No. 660329 and Japanese Pat. No. 2904583.

^(b)U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217, Australian Pat. No. 646803 and Japanese Pat. Nos. 3009458 and 3366596.

^(c)Any use of the product for diagnostics requiring clearance or approval by the FDA may require a license under Mayo Clinic U.S. Pat. Nos. 6,027,913.

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