TnT® Coupled Wheat Germ Extract Systems

Technical Bulletin No. 165

INSTRUCTIONS FOR USE OF PRODUCTS L4120, L4130, L4140, L5030, AND L5040.
PLEASE DISCARD PREVIOUS VERSIONS.
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I. Description

The TNT® Coupled Wheat Germ Extract Systems[a,b,c,d] offer researchers an alternative for eukaryotic in vitro translation: a one-tube, coupled transcription/translation system. The TNT® Extract Systems greatly simplify the process and reduce the time required to obtain in vitro translation results (Figure 1). Standard wheat germ extract translations (1) commonly use RNA synthesized in vitro (2) from SP6, T3 or T7 RNA polymerase promoters. This entire process requires separate reactions with several steps between each reaction. The TNT® Extracts bypass many of these steps by incorporating transcription directly in the translation mix. Additionally, the TNT® Extract reactions often produce significantly more protein (2- to 6-fold) in a 1.5-hour reaction than do standard in vitro wheat germ extract translations using RNA templates.

Potential applications of the TNT® Coupled Wheat Germ Extract Systems include:

- Truncation mutation analysis (e.g., the Protein Truncation Test (PTT))
- Drug screening (affecting translation rates)
- Mutation detection and analysis (i.e., enzyme kinetics)
- Protein-protein interactions (using GST fusion proteins)
- Immunoprecipitation of protein complexes
- Protein dimerization assays
- Ligand-binding region determination/confirmation/competition assays
- Protein structure analysis
- Electrophoretic mobility shift assays (EMSAs) for DNA-protein interactions
- DNA footprinting and protein crosslinking studies
- Protein-RNA binding assays
- Post-translational modification tests
- In vitro expression cloning (functional genomics) (3)
- Verification/characterization of cloned gene products

Do Not use extracts, lysates, polymerases or buffers from other lots or systems. Use of reagents or reagent combinations other than those verified for the TNT® Coupled Wheat Germ Extract Systems may result in suboptimal coupled transcription/translation.

The TNT® Wheat Germ Extract Systems are available in five configurations for transcription and translation of genes cloned downstream from the SP6, T3 or T7 RNA polymerase promoter. With this system, a 50µl reaction is programmed with 0.2–2µg of template and incubated for 1.5 hours at 30°C. The following templates can be used with this system:

- Circular plasmid DNA containing a T3 or SP6 RNA polymerase promoter
- Linearized plasmid DNA containing a T7 RNA polymerase promoter
- Circular plasmid DNA containing both a T7 RNA polymerase promoter and T7 transcription terminator (see Notes at end of Section III.A)

Note: All components of the TNT® Coupled Wheat Germ Extract Systems are quality tested and verified for coupled transcription/translation in the lot-specific combinations represented in each prepackaged system.

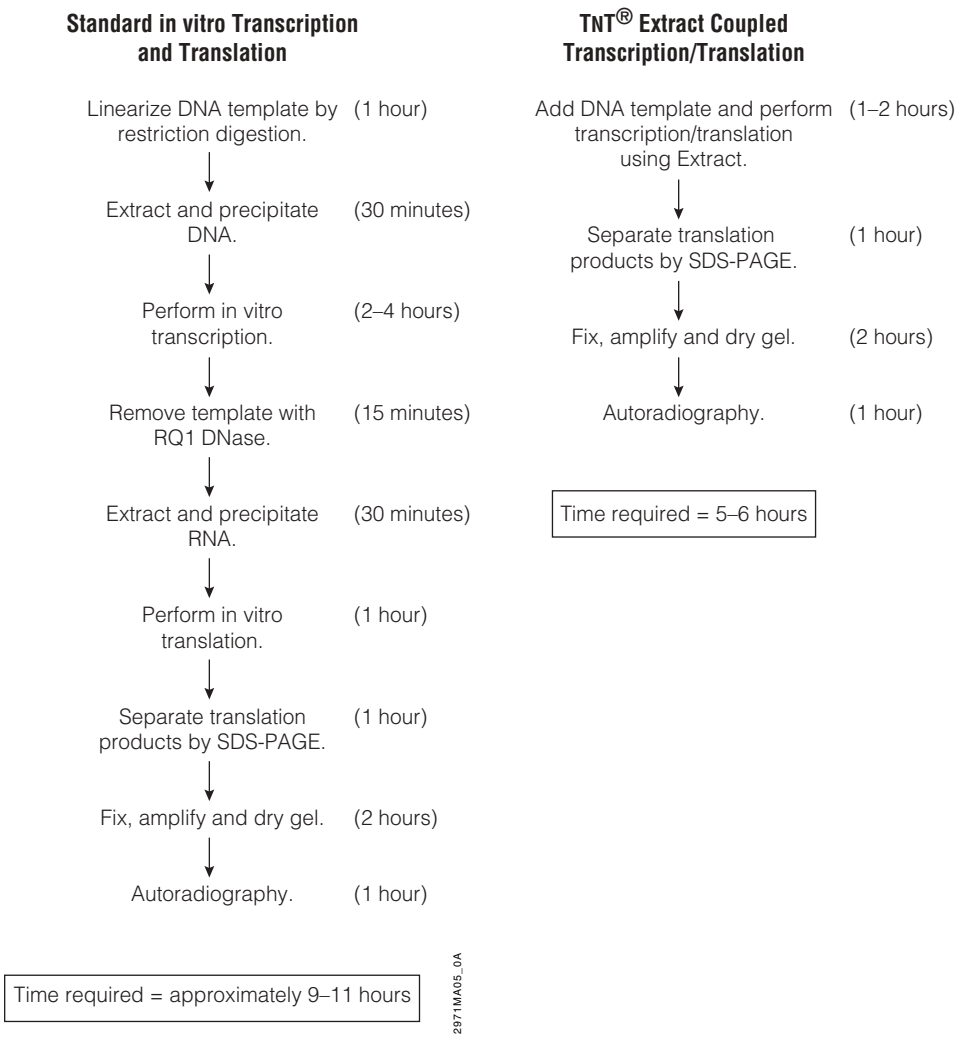

Figure 1. Comparison of standard in vitro transcription and translation procedures to the TNT® Extract coupled transcription/translation protocol.

II. Product Components

Product	Size	Cat.#
TnT® T3 Coupled Wheat Germ Extract System	40 reactions	L4120
TnT® SP6 Coupled Wheat Germ Extract System	40 reactions	L4130
TnT® T7 Coupled Wheat Germ Extract System	40 reactions	L4140
TnT® T7/SP6 Coupled Wheat Germ Extract System	40 reactions	L5030
TnT® T7/T3 Coupled Wheat Germ Extract System	40 reactions	L5040

For Laboratory Use. TnT® Wheat Germ Extract is supplied in 200µl aliquots. Each system contains sufficient reagents to perform approximately 40 × 50µl translation reactions. Includes:

- 1ml TnT® Wheat Germ Extract
- 90µl TnT® Reaction Buffer
- 60µl TnT® RNA Polymerase (SP6, T3 or T7, individually or 2 × 30µl when two polymerases are included)
- 5µg Luciferase Control DNA, 0.5mg/ml (for each polymerase purchased, SP6, T3 or T7)[a]
- 50µl Amino Acid Mixture, Minus Methionine, 1mM
- 50µl Amino Acid Mixture, Minus Leucine, 1mM
- 50µl Amino Acid Mixture, Minus Cysteine, 1mM
- 250µl Luciferase Assay Reagent[d,e,f]
- 1 set Luciferase Assay Wells (1 set of 3 wells)
- 1 Protocol

Storage and Stability: Store all components at –70°C (except the Luciferase Assay Wells, which should be stored at room temperature). The product is sensitive to CO_2 (avoid prolonged exposure) and multiple freeze-thaw cycles, which may have an adverse effect on activity/performance. LAR is stable for at least 12 months if stored and handled properly.

III. Translation Procedure

Materials to Be Supplied by the User

- RNasin® Ribonuclease Inhibitor[b] (Cat.# N2111 or N2511[b,g])
- Nuclease-Free Water (Cat.# P1193)
- radiolabeled amino acid (for radioactive detection; see Table 1) or Transcend™ tRNA (Cat.# L5061) (for non-radioactive detection)

A. General Protocol for TnT® Wheat Germ Extract Coupled Transcription/Translation Reactions

This section contains the protocol for coupled transcription/translation using the TnT® Wheat Germ Extract. Also provided is an example of a standard reaction using [^{35}S]methionine (radioactive). For more information on non-radioactive detection consult the *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin*, #TB182. Using the Transcend™ Systems, biotinylated lysine residues are incorporated into nascent proteins during translation. The biotinylated lysine is added to the translation reaction as a precharged ε-labeled biotinylated

lysine tRNA complex (Transcend™ tRNA), rather than a free amino acid. Please note that Wheat Germ Extract contains endogenously biotinylated proteins (see Note 9) that may be difficult to distinguish from biotinylated translation products when using the Transcend™ tRNA and Transcend™ Translation Detection Systems. It is important to include a negative control reaction (without added DNA) to identify any endogenously biotinylated proteins in the Wheat Germ Extract.

Radioactive and non-radioactive control reactions for the production of luciferase are described in Section IV.

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. We recommend the addition of RNasin® Ribonuclease Inhibitor to all TNT® Extract reactions to prevent degradation of RNA.

1. Remove the reagents from storage at –70°C. Immediately place the TNT® RNA Polymerase on ice. Rapidly thaw the TNT® Wheat Germ Extract by hand warming and then place on ice. The other components can be thawed at room temperature and then stored on ice.

Note: The TNT® Reaction Buffer may contain a precipitate after thawing on ice. Redissolve the precipitate by vortexing at room temperature for 30 seconds.

2. Following the example below, assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube. **Gently** mix the extract with a pipette tip upon addition of each component. If necessary, centrifuge briefly to collect the reaction at the bottom of the tube.

Example of TNT® Wheat Germ Extract Reactions:

Component	Standard Reaction Using [35S]methionine
TNT® Wheat Germ Extract	25µl
TNT® Reaction Buffer	2µl
TNT® RNA Polymerase (SP6, T3 or T7)	1µl
Amino Acid Mixture, Minus Methionine, 1mM	1µl
[35S]methionine (see Note 1)	2µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
DNA template (0.5µg/µl; see Note 2)	2µl
Nuclease-Free Water to a final volume of	50µl

Note: A 50% extract concentration is optimal for most TNT® Wheat Germ Extract reactions.

Small-scale reactions may be performed by reducing volumes proportionately.

Note: Multiple proteins can be expressed from different promoters in the same reaction by using multiple TNT® RNA Polymerases. This allows greater flexibility in designing experiments for coexpression of multiple genes (4). T7/SP6 or T7/T3 polymerase may be added to the same reaction if they are from the same lot. In vitro-translated proteins expressed simultaneously in TNT® Systems can be used to study protein-protein interactions. When using two DNA templates, add approximately 0.5–1.0µg of each template, keeping the total amount of DNA added to 2µg or less.

Note: All components of the TNT® Coupled Wheat Germ Extract Systems are quality tested and verified for coupled transcription/translation in the lot-specific combinations represented in each prepackaged system.

3. Incubate the reaction at 30°C for 60–120 minutes (see Note 3).

4. Analyze the results of translation. Procedures are provided for incorporation assays (Section V.A) and gel analysis of translation products (Section V.B). When using the Transcend™ Translation Detection Systems, see Note 9.

Notes:

1. We recommend using a translational grade [^{35}S]methionine, such as Amersham Pharmacia Redivue™ (Amersham Pharmacia Cat.# AG1094), which can be stored without aliquoting at 4°C. ^{35}S-labeled amino acids are easily oxidized to translation-inhibiting sulfoxides and should be stored in aliquots at –70°C in buffer containing DTT.

   Other radiolabeled amino acids can be used with the TnT® Extracts. Table 1 lists these amino acids and the appropriate amount to use.

   Table 1. Recommended Concentrations of Alternative Radiolabeled Amino Acids.

Amino Acid	Final Concentration in Reaction	Volume to Add to Reaction
[^{3}H]leucine (100–200Ci/mmol)	0.5mCi/ml	5µl
[^{14}C]leucine (300mCi/mol)	5µCi/ml	5µl
[^{35}S]cysteine (600mCi/mol)	1mCi/ml	5µl

2. DNA template considerations:

   a. Although circular plasmid DNA gives the best translation results in the SP6 and T3 TnT® Wheat Germ Extract Systems, **linear DNA templates are recommended for the T7 TnT® Extract System.** In the T7 TnT® Extract System, protein production levels will drop if the plasmid construct does not contain a T7 terminator or if the construct has not been linearized. Linearized templates should be phenol:chloroform extracted and ethanol precipitated before use in the translation reaction.

   b. DNA that is prepared using the Promega Wizard® *Plus* SV DNA Isolation System[h,i] (Cat.# A1330) or by the standard alkaline lysis method described by Sambrook *et al.* (5) is sufficiently clean for TnT® Extract reactions.

   c. Optimal results are obtained when 1µg of plasmid DNA template is used. However, we have used from 0.2–2.0µg of DNA template and obtained satisfactory levels of translation. The use of more than 1µg of plasmid does not necessarily increase the amount of protein produced. When simultaneously expressing from two DNA templates, add approximately 0.5–1.0µg of each template, keeping the total amount of DNA added to ≤2µg.

   d. Two plasmid constructs can be simultaneously translated in the TnT® Extracts. However, the amounts of protein produced from the two different constructs may not be equal and may be less than the quantity of protein produced from a single plasmid.

   e. Residual ethanol should be removed from DNA preparations before they are added to the translation reaction.

   f. The sequence of the DNA template should be checked for the presence of additional upstream initiation codons. During translation, the ribosome is thought to scan the 5´ end of the DNA and begin translation at the first AUG encountered. Thus, and AUGs within the transcribed portion of the vector or untranslated sequence of the insert may cause translation initiation to occur prior to the desire start codon, leading to a shift in reading frame or production of a larger protein than expected.

3. Using the T7 or T3 promoter, optimal translation will occur in 60–90 minutes at 30°C, while translation using the SP6 promoter may continue for up to 120 minutes.
4. Except for the actual translation incubation, all handling of the extract components should be done at 4°C or on ice. Any unused extract should be refrozen in an ethanol/dry ice bath as soon as possible after thawing, to minimize loss of translational activity. Do not expose the extracts to more than two freeze-thaw cycles.
5. Avoid adding calcium to the translation reaction. Calcium may reactivate the micrococcal nuclease used to destroy endogenous RNA in the extract and result in degradation of DNA or RNA templates.
6. Use capped plastic vials for reaction incubation. This avoids changes in reaction volume due to evaporation, which may affect the concentration of the components, and thus the TnT® reaction performance.
7. The luciferase control reaction usually produces 50–500ng of protein per 50µl reaction as deduced from luciferase activity. Do not use more than one polymerase per control reaction.
8. The TnT® Wheat Germ Extract System is not recommended for use with Canine Microsomal Membranes for post-translational processing. In our experience, the addition of microsomal membranes inhibits overall expression.
9. Wheat Germ Extracts contain endogenously biotinylated proteins, which may be detected when translation products are analyzed by SDS-PAGE electroblotting and streptavidin-AP or streptavidin-HRP detection. Wheat Germ Extract contains 5 major endogenous biotinylated proteins, migrating at 200kDa, 80kDa and 32kDa, with a doublet 17kDa. Comparison of translation products to a control reaction without template will enable distinction between endogenously biotinylated proteins and newly synthesized biotinylated translation product.

DO NOT
...expose the extracts to more than two freeze-thaw cycles.

IV. Positive Control Translation Reactions Using Luciferase

The assay for firefly luciferase activity is extremely sensitive, rapid and easy to perform. It is an excellent control for in vitro translations because only full-length luciferase is active. Additionally, luciferase is a monomeric protein (approximately 61kDa) that does not require post-translational processing or modification for enzymatic activity. The control reaction can be performed with or without the addition of radiolabeled amino acids. For the Luciferase Control, usually 50–500ng of protein per 50µl reaction is produced as deduced from luciferase activity.

A. Radioactive Luciferase Control Reaction

Materials to Be Supplied by the User

- RNasin® Ribonuclease Inhibitor (Cat.# N2111 or N2511)
- Nuclease-Free Water (Cat.# P1193)
- radiolabeled amino acid (for Section IV.A only)

The following example uses [35S]methionine in the luciferase control reaction. For use of other radiolabeled amino acids, see Section III.A, Step 2.

Note: A 50% extract concentration is optimal for most TnT® Wheat Germ Extract reactions.

Note: The TnT® Reaction Buffer may contain a precipitate after thawing on ice. Redissolve the precipitate by vortexing at room temperature for 30 seconds.

TnT® Wheat Germ Extract	25µl
TnT® Reaction Buffer	2µl
TnT® RNA Polymerase (SP6, T3 or T7)	1µl
Amino Acid Mixture, Minus Methionine, 1mM	1µl
[35S]methionine (1,000Ci/mmol) at 10mCi/ml (see Note 1, Section III.A)	4µl
RNasin® Ribonuclease Inhibitor, 40u/µl	1µl
Luciferase Control DNA, 0.5mg/ml	2µl
Nuclease-Free Water to a final volume of	50µl

1. Incubate the reaction at 30°C for 60–120 minutes (see Note 3, Section III.A).
2. Analyze the results of translation by measuring direct incorporation of radiolabel (Section V.A) and/or gel analysis of translation products (Section V.B).
3. Store the luciferase control reactions at –20°C for up to 2 months or at –70°C for up to 6 months.

B. Non-Radioactive Luciferase Control Reaction

Both Amino Acid Mixture, Minus Leucine, and Amino Acid Mixture, Minus Methionine, are used in this reaction. By using both incomplete mixes, a sufficient concentration of all amino acids is provided. As an alternative to assaying luciferase activity, this reaction may be performed using the Transcend™ tRNA and Non-Radioactive Detection Systems. For more information on these products request Technical Bulletin #TB182.

Note: A 50% extract concentration is optimal for most TnT® Wheat Germ Extract reactions.

Note: The TnT® Reaction Buffer may contain a precipitate after thawing on ice. Redissolve the precipitate by vortexing at room temperature for 30 seconds.

TnT® Wheat Germ Extract	25µl
TnT® Reaction Buffer	2µl
TnT® RNA Polymerase (SP6, T3 or T7)	1µl
Amino Acid Mixture, Minus Leucine, 1mM	0.5µl
Amino Acid Mixture, Minus Methionine, 1mM	0.5µl
RNasin® Ribonuclease Inhibitor, 40u/µl	1µl
Luciferase Control DNA, 0.5mg/ml	2µl
Nuclease-Free Water to a final volume of	50µl

1. Incubate the translation reaction at 30°C for 60–120 minutes (see Note 3, Section III.A).
2. Test for the synthesis of functional luciferase using either the standard luciferase assay (Section VI.A) or qualitative visual determination of luciferase activity (Section VI.B).
3. Store the luciferase control reactions at –20°C for up to 2 months or at –70°C for up to 6 months.

V. Post-Translational Analysis

Upon completion of translation, the percent incorporation of radioactive label should be calculated as described in Section V.A. Analysis of translation products by denaturing gel electrophoresis is described in Section V.B.

Materials to Be Supplied by the User

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

- 1M NaOH
- 25% TCA/2% casamino acids (Difco brand, Vitamin Assay Grade)
- 5% TCA
- 30% acrylamide solution
- separating gel 4X buffer
- stacking gel 4X buffer
- SDS polyacrylamide 10X running buffer
- SDS sample buffer
- acetone
- **optional:** precast gels (e.g., NOVEX® 4-20% Tris-Glycine gradient gels, Invitrogen Cat.# EC6025BOX, EC60355BOX and Bio-Rad® Ready Gel 4-20% Tris-Glycine Gel, 10-well, Cat.# 161-0903)
- Whatman® GF/A glass fiber filters (Whatman® Cat.# 1820 021)
- Whatman® 3MM filter paper
- fixing solution

A. Determination of Percent Incorporation of Radioactive Label

1. After the 50µl translation reaction is completed, remove 2µl from the reaction and add it to 98µl of 1M NaOH.
2. Vortex briefly and incubate at 37°C for 10 minutes.
3. At the end of the incubation, add 900µl 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 (Whatman® Cat.# 1820 021) with a small amount of cold 5% TCA. Collect the precipitated translation product by vacuum filtering 250µl 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. To determine the ^{35}S incorporation, put the filter into 1–3ml of 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 5µl 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 2µl from a 50µl translation reaction without DNA and proceed as described in Steps 1–5.
8. Perform the following calculation to determine percent incorporation:

$$\frac{\text{cpm of washed filter (Step 4)}}{\text{cpm of unwashed filter (Step 6)} \times 50} \times 100 = \text{percent incorporation}$$

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

$$\frac{\text{cpm of washed filter (Step 4)}}{\text{cpm of “no DNA control reaction” washed filter (Step 7)}} = \text{fold stimulation}$$

B. Denaturing Gel Analysis of Translation Products

Precast polyacrylamide gels are available from a number of manufacturers. For protein analysis, Invitrogen NOVEX® 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 NOVEX® 4–20% Tris-Glycine gradient gels (Invitrogen Cat.# EC6025BOX or EC60355BOX) and the Bio-Rad® Ready Gel 4–20% Tris-Glycine Gel, 10-well (Bio-Rad® 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 50µl translation reaction is complete (or at any desired timepoint), remove a 5µl aliquot and add it to 20µl of SDS sample buffer. The remainder of the reaction may be stored at –20°C.

Note: In some cases, high molecular weight complexes are formed at 100°C and denaturation may need to be performed at lower temperatures (e.g., 20 minutes at 60°C or 3–4 minutes at 80–85°C).

2. Cap the tube and heat at 100°C for 2 minutes to denature the proteins.
3. Load a small aliquot (5–10µl) of the denatured sample onto an SDS-polyacrylamide gel or store at –20°C. It is not necessary to separate labeled polypeptides from free amino acids by acetone precipitation.
4. Typically, electrophoresis is carried out 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. Because the dye front also contains the free labeled amino acids, disposal of unincorporated label may be easier if the gel is stopped while the dye front remains in the gel. If transferring the gel to a membrane filter for Western blot analysis, proceed to **Step 7**.
5. Place the polyacrylamide gel in a plastic box and cover the gel with fixing solution (as prepared in Section IX.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 ^{3}H-labeled proteins and is recommended for the 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 so increases the proportion of energy that may be detected by X-ray film. Commercial reagents, such as Amplify™ (Amersham) can conveniently be used for fluorographic enhancement of signal.

Alternatively: The fixed gel can be exposed to a phosphorimaging screen. These systems provide greater sensitivity and speed and the ability to quantitate the radioactive bands.

6. Dry the gel prior to exposure to film as follows: Soak the gel in 7% acetic acid, 7% methanol and 1% glycerol for 5 minutes to prevent 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 may also be dried overnight, using the Promega Gel Drying Kit (Cat.# V7120). To decrease the likelihood of cracking gradient gels, dry them with the wells pointing down. It is advantageous to cut or mark one corner of the filter paper to help in discerning the gel orientation on the filter. Expose the gel on X-ray film for 1–6 hours at –70°C (for fluorography) or 6–15 hours at room temperature (for autoradiography).

7. For Western blot analysis of proteins, transfer (immobilize) the protein from the gel onto nitrocellulose or PVDF membrane (6,7). Usually Western blots are made by electrophoretic transfer of proteins from SDS-polyacrylamide gels. Detailed procedures for electrophoretic blot analysis are often included with commercial devices and can be found in references 6, 8–10. A general discussion of Western blot analysis 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 may then be subjected to immunodetection analysis. For more information, refer to the Promega *Protocols and Applications Guide*, Third Edition (12). For detection of biotinylated protein using the Transcend™ Non-Radioactive Translation Detection Systems, see Technical Bulletin #TB182.

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

VI. Positive Control Luciferase Assays

Light intensity is a measure of the rate of catalysis by luciferase and is therefore dependent upon temperature. The optimum temperature for luciferase activity is approximately room temperature (20–25°C). It is important that the Luciferase Assay Reagent be fully equilibrated to room temperature before beginning measurements. To ensure temperature equilibration, place a thawed aliquot of the Luciferase Assay Reagent in a sealed tube into a water bath maintained at ambient temperature, and equilibrate for at least 30 minutes. The sample to be assayed should also be at ambient temperature.

Either a luminometer or a scintillation counter can be used for quantitation. (There is usually insufficient light output for qualitative visual detection.) A luminometer can measure as little as 10^{-20} moles (0.001pg) of luciferase, whereas a scintillation counter typically has a less sensitive detection limit. However, the limits of sensitivity may vary depending upon the particular instrument used. The assay should be linear in some portion of the detection range of the instrument. Please consult your instrument operator's manual for general operating instructions.

A. Using a Luminometer

1. Dispense 50µl of the Luciferase Assay Reagent into luminometer tubes, one tube per sample.
2. Program the luminometer to perform a 2-second measurement delay followed by a 10-second measurement read for luciferase activity. The read time may be shortened if sufficient light is produced.
3. Add 2.5µl of cell lysate to a luminometer tube containing the Luciferase Assay Reagent. Mix by pipetting 2–3 times or vortex briefly.
4. Place the tube in the luminometer and initiate reading.
5. If the luminometer is not connected to a printer or computer, record the reading.

(!) The Luciferase Assay Reagent and samples should be at ambient temperature prior to performing a luciferase assay.

B. Using a Scintillation Counter

Ideally, the coincidence circuit of the scintillation counter should be turned off. Usually, this is achieved through an option of the programming menu or by a switch within the instrument. If the circuit cannot be turned off, a linear relationship between luciferase concentration and cpm still can be produced by calculating the square root of measured counts per minute (cpm) minus background cpm (i.e., $[\text{sample} - \text{background}]^{1/2}$). To measure background cpm, use water or Luciferase Assay Reagent as a blank.

Do not add scintillant because it will inactivate the luciferase and is not needed.

Use the same protocol as luciferase assays using a luminometer (Section VI.A). The sample may be placed directly in the scintillation vial if it completely covers the bottom of the vial (clear or translucent vials are acceptable). Do not add scintillant, because it will inactivate luciferase. Alternatively, place the sample in a microcentrifuge tube, and then place the tube in the scintillation vial. To ensure consistency when working with multiple samples, place each microcentrifuge tube at the same relative position within the scintillation vial.

For consistency in measuring luciferase activity, use the scintillation counter in manual mode. Initiate each sample reaction immediately before measurement and read the samples one at a time. Because the enzymatic reaction produces light at all wavelengths, read the samples with all channels open (open window). To reduce background counts, it may be necessary to wait 10–30 seconds before counting. Read individual samples for 1–5 minutes.

C. Qualitative Visual Detection of Luciferase Activity

For qualitative determination of luciferase activity, the reactions may be visualized by eye in a dark room after acclimation to the dark. Most individuals should be able to see the reaction after a minute or two of acclimation, although individuals may differ in their ability to detect low light output.

VII. Troubleshooting

Symptoms	Possible Causes	Comments
The control reaction did not produce luciferase	Loss of activity of the reaction components	The extracts should not be used after more than two freeze-thaws.
		Do not use reagents after the expiration date.
The control reaction worked but the sample reaction did not	Ethanol or salt is present in the translation reaction	Ethanol or salt may inhibit translation.
Low translation efficiency	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 translation reaction	Residual ethanol should be removed from preparations and labeled amino acids before they are added to the translation reaction.
Unexpected bands are present at higher molecular weights	Denaturing temperature is too high	Denature sample at a lower temperature (e.g., 60–80°C).
Unexpected bands are present on the gel	Proteolysis of translation product	Add a protease inhibitor, such as leupeptin, α-macroglobulin or chymostatin.
	More than one peptide is translated from the template	Leaky scanning for translation initiation can result in translation initiating at internal downstream methionines.

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**

VII. Troubleshooting (continued)

Symptoms	Possible Causes	Comments
Unexpected bands are present on the gel (continued)	^{35}S-labeled amino acid is beyond its expiration date	Older ^{35}S may dissociate from the amino acid and label other proteins in the lysate. Use fresh ^{35}S-labeled amino acids.
	The [^{35}S]methionine used is not of translational grade	We recommend Amersham International Redivue™ [^{35}S]methionine (Amersham Cat.# AG1094).
	Aminoacyl tRNAs may produce background bands	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-mercapto-ethanol is present or not enough SDS in loading buffer	Use loading buffer that contains 2% SDS and 100mM DTT.
Low protein yield	Incubation of the reaction at 37°C can decrease protein synthesis	Incubate the reaction at 30°C.
There is smearing on the gel	Gel is not clean	Gel must be washed before placing onto film. Once gel 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 is too low to resolve the proteins	Acrylamide concentration can be increased to 12%.
	Ethanol is present in the sample	Ethanol present in the sample can cause smearing on the gel.

VIII. References

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IX. Appendix

A. Composition of Buffers and Solutions

fixing solution

50%	methanol
10%	glacial acetic acid
40%	water

1X SDS gel-loading buffer

50mM	Tris-HCl (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 10X running buffer

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

Bring to a final volume of 1L. Store at room temperature.

separating gel 4X buffer

18.17g	Tris base
4ml	10% SDS

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

stacking gel 4X buffer

6.06g	Tris base
4ml	10% SDS

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

B. TnT® Luciferase SP6/T7/T3 Control DNAs[e]

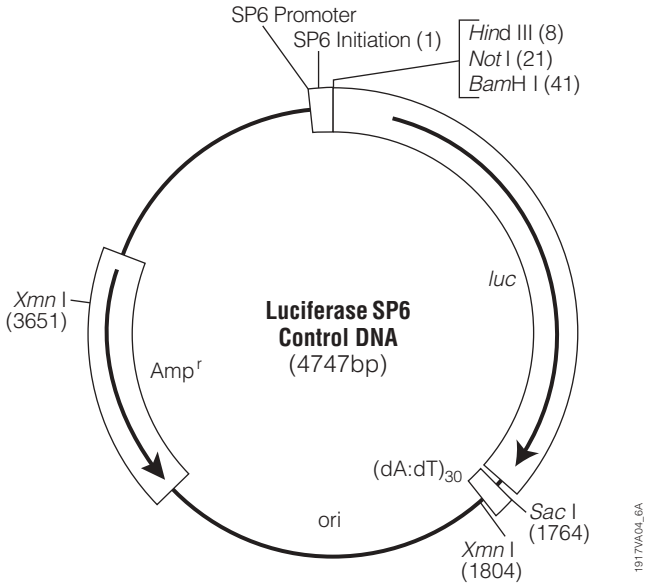

Figure 2. Luciferase SP6 Control DNA circle map and sequence reference points. Additional description: Amp^r, β-lactamase gene (resistant to ampicillin); ori, origin of plasmid replication.

Sequence reference points:

SP6 RNA polymerase initiation	1
GL Primer 2	49–71
Luciferase gene	48–1697
Poly(A) $(dA)_{30}$	1767–1796
pUC/M13 reverse primer (17mer)	1833–1817
pUC/M13 reverse primer (22mer)	1838–1817
β-lactamase gene (Amp^r)	3838–2975
SP6 RNA polymerase promoter primer	4731–1
SP6 RNA polymerase promoter	4731–3

Note: there is a single base mismatch at the 5´ end of the SP6 RNA polymerase promoter primer.

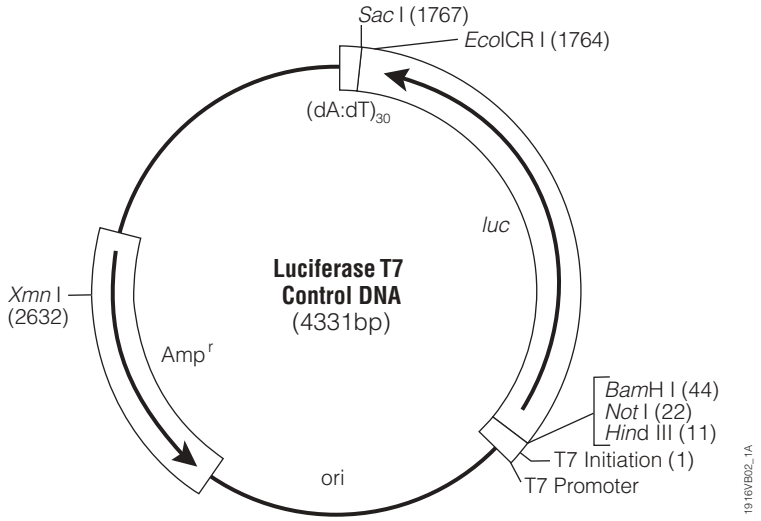

Figure 3. Luciferase T7 Control DNA circle map and sequence reference points. The Luciferase T7 Control DNA supplied with the TNT® Wheat Germ Extract System has been linearized with *Eco*ICR I. Additional description: Amp^r, β-lactamase gene (resistant to ampicillin); ori, origin of plasmid replication.

Sequence reference points:

T7 RNA polymerase initiation	1
GI Primer 2	52–74
Luciferase gene	51–1700
Poly(A) $(dA)_{30}$	1770–1799
β-lactamase gene (Amp^r)	2444–3301
T7 RNA polymerase promoter	4315–3
T7 RNA polymerase promoter Primer	4315–3

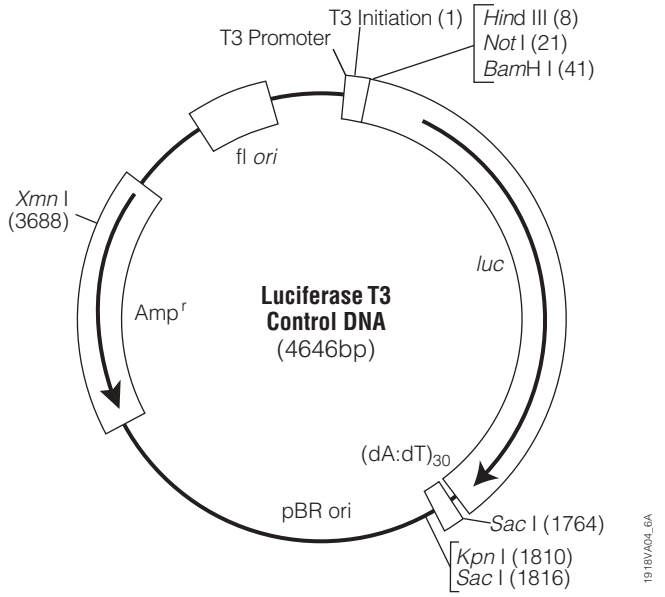

Figure 4. Luciferase T3 Control DNA circle map and sequence reference points. Additional description: Amp^r, β-lactamase gene (resistant to ampicillin); f1 ori, origin of replication; pBR ori, origin of plasmid replication.

Sequence reference points:

T3 RNA polymerase initiation	1
GI primer 2	49–71
Luciferase gene	48–1697
Poly(A) $(dA)_{30}$	1767–1796
β-lactamase gene (Amp^r)	3875–3012
T7 RNA polymerase promoter (–17 to +2)	1840–1822
pUC/M13 reverse primer (17mer)	1870–1854
pUC/M13 reverse primer (22mer)	1875–1854
f1 origin	4006–4461
pUC/M13 forward primer (24mer)	4576–4599
pUC/M13 forward primer (17mer)	4583–4599
T3 RNA polymerase promoter	4631–4
T3 RNA polymerase promoter Primer	4631–4

Note: the T7 Sequencing primer has a 3´ mismatch and won't bind.

C. Related Products

The in vitro synthesis of proteins is a popular method in biological research. Among other applications, translation systems are used to characterize plasmid clones, study structural mutations and examine translational signals.

Two basic approaches to in vitro protein synthesis are available: 1) in vitro systems programmed with RNA (translation systems) and 2) those programmed with DNA (coupled transcription/translation systems). Several general considerations to assist in selection of the appropriate Promega product(s) are given below. Please see our product catalog, available upon request from Promega, or visit our website at www.promega.com/catalog for a complete listing of our in vitro translation systems.

Translation Systems

A number of cell-free protein synthesizing systems have been developed for the translation of mRNA isolated from tissue or generated in vitro. Promega offers several Rabbit Reticulocyte Lysate and Wheat Germ Extract Systems. All are reliable, convenient and easy-to-use systems to initiate translation and produce full-size polypeptide products. Rabbit Reticulocyte Lysate is appropriate for the translation of larger mRNA species and is generally recommended when microsomal membranes are to be added for cotranslational processing of translation products. Promega's Flexi® Rabbit Reticulocyte Lysate[a,c,d] is recommended where optimization of translation of particular RNAs through adjustments to salt and DTT concentrations is required. Wheat Germ Extract readily translates a variety of RNA preparations, including those containing low concentrations of double-stranded RNA (dsRNA) or oxidized thiols, which are inhibitory to reticulocyte lysate.

Coupled Transcription/Translation Systems

DNA sequences cloned in plasmid vectors also may be expressed directly using either Promega's TNT® Coupled Wheat Germ Extract Systems, Rabbit Reticulocyte Lysate Systems or *E. coli* S30 Coupled Transcription/Translation Systems[a,c]. The TNT® Systems are used to direct eukaryotic translation, whereas the S30 Systems are under prokaryotic translational controls. The TNT® Systems require plasmid constructs containing a prokaryotic phage RNA polymerase promoter (SP6, T3 or T7) for the initiation of transcription, but translation in this system is under eukaryotic controls. Optimal translation will occur if the AUG initiation codon is in a "Kozak consensus" context (A/GCCAUGG) (13) in the absence of inhibiting secondary structure. Recent literature suggests that there is polymorphism within the Kozak sequence, and certain sequences show increased translational efficiency in vitro and in vivo (14). The template DNA to be expressed in the S30 Systems must contain *E. coli* promoter sequences and prokaryotic ribosome binding sites (GGAGG) for translation. The TNT® and *E. coli* S30 Systems can use either circular or linear DNA templates, depending on the system.

Wheat Germ Extract

Wheat Germ Extract is provided as 200µl aliquots. Each 1ml order also includes 5µg of BMV Control RNA, Potassium Acetate, a choice of one Amino Acid Mixture and a Wheat Germ Extract Systems Technical Manual.

(a)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. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

(b)U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

(c)U.S. Pat. Nos. 5,324,637 and 5,492,817, European Pat. No. 0 566 714 B1, Australian Pat. No. 660329 and Japanese Pat. No. 2904583 have been issued to Promega Corporation for coupled transcription/translation systems that use RNA polymerases and eukaryotic lysates.

(d)U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289, European Pat. No. 0 553 234 and Japanese Pat. No. 3171595 have been issued to Promega Corporation for a beetle luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.

(e)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.

(f)Certain applications of this product may require licenses from others.

(g)U.S. Pat. No. 5,552,302, European Pat. No. 0 422 217, Australian Pat. No. 646803 and Japanese Pat. Nos. 3009458 and 3366596 have been issued to Promega Corporation for the methods and compositions for production of human recombinant placental ribonuclease inhibitor.

(h)Australian Pat. No. 730718 and Singapore Pat. No. 64532 have been issued to Promega Corporation for an improved filtration system and method. Other patents are pending.

(i)U.S. Pat. No. 5,981,235 and Australian Pat. No. 729932 have been issued to Promega Corporation for methods for isolating nucleic acids using alkaline protease. Other patents are pending.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.