

Flexi[®] Rabbit Reticulocyte Lysate System

INSTRUCTIONS FOR USE OF PRODUCT L4540.

Flexi[®] Rabbit Reticulocyte Lysate System

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1. Description

The rabbit reticulocyte in vitro translation system, as described by Pelham and Jackson (1), is widely used to identify mRNA species and characterize their products. This eukaryotic system has been reported to post-translationally modify proteins via phosphorylation, acetylation and isoprenylation (2). Signal peptide cleavage and core glycosylation also can be achieved in the Rabbit Reticulocyte Lysate System with the addition of Canine Pancreatic Microsomal Membranes (3,4).

The Flexi® Rabbit Reticulocyte Lysate System^(a,b) provides greater flexibility of reaction conditions than standard rabbit reticulocyte lysate systems. As with Promega standard Rabbit Reticulocyte Lysate, the Flexi® Rabbit Reticulocyte Lysate is optimized for translation by the addition of:

- Hemin, to prevent inhibition of initiation factor eIF-2 α
- An energy-generating system consisting of pretested phosphocreatine kinase and phosphocreatine
- Calf liver tRNAs, to balance the endogenous tRNA populations, thus optimizing codon usage and expanding the range of mRNAs that can be translated efficiently
- Micrococcal nuclease, to eliminate endogenous mRNA, thus reducing background translation

mRNAs commonly exhibit differing salt requirements for optimal translation. Small variations in salt concentration can lead to dramatic differences in translation efficiency. The Flexi® Rabbit Reticulocyte Lysate System allows translation reactions to be optimized for a wide range of parameters, including Mg²⁺ and K⁺ concentrations and the choice of adding DTT. To help optimize Mg²⁺ for a specific message, the endogenous Mg²⁺ concentration of each lysate batch is stated in the product information included with this product. The Flexi® System also offers the choice of three amino acid mixtures and includes a control RNA encoding the firefly luciferase gene. The moderate translation efficiency of the uncapped Luciferase Control RNA^(b) is more representative of a typical in vitro runoff transcript than the highly efficient viral genome mRNAs such as BMV (brome mosaic virus) or TMV (tobacco mosaic virus). More importantly, synthesis of full-length, functional luciferase protein can be monitored easily, quickly (<1 minute) and non-radioactively using the assay reagents supplied.

Potential applications of the Flexi® System include:

- Drug screening (affecting translation rates)
- Mutation and detection analysis (i.e., enzyme kinetics)
- Protein:protein interactions (e.g., 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 cross-linking studies
- Protein:RNA binding assays
- Post-translational modification tests
- Verification/characterization of cloned gene products

2. Product Components and Storage Conditions

Product	Size	Cat.#
Flexi® Rabbit Reticulocyte Lysate System	1ml	L4540

Bulk Flexi® Lysate is available from Promega. Flexi® Rabbit Reticulocyte Lysate is provided in 5 × 200 μ l aliquots. Each system contains sufficient reagents for 30 × 50 μ l translation reactions. Includes:

- 1ml Flexi® Rabbit Reticulocyte Lysate
- 100 μ l Magnesium Acetate, 25mM
- 200 μ l Potassium Chloride, 2.5M
- 100 μ l DTT, 100mM
- 50 μ l Amino Acid Mixture, Minus Methionine, 1mM
- 50 μ l Amino Acid Mixture, Minus Leucine, 1mM
- 50 μ l Amino Acid Mixture, Minus Cysteine, 1mM
- 10 μ l Luciferase Control RNA, 1mg/ml
- 250 μ l Luciferase Assay Reagent^(a,b)
- 1 Luciferase Assay Wells, set of 3

Storage and Stability: Store all components at -70°C (except the 100mM DTT, which should be stored at the temperature noted on its label, and the Luciferase Assay Wells, which should be stored at room temperature). Product is sensitive to CO₂ (avoid prolonged exposure) and multiple freeze-thaw cycles, which may adversely affect activity and performance. Luciferase Assay Reagent (LAR) is stable for at least 12 months if stored and handled properly.

Do not store the lysate in the presence of dry ice. Prolonged exposure to dry ice can cause significant loss of lysate activity.

Do not freeze-thaw the lysate more than two times.

3. Translation Procedure

The following is a general guideline for setting up a translation reaction. Examples are provided for standard reactions using [³⁵S]methionine (radioactive), FluoroTect™ Green_{Lys} in vitro Translation Labeling System or Transcend™ Non-Radioactive Detection Systems. Using the Transcend™ or FluoroTect™ Systems, biotinylated or fluorescently labeled lysine residues are incorporated into nascent proteins during translation, eliminating the need to label with [³⁵S]methionine or other radioactive amino acids. 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. The FluoroTect™ System uses a modified charged lysine transfer RNA labeled with the fluorophore BODIPY®-FL. For more information on the Transcend™ Systems, refer to the *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin #TB182*. For more information on the FluoroTect™ Green_{Lys} in vitro Translation Labeling System, refer to the *FluoroTect™ Green_{Lys} in vitro Translation Labeling System Technical Bulletin #TB285*. These documents are available online at: www.promega.com

Several measures should be taken to reduce the chance of RNase contamination. Gloves should be worn throughout the experiment. Use microcentrifuge tubes and pipette tips that have been exposed only to gloved handling. Addition of RNasin® Ribonuclease Inhibitor to the translation reaction is recommended but not required. RNasin® Ribonuclease Inhibitor prevents degradation of sample mRNAs by contaminating RNases.

Materials to Be Supplied by the User

- RNasin® Ribonuclease Inhibitor (Cat.# N2111)
- Nuclease-Free Water (Cat.# P1193)
- appropriately labeled amino acid
- Transcend™ Colorimetric (Cat.# L5070) or Chemiluminescent (Cat.# L5080) Translation Detection System (for non-radioactive detection) or Transcend™ tRNA (Cat.# L5061; for non-radioactive detection) or FluoroTect™ Green_{Lys} in vitro Translation Labeling System (Cat.# L5001)

1. Remove the reagents from -70°C storage. Rapidly thaw the Flexi® Rabbit Reticulocyte Lysate by hand-warming, and immediately store on ice. The other components can be thawed at 37°C, then stored on ice.
2. Denature the template mRNA at 65°C for 3 minutes, and immediately cool in an ice-water bath. This increases the efficiency of translation, especially of GC-rich mRNA, by denaturing local regions of secondary structure.

Note: We recommend including a control reaction containing no mRNA. This allows measurement of any background incorporation of labeled amino acids.

3. Assemble the reaction components, appropriate for the label being used, in a 0.5ml polypropylene microcentrifuge tube. After all components are added, gently mix the lysate by pipetting and stirring the reaction with the pipette tip. If necessary, centrifuge briefly to return the sample to the bottom of the tube.

Examples of Standard Reactions

Component	[³⁵ S]methionine Reaction	Transcend™ tRNA Reaction	FluoroTect™ tRNA Reaction
Flexi® Rabbit Reticulocyte Lysate (see Note 1)	33µl	33µl	33µl
Amino Acid Mixture, Minus Leucine, 1mM	—	0.5µl	0.5µl
Amino Acid Mixture, Minus Methionine, 1mM	1µl	0.5µl	0.5µl
[³⁵ S]methionine (1,200Ci/mmol, 10mCi/ml; see Notes 2, 3)	2µl	—	—
Magnesium Acetate, 25mM (see Note 4)	0-4µl	0-4µl	—
Potassium Chloride, 2.5M (see Note 5)	1.4µl	1.4µl	—
DTT, 100mM (see Notes 6, 7)	0-1µl	0-1µl	—
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl	1µl	1µl
Transcend™ tRNA (see Note 8)	—	1-2µl	—
FluoroTect™ Green _{Lys} tRNA (see Notes 9, 10)	—	—	1µl
RNA substrate (see Note 10)	<u>1-12µl</u>	<u>1-12µl</u>	<u>1-12µl</u>
Nuclease-Free Water to a final volume of	50µl	50µl	50µl

These reactions may be scaled up or down. We have successfully scaled up to a 500µl final volume. A 25µl translation reaction may be performed by reducing the recommended volumes by one-half.

4. Immediately incubate the translation reaction at 30°C for 90 minutes.
5. Analyze the results of translation. Procedures are provided for incorporation assays (Section 5.A), gel analysis of translation products (Section 5.B) and an assay for luciferase production in the control reactions (Section 7). For analysis of reactions using Transcend™ tRNA, refer to the *Transcend™ Non-Radioactive Detection Systems Technical Bulletin #TB182*. For analysis of reactions using FluoroTect™ Green_{Lys} tRNA, refer to the *FluoroTect™ Green_{Lys} in vitro Translation Labeling System Technical Bulletin #TB285*.

See the following pages for Notes 1-18, which provide recommendations for optimizing your translation reactions using the Flexi® Lysate System.

3. Translation Procedure (continued)

Notes:

- The lysate is diluted to 66% of its original concentration in the standard reaction. This is optimal for most applications. If desired, the lysate can be diluted to 50–60% of its original concentration without a substantial reduction in translational efficiency. If optimal expression is desired in a reduced lysate concentration reaction, then the reduced endogenous levels of Mg²⁺ and K⁺ must be compensated. The endogenous Mg²⁺ concentration of each lysate batch is listed in the product information included with this product. Because the endogenous K⁺ concentration of each lysate batch is not determined, the optimal amount of K⁺ must be determined empirically. Note that Mg²⁺ concentration is critical and has a narrow (within 0.1mM) optimal peak, while the range for K⁺ is much broader and less critical (see Table 1).

Table 1. Guidelines for KCl Concentration.

RNA Construct (see Figure 1)	Concentration of Added KCl	Volume of 2.5M KCl to Add
Poly(A) ⁺ containing transcript (e.g., pSP64 Poly(A) Vector, Cat.# P1241)	60–90mM	1.2–1.8µl
Transcript containing EMCV UTR (untranslated region)	100–120mM	2.0–2.4µl
Basic “run-off” transcript	40–70mM	0.8–1.4µl
Capped RNAs	70–100mM	1.4–2.0µl

Although each RNA transcript will have its own optimal KCl concentration for translation, these recommendations can be used as a rough guideline. The volumes recommended are for a 50µl final reaction volume.

- We recommend using 1–4µl of [³⁵S]methionine ([³⁵S]met, 1,200Ci/mmol at 10mCi/ml). Depending upon the translational efficiency of the experimental RNA and number of methionines present in the protein, the amount of [³⁵S]methionine can be adjusted to balance exposure time versus cost of label.
- We recommend using a grade of [³⁵S]methionine, PerkinElmer EasyTag™ L-[³⁵S]methionine (PerkinElmer Cat.# NEG709A), which does not cause the background labeling of the rabbit reticulocyte lysate 42kDa protein that can occur using other grades of label (5). In addition, a stabilizer is added to increase the stability above that of conventional radiolabeled amino acids, so that the release of volatile gases is reduced substantially. This [³⁵S]methionine may be stored at 4°C without dividing into aliquots. Other types of ³⁵S-labeled amino acids may be oxidized easily to translation-inhibiting sulf-oxides and should be stored in aliquots in buffer containing DTT at -70°C. Other radiolabeled amino acids can be used with the Flexi® System. See Table 2 for recommendations.

When using other radiolabeled amino acids, be sure to substitute the appropriate amino acid mixture for the Amino Acid Mixture, Minus Methionine, 1mM. When using radiolabeled leucine, use the Amino Acid Mixture, Minus Leucine, 1mM, and when using radiolabeled cysteine, use the Amino Acid Mixture, Minus Cysteine, 1mM.

Table 2. Recommended Concentrations of Radiolabeled Amino Acids in Reaction.

Amino Acid	Final Concentration	Volume to Add
[³ H]leucine (100–200Ci/mmol)	0.5mCi/ml	5µl
[¹⁴ C]leucine (300mCi/mmol)	5µCi/ml	5µl
[³⁵ S]cysteine (1,200Ci/mmol)	0.3mCi/ml	5µl

- Mg²⁺ is one of the most critical components affecting translation. The range of Mg²⁺ for optimal translation is very narrow; therefore, small changes in Mg²⁺ concentration can affect the efficiency of translation dramatically. Furthermore, each RNA transcript has an individual optimal Mg²⁺ concentration. The endogenous Mg²⁺ concentration of each lysate batch is stated in the product information included with this product. For many RNA transcripts, this endogenous level should be very close to the optimal concentration. To optimize Mg²⁺ for a specific transcript, add 0–4µl (0–2mM) of the provided Magnesium Acetate to the standard 50µl reaction. High Mg²⁺ concentrations may reduce the fidelity of translation and should be avoided (6).

Addition of polyamines, such as spermidine (0.1–1.5mM) and certain diamines (0.1–40mM), has been shown to stimulate translation (6). Less Mg²⁺ is required for the translation reaction when polyamines are used.

- There have been varying reports in the literature concerning which potassium salt should be used in rabbit reticulocyte translation reactions. Potassium acetate (KOAc) was often preferred over potassium chloride (KCl) because the chloride ion was shown to be inhibitory to translation while the acetate ion was not (7). However, there are advantages of adding potassium chloride to rabbit reticulocyte translation reactions. Both KCl and KOAc may improve the fidelity of initiation from capped messages (8), but uncapped, in vitro-generated RNAs are translated with greater initiation fidelity using KCl (9). For further discussion of the translation of capped and uncapped mRNA, see Section 8.B. Another effect of KCl is that addition of high (120mM) levels of KCl may produce greatly enhanced translational efficiency of EMCV (encephalomyocarditis virus) RNA (Figure 1, lanes 5 and 10) (5). KCl is provided with the Flexi® System. Suggested concentrations are provided in Table 1.

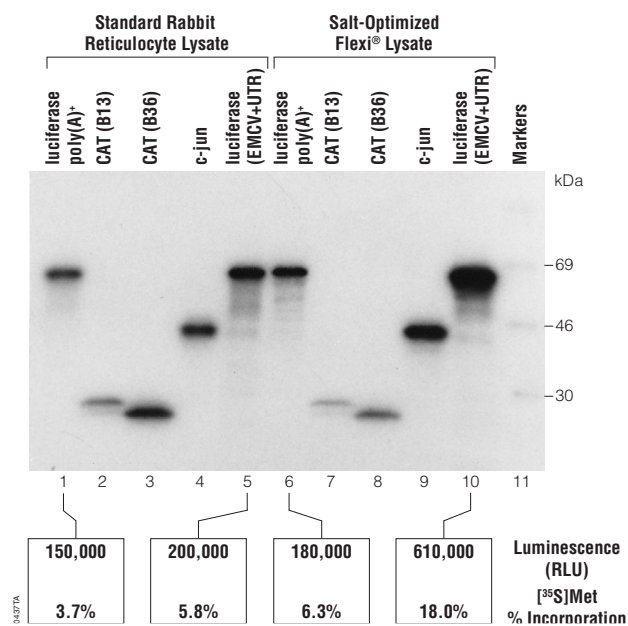


Figure 1. Comparison of standard and salt-optimized rabbit reticulocyte lysate translations. Samples from either standard (lanes 1-5) or Flexi® Lysate (lanes 6-10) reactions were resolved on a 4-15% gradient SDS-polyacrylamide gel and analyzed by autoradiography. Each 50µl reaction contained 1µg RNA, 20µCi [³⁵S]methionine and Amino Acid Mixture Minus Methionine. Reactions were incubated at 30°C for 90 minutes. The translation reactions were programmed with five different RNAs generated from the following plasmid constructs: lanes 1 and 6, pPOLY(A)-*luc*; lanes 2 and 7, B13 (CAT); lanes 3 and 8, B38 (CAT); lanes 4 and 9, c-jun (transcription factor); lanes 5 and 10, pEMCV UTR-*luc*. Lane 11 contained [¹⁴C] size standards. Two measures of luciferase production are listed below the luciferase-producing reactions: Luminescence measured in relative light units (RLU) (2.5µl of translation reaction + 50µl Luciferase Assay Reagent, 10-second integration) and percent incorporation of [³⁵S]methionine (determined by TCA precipitation of 0.5µl of the translation reaction).

Template Derivations, Figure 1: pGEM®-*luc* Vector was derived by cloning the luciferase gene into the center of the multiple cloning region of pGEM®-11Zf(-)Vector. pPOLY(A)-*luc* was derived by cloning the luciferase gene from pGEM®-*luc* into pSP64 Poly(A) (which added 30 adenine residues to the 3' end). pEMCV UTR-*luc* was derived by cloning the luciferase gene from pGEM®-*luc* into pCITE®-1 (Novagen), which placed the encephalomyocarditis 5'-untranslated region upstream of the luciferase gene. CAT(B13) is a chloramphenicol acetyltransferase (CAT) construct with the first AUG in good "Kozak" context for translation initiation. CAT(B38) is the same as CAT(B13) with the second AUG in good "Kozak" context.

3. Translation Procedure (continued)

- No DTT is added to the lysate. We find no differences in translational efficiency from lysates prepared with or without DTT. If desired, 1µl of the provided 100mM DTT can be added to a 50µl lysate reaction to provide the identical DTT concentration found in our standard Rabbit Reticulocyte Lysate reaction (2mM).
- DTT can prevent formation of disulfide bridges in proteins, possibly inactivating the protein. We recommend that DTT not be added initially to translation reactions designed to study protein activity.
- The level of added Transcend™ tRNA can be increased (1-4µl) to allow more sensitive detection of proteins that contain few lysines or are poorly expressed.
- Fluorescent labeling of poorly expressed proteins containing few lysines can be increased by adding greater amounts of FluoroTect™ Green_{Lys} tRNA in a 50µl reaction.
- With FluoroTect™ labeling there is a 30kDa endogenous fluorescent band. There is also an endogenous fluorescent band from hemoglobin that migrates at or below 12-15kDa. An 18kDa endogenous fluorescent band from charged tRNA can be removed by treatment with RNase ONE™ Ribonuclease (5 units/50µl reactions, incubated for 5 minutes at 37°C) or RNase A (4mg/ml) treatment (dilute RNase A at a ratio of 1:10 to 1:20 in water, and use 1µl/5µl translation reaction incubated for 5 minutes at 37°C).
- Avoid adding calcium to the translation reaction. Calcium may reactivate the micrococcal nuclease used to destroy endogenous mRNAs, resulting in degradation of the RNA template.
- RNase A can be added to the completed translation reaction to digest aminoacyl tRNAs, which sometimes produce background bands through interactions with endogenous lysate proteins. Add RNase A to a final concentration of 0.2mg/ml for 5 minutes at 30°C.
- In some cases, addition of 1-10mM adenosine-3',5'-cyclic monophosphate (cAMP) may stimulate translation for a short period by stabilizing the activity of the initiation factor eIF-2B (10).

3. Translation Procedure (continued)

14. An unfractionated total cytoplasmic RNA preparation is 60–70% rRNA, resulting in poor translation of mRNA. Usually such preparations yield no better than 20–30% of the maximum incorporation attainable, and high final concentrations of RNA (100–200µg/ml) are needed to stimulate translation. In contrast, viral RNAs and poly(A)+ mRNAs (including mRNA transcribed in vitro) can be used at much lower concentrations. Using in vitro transcripts produced with the RiboMAX™ Large Scale RNA Production Systems (Cat.# P1280, P1300), a final concentration of 5–80µg/ml of in vitro transcript may be used for translation. Using the RiboMAX™ Systems, milligram quantities of RNA can be produced (see Section 8.A). RNA from other standard transcription procedures may contain components at concentrations that inhibit translation. Therefore, a lower concentration (5–20µg/ml) of in vitro transcript should be used.

To prepare template DNA for run-off RNA transcript production, the Wizard® Plus SV DNA Purification System (Cat.# A1330, A1340, A1460, A1470) or Wizard® PCR Preps DNA Purification System (Cat.# A7170, A2180) is recommended. The SV Total RNA Isolation System (Cat.# Z3100, Z3101) is ideal for purifying total RNA, free from DNA contamination. The PolyATtract® mRNA Isolation Systems (Cat.# Z5300, Z5310), using MagneSphere® technology, may be used to purify run-off RNAs or isolate poly(A)+ mRNA free from other nucleic acid contamination in approximately 45 minutes.

15. Adding glucose-6-phosphate to a final concentration of 0.5mM may stimulate translation in some cases by regulating the activity of eIF-2B (10).
16. Except for the actual translation incubation, all handling of lysate components should be done at 4°C. Any unused lysate should be refrozen in liquid nitrogen as soon as possible after thawing to minimize loss of translational activity. Do not freeze-thaw the lysate more than 2 times.
17. Lysate contains approximately 100–200mg/ml of endogenous protein (using BSA as a standard).
18. Use capped plastic vials or covered multiwell plates to avoid changes in reaction volume, which may affect the concentration of important components.

4. Cotranslational Processing Using Canine Pancreatic Microsomal Membranes

The following protocol should be used with the Flexi® Rabbit Reticulocyte Lysate System (Cat.# L4540). For more information on using Canine Pancreatic Microsomal Membranes, see the *Canine Pancreatic Microsomal Membranes Technical Manual* #TM231. See Notes 1–5 following this protocol for important information on the use of Canine Microsomal Membranes.

Materials to Be Supplied by the User

- double-distilled, RNase-free water
- RNasin® Ribonuclease Inhibitor
- isotopically labeled amino acids, typically [³⁵S]methionine, [³⁵S]cysteine, [³H]leucine or [¹⁴C]leucine
- Canine Pancreatic Microsomal Membranes (Cat.# Y4041)

1. Remove the reagents from the freezer, and allow them to thaw on ice.
2. Mix the following components on ice, in the order given, in a sterile microcentrifuge tube:

Flexi® Rabbit Reticulocyte Lysate	16.5µl
Amino Acid Mixture, Minus Methionine, 1mM	0.5µl
[³⁵ S]methionine (1,200Ci/mmol at 10mCi/ml)	2.0µl
Canine Microsomal Membranes (see Note 1)	1–3µl
RNA substrate in water (pre-β-lactamase and α-factor control mRNA at 0.1µg/µl; see Note 2)	1.0µl
RNase-Free Water to a final volume of	25.0µl

Note: RNasin® Ribonuclease Inhibitor (approximately 40 units) may be added to the reaction.

3. Incubate at 30°C for 60 minutes.
4. Analyze the results of translation and processing by SDS-PAGE as described in Section 5.B.

Notes:

1. The amount of Canine Microsomal Membranes used in the reaction may need to be titrated. While these reaction conditions will be suitable for most applications, the efficiency of processing using membranes may vary. Thus, reaction parameters may need to be altered to suit individual requirements. In general, increasing the amount of membranes in the reaction increases the proportion of polypeptides that are processed but reduces the total amount of polypeptide synthesized.

4. **Cotranslational Processing Using Canine Pancreatic Microsomal Membranes (continued)**
2. The pre- β -lactamase and α -factor control mRNA are included with the Canine Microsomal Membranes. When analyzed by SDS gel electrophoresis, the precursor for β -lactamase migrates at 31.5kDa and the processed β -lactamase at 28.9kDa. The precursor for the α -factor migrates at 18.6kDa, and the core-glycosylated α -factor has a molecular weight of 32.0kDa, but it will migrate faster than the β -lactamase precursor.
3. Depending on the mRNA under examination, translation efficiency can be expected to drop between 10–50% in the presence of Canine Microsomal Membranes.
4. Storage buffer for the Canine Microsomal Membranes consists of 50mM triethanolamine, 2mM DTT and 250mM sucrose.
5. In some cases, it is difficult to determine by gel analysis alone if efficient processing or glycosylation has occurred. For alternative procedures for determining if cotranslational processing has occurred, see the *Canine Pancreatic Microsomal Membranes Technical Manual #TM231*.

5. Post-Translational Analysis

Materials to Be Supplied by the User

(Solution compositions are provided in Section 11.B.)

- 1M NaOH/2% H₂O₂
- 25% TCA/2% casamino acids (Difco® brand, Vitamin Assay Grade)
- 5% TCA
- Whatman® GF/C glass fiber filter (Whatman® Cat.# 1822A021)
- acetone
- 30% acrylamide solution
- SDS sample buffer
- separating gel 4X buffer
- stacking gel 4X buffer
- SDS polyacrylamide 10X running buffer
- fixing solution
- Whatman® 3MM filter paper
- **optional:** precast polyacrylamide gels

5.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/C glass fiber filter with a small amount of cold 5% TCA. Collect the precipitate by vacuum filtering 250 μ l of the TCA reaction mix (Step 3). 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 ³⁵S incorporation, put the filter in 1–3ml of 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. The measured counts per minute (cpm) are the “cpm of unwashed filter”.
7. To determine background, remove 2 μ l from a 50 μ l translation reaction containing no RNA, and proceed as described in Steps 1–5.
8. The following is a calculation to determine percent incorporation:

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

5.A. Determination of Percent Incorporation of Radioactive Label (continued)

9. The following is a calculation to determine the amount of stimulation above background levels:

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

5.B. Denaturing Gel Analysis of Translation Products

For information on preparing SDS-polyacrylamide gels and separating proteins by electrophoresis, refer to the *Protocols and Applications Guide, Online Edition* (11). Alternatively, precast polyacrylamide gels are available from a number of manufacturers. For protein analysis, 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 a variety of Tris-Glycine, Tricine and Bis-Tris gels for resolution of proteins under different conditions and over a broad spectrum of sizes. The NOVEX® 4-20% Tris-Glycine gradient gels (NOVEX® Cat.# EC6025 or EC60355) and Bio-Rad® Ready Gel 4-20% Tris-Glycine Gel, 10-well (Bio-Rad® Cat.# 161-1105EDU) are convenient for resolving proteins over a wide range of molecular weights. In addition to convenience and safety, precast gels give 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.
2. Cap the tube, and heat at 70°C for 15 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 usually is performed until the bromophenol blue dye has run off the bottom of the gel. However, the dye front also contains the free labeled amino acids, so disposal of unincorporated label may be easier if the gel is stopped while the dye front remains in the gel. Proceed to Step 7 for Western blotting analysis.

5. Place the polyacrylamide gel in a plastic box, and cover the gel with fixing solution (as prepared in Section 11.B). Agitate slowly on an orbital shaker for 30 minutes. 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 ³⁵S-, ¹⁴C- and ³H-labeled proteins. The increased 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 for exposure to film as follows: Soak the gel in a mixture of 7% acetic acid, 7% methanol, 1% 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. Cut or mark one corner of the filter to help discern the gel orientation on the filter. Expose the gel on X-ray film for 1-6 hours at -70°C (with fluorography) or 6-15 hours at room temperature (with autoradiography).
7. For Western blot analysis of proteins, transfer the protein from the gel onto nitrocellulose or PVDF membrane (12,13). Usually Western blots are made by electrophoretic transfer of proteins from SDS-polyacrylamide gels. Detailed procedures for electrophoretic blotting usually are included with commercial devices and can be found in references 12, 14, 15 and 16. A general discussion of Western blotting with PVDF membranes is found in reference 17. PVDF membranes must be prewet in methanol or ethanol before equilibrating in transfer buffer. The blot then may be subjected to immunodetection analysis or exposed to X-ray film. To detect biotinylated protein using the Transcend™ Non-Radioactive Translation Detection Systems, refer to Technical Bulletin #TB182.

6. Luciferase Positive Control Translation Reaction

The assay for firefly luciferase activity is extremely sensitive, rapid and easy to perform. It is a good control for in vitro translation reactions because only full-length luciferase is active (see Figure 1). Additionally, luciferase is a monomeric protein (61kDa) that does not require post-translational processing or modification for enzymatic activity. Promega has developed the Luciferase Assay System to provide an assay that is substantially improved over conventional assays in both sensitivity and simplicity (18).

6.A. 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 amino acid mixtures, a sufficient concentration of all amino acids is provided. As an alternative to assaying luciferase activity, a non-radioactive luciferase control may be performed using the Transcend™ tRNA and Transcend™ Non-Radioactive Detection Systems. For more information on these systems, request Technical Bulletin #TB182.

1. Assemble the following reaction:

Flexi® Rabbit Reticulocyte Lysate (see Note 1, Section 3)	35µl
Amino Acid Mixture, Minus Leucine, 1mM	0.5µl
Amino Acid Mixture, Minus Methionine, 1mM	0.5µl
Potassium Chloride, 2.5M (see Note 5, Section 3)	1.4µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
Luciferase Control RNA, 1mg/ml	1µl
Nuclease-Free Water	10.6µl
final volume	50µl

2. Incubate the translation reactions at 30°C for 60–90 minutes.
3. The luciferase control reactions can be stored at –20°C for up to 2 months or at –70°C for up to 6 months with little loss of luciferase activity.
4. The reaction can be tested for the synthesis of functional luciferase using the standard luciferase assay (see Section 7).

6.B. Radioactive Luciferase Control Reaction

For use of radiolabeled amino acids other than [³⁵S]methionine, see Table 1, Section 3.

1. Assemble the following reaction:

Flexi® Rabbit Reticulocyte Lysate (see Note 1, Section 3)	35µl
Amino Acid Mixture, Minus Methionine, 1mM	1µl
[³⁵ S]methionine (1,200Ci/mmol) at 10mCi/ml (see Note 2, Section 3)	2µl
Potassium Chloride, 2.5M (see Note 5, Section 3)	1.4µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
Luciferase Control RNA, 1mg/ml	1µl
Nuclease-Free Water to a final volume of	50µl

2. Follow Steps 2 through 4, Section 6.A.

7. 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 also should be at ambient temperature.

Either a luminometer or 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.

7.A. Using a Luminometer

1. Dispense 50µl of the Luciferase Assay Reagent into luminometer tubes, one tube per sample.

Note: The Luciferase Assay Reagent should be at ambient temperature prior to performing a luciferase assay.

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 vortexing 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.

7.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 can still 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; it will inactivate the luciferase and is not needed.

Use the same protocol as luciferase assays using a luminometer (Section 7.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, 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.

8. Related Procedures

8.A. Synthesis of Milligram Quantities of In Vitro Transcripts

In vitro transcription reactions are used widely to synthesize microgram amounts of RNA probes from recombinant DNA templates. The RiboMAX™ Large Scale RNA Production Systems produce milligram amounts of RNA. These systems consistently produce 2–5mg/ml of RNA in a 1ml reaction, about 10- to 20-fold more RNA than is produced with a standard Riboprobe® transcription reaction. The T7 RiboMAX™ Express System uses an optimized enzyme mix and transcription buffer to generate yields of 5–8.5mg/ml in 30 minutes.

An additional advantage of the RiboMAX™ Systems is that RNA synthesized using these systems is of higher quality for in vitro translation in the Flexi® Rabbit Reticulocyte Lysate System than RNA synthesized by standard methods (19). This is especially evident at high RNA concentrations, which normally inhibit in vitro translation (20,21). These systems are useful to researchers wishing to produce large amounts of RNA for in vitro translation. The reduction of components inhibiting translation also may be advantageous for other applications requiring biologically active RNA.

An important consideration when preparing DNA templates for transcription is to avoid linearizing the DNA with restriction enzymes that leave 3', 4-base overhangs (PstI, KpnI, SacI, SacII, BstXI, NsiI, ApaI and AatII), as aberrant transcription products can be produced (22). If no alternative enzyme is available, 3'-overhangs can be removed to produce blunt ends using DNA Polymerase I Large (Klenow) Fragment (Cat.# M2201, M2206) prior to transcription.

For a protocol and further information regarding use of the RiboMAX™ RNA Production System, refer to the *RiboMAX™ Large Scale RNA Production Systems Technical Bulletin #TB166*. This document is available online at: www.promega.com/tbs/

8.B. In Vitro Synthesis of Capped RNA Transcripts

Most eukaryotic mRNAs contain a m⁷G(5')ppp(5')G cap at the 5'-end, which is important for binding of translation initiation factors and contributes to mRNA stability. The use of capped RNA is suggested for programming particular translation systems, such as *Xenopus* oocytes. Many transcripts do not require a cap structure for efficient translation in the Flexi® Rabbit Reticulocyte Lysate System, but enhanced translation of certain capped transcripts has been observed (20,23,24).

Increasingly, uncapped messages are being used effectively in reticulocyte lysate systems, provided the proper concentration of the appropriate potassium salt is supplied (24,25). In rabbit reticulocyte lysate, potassium chloride (not potassium acetate) at levels 20mM above the maximal stimulatory level have been shown to provide optimal conditions for the synthesis of authentic products from uncapped mRNA (9). Optimal translation efficiency for capped or uncapped transcripts can be obtained with potassium chloride titrations.

8.B. In Vitro Synthesis of Capped RNA Transcripts (continued)

The Ribo m⁷G Cap Analog (Cat.# P1711) can be used in combination with the Riboprobe® or RiboMAX™ Systems to prepare RNA transcripts with a 5' m⁷G cap structure. For further information, including a protocol for synthesis of capped RNA transcripts in vitro, refer to the *Riboprobe® In Vitro Transcription Systems Technical Manual #TM016*.

9. 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
The control reaction does not produce luciferase	Loss of activity of components. Do not use the lysate after more than two freeze-thaw cycles. Do not use reagents after the expiration date.
Low protein yield	<p>RNA concentration was not optimized. The optimal RNA concentration for translation should be determined prior to performing definitive experiments. To determine the optimal concentration, serially dilute your RNA template first, then add the same volume of RNA to each reaction to ensure that other variables are kept constant. For more information on RNA concentration, see Section 3, Note 8.</p> <p>Incubation of the translation reaction at 37°C can decrease protein synthesis. Incubate at 30°C.</p> <p>Addition of spermidines and certain diamines can stimulate translation (6). With the addition of spermidines and diamines, the optimal Mg²⁺ concentration will change. Therefore, these components must be co-optimized.</p> <p>Potassium or magnesium concentration was not optimized. Optimal potassium concentration varies at 30–120mM, depending on the mRNA used. Additional potassium can be added if the initial translation results are poor. Specific mRNAs may require altered magnesium concentrations. A range of 0.5–2.5mM of magnesium in addition to that endogenously present in the lysate generally is sufficient for the majority of mRNAs used (26).</p>

Symptoms	Causes and Comments
Low protein yield (continued)	<p>Inhibitors were present in the translation reaction. To determine if inhibitors are present in the mRNA preparation, mix mRNA with Luciferase Control RNA and determine if translation of the Luciferase Control RNA is inhibited relative to a control translation containing Luciferase Control RNA alone. Oxidized thiols, low concentrations of double-stranded RNA and polysaccharides are typical inhibitors of translation (2).</p> <p>Calcium was 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 mRNA template.</p> <p>Ethanol or salt was present in the translation reaction. Ethanol or salt in the RNA preparation or labeled amino acids may inhibit translation.</p>
Unexpected bands present at higher molecular weights	Denaturing temperature was too high. Denature sample at a lower temperature (e.g., 60–80°C).
Unexpected bands present on the gel	<p>Proteolysis of translation product. Add a protease inhibitor, such as α-macroglobulin, leupeptin or chymostatin.</p> <p>More than one peptide was translated from the RNA template. Leaky scanning for translation initiation can result in translation initiating at internal downstream methionines.</p> <p>³⁵S-labeled amino acid was beyond its expiration date. Older ³⁵S may dissociate from the amino acid and label other proteins in the lysate. Use fresh ³⁵S.</p> <p>The [³⁵S]methionine used was not of translational grade. There are reports of a 42kDa band with some grades of [³⁵S]methionine (3). We recommend EasyTag™ L-[³⁵S]methionine (PerkinElmer Cat.# NEG709A) to avoid this 42kDa band.</p> <p>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.</p>

9. Troubleshooting (continued)

Symptoms	Causes and Comments
Unexpected bands present on the gel (continued)	Aminoacyl tRNAs may produce background bands (~25kDa). Add RNase A to the reaction (after completion) to a final concentration of 0.2mg/ml. Incubate for 5 minutes at 30°C. Oxidized β -mercaptoethanol was present or not enough SDS in the loading buffer. Use a loading buffer that contains 2% SDS and 100mM DTT.
Smearing on the gel	Gel was 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 was loaded on the gel. Check the amount of sample loaded on the gel and the amount of loading buffer. Too much protein loaded onto the gel can cause smearing. Acrylamide concentration was too low to resolve proteins. Acrylamide concentration can be increased to 12%. Ethanol was present in the sample. Ethanol present in the sample can cause smearing on the gel.

10. References

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11. Appendix

11.A. Approximate Endogenous Amino Acid Pools (μM) of the Flexi[®] Lysate

Amino Acid	Concentration (μM)	Amino Acid	Concentration (μM)	Amino Acid	Concentration (μM)
Ala	157	Gly	1050	Pro	87
Asn	51	His	14	Ser	93
Asp	1093	Ile	9	Thr	59
Arg	41	Leu	5	Trp	1
Cys	2	Lys	51	Tyr	3
Gln	200	Met	5	Val	30
Glu	260	Phe	4		

11.B. 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 sample buffer

50mM Tris-HCl (pH6.8)
2% SDS
0.1% bromophenol blue
10% glycerol
100mM dithiothreitol

1X SDS sample 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

Bring to a 1 liter final volume.

stacking gel 4X buffer

6.06g Tris base
4ml 10% SDS

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

separating gel 4X buffer

18.17g Tris base
4ml 10% SDS

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

11.C. Related Products

RNA Transcription Systems

Product	Size	Cat.#
T7 RiboMAX [™] Express Large Scale RNA Production System	1 system	P1320
RiboMAX [™] Large Scale RNA Production System – SP6	50 reactions	P1280
RiboMAX [™] Large Scale RNA Production System – T7	50 reactions	P1300
Riboprobe [®] System – SP6	25 reactions	P1420
Riboprobe [®] System – T3	25 reactions	P1430
Riboprobe [®] System – T7	25 reactions	P1440
Riboprobe [®] Combination System – T3/T7 RNA Polymerase	25 reactions	P1450
Riboprobe [®] Combination System – SP6/T7 RNA Polymerase	25 reactions	P1460

For Laboratory Use.

RNasin[®] Ribonuclease Inhibitor

Product	Size	Cat.#
RNasin [®] Ribonuclease Inhibitor	2,500u	N2111
	10,000u	N2115
Recombinant RNasin [®] Ribonuclease Inhibitor*	2,500u	N2511
	10,000u	N2115
RNasin [®] Plus RNase Inhibitor*	2,500u	N2611
	10,000u	N2615

*For Laboratory Use.

Enzymes and Ribonucleotide Triphosphates

Product	Size	Cat.#
SP6 RNA Polymerase (High Concentration)*	2,500u	P4084
T7 RNA Polymerase (High Concentration)*	10,000u	P4074
RQ1 RNase-Free DNase*	1,000u	M6101
rATP, 100mM*	0.4ml	E6011
rCTP, 100mM*	0.4ml	E6041
rGTP, 100mM*	0.4ml	E6031
rUTP, 100mM*	0.4ml	E6021
Ribo m ⁷ G Cap Analog	10 A ₂₅₄ units	P1711
	25 A ₂₅₄ units	P1712

*For Laboratory Use.

11.C. Related Products (continued)

Rabbit Reticulocyte Lysate/Wheat Germ Extract Systems

Product	Size	Cat.#
Rabbit Reticulocyte Lysate, Nuclease-Treated*	30 × 50µl reactions	L4960
Rabbit Reticulocyte Lysate, Untreated	30 × 50µl reactions	L4151

Bulk Rabbit Reticulocyte Lysate is available from Promega. *For Laboratory Use.

TnT® Quick Coupled Transcription/Translation Systems

Product	Size	Cat.#
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® T7 Quick Coupled Transcription/Translation System*	40 × 50µl reactions	L1170
TnT® T7 Quick Coupled Transcription/Translation System Trial Size*	5 × 50µl reactions	L1171
TnT® T7 Quick for PCR DNA*	40 × 50µl reactions	L5540
TnT® SP6 High-Yield Wheat Germ Protein Expression System	40 × 50µl reactions	L3260
	5 × 50µl reactions	L3261

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Protein Expression System

Product	Size	Cat.#
S30 T7 High-Yield Protein Expression System	24 reactions	L1110
	8 reactions	L1115

Co-Translation Labeling Systems

Product	Size	Cat.#
Transcend™ Colorimetric Non-Radioactive Translation Detection System	30 × 50µl reactions	L5070
Transcend™ Chemiluminescent Non-Radioactive Translation Detection System	30 × 50µl reactions	L5080
Transcend™ Biotinylated tRNA	30µl	L5061
FluoroTect™ Green _{LV8} in vitro Translation Labeling System	40 reactions	L5001

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