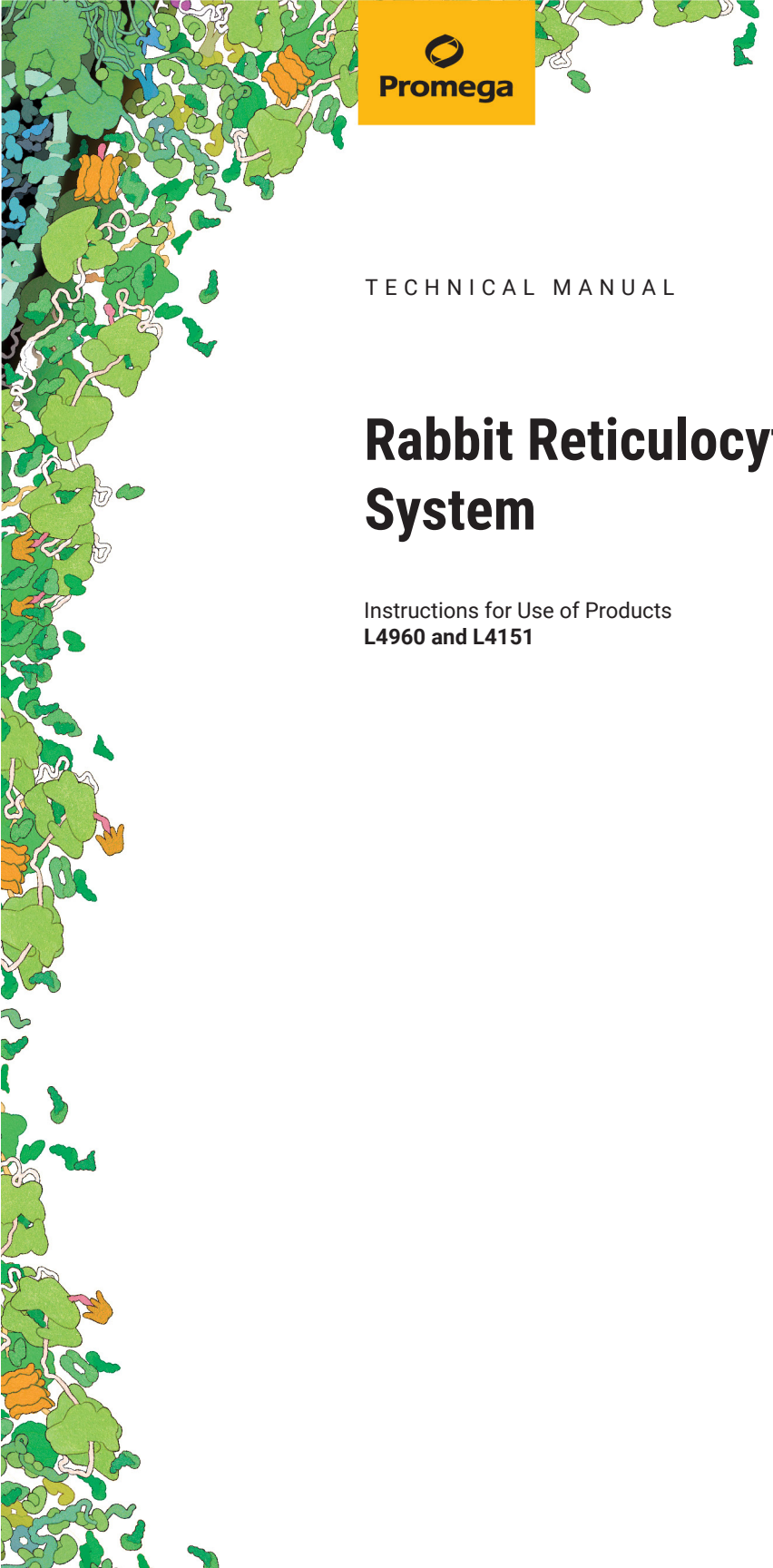

TECHNICAL MANUAL

Rabbit Reticulocyte Lysate System

Instructions for Use of Products
L4960 and L4151

Revised 9/25
TM232

Rabbit Reticulocyte Lysate System

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

Promega

1. Description

1.A. Rabbit Reticulocyte Lysate, Nuclease Treated

Rabbit Reticulocyte Lysate Translation Systems are used for characterizing protein products from RNA transcripts and investigating transcriptional and translational control. Rabbit Reticulocyte Lysate is prepared from New Zealand white rabbits using a standard protocol (1) that ensures reliable and consistent reticulocyte production in each lot. The reticulocytes are purified to remove contaminating cells, which could otherwise alter the translational properties of the final extract. After the reticulocytes are lysed, the extract is treated with micrococcal nuclease to destroy endogenous mRNA and thus reduce background translation to a minimum (1). The lysate contains the cellular components necessary for protein synthesis (tRNA, ribosomes, amino acids, initiation, elongation and termination factors). Rabbit Reticulocyte Lysate is optimized further for mRNA translation by the addition of the following:

- an energy-generating system consisting of prequalified phosphocreatine and phosphocreatine kinase
- a mixture of tRNAs to expand the range of mRNAs that can be translated
- hemin, to prevent inhibition of initiation
- potassium acetate and magnesium acetate

Rabbit Reticulocyte Lysate may contain a variety of post-translational processing activities, including acetylation, isoprenylation and some phosphorylation activity that will vary from lot to lot (2).

1.B. Rabbit Reticulocyte Lysate, Untreated

Untreated Rabbit Reticulocyte Lysate contains the cellular components necessary for protein synthesis (tRNA, ribosomes, amino acids, initiation, elongation and termination factors) but has not been treated with micrococcal nuclease. Untreated lysate is used primarily for the isolation of these components and as an abundant source of endogenous globin mRNA. Untreated lysate is prepared from New Zealand white rabbits in the same manner as treated lysates with the exception that the lysate is not treated with micrococcal nuclease (1). The reticulocytes are purified to remove contaminating cells. The packed cells then are lysed by the addition of water. Untreated Rabbit Reticulocyte Lysate is provided in 1ml aliquots.

Note: Untreated Rabbit Reticulocyte Lysate is not supplemented with tRNA, creatine phosphate, creatine phosphokinase, DTT, potassium acetate, magnesium chloride or hemin. Untreated Rabbit Reticulocyte Lysate is not recommended for in vitro translation without further supplements.

When supplemented with hemin (20µM final concentration), Untreated Rabbit Reticulocyte Lysate synthesizes protein from added mRNA templates at approximately the same rate as treated reticulocyte lysate for up to 60 minutes. The primary disadvantage of such a system for in vitro translation is that the template mRNA is translated in competition with endogenous globin mRNA, making quantitation of the activity of the exogenous mRNA very difficult.

The lysate may be treated with micrococcal nuclease to destroy endogenous mRNA (6). In the presence of calcium as a cofactor, nuclease treatment is very effective in eliminating globin mRNA using incubation times as short as 20 minutes. EGTA is then added to chelate the calcium and thereby inactivate the nuclease. Untreated Rabbit Reticulocyte Lysate may be optimized further by adding the components listed in Table 2, Section 4.A. Standard stock solutions and their preparation are given in Section 10.A. Please see Section 4.A for a standard translation protocol.

For applications, see reference 3.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Rabbit Reticulocyte Lysate System, Nuclease Treated	**5 × 200µl**	**L4960**

Rabbit Reticulocyte Lysate is provided in 200µl aliquots. Each system contains sufficient reagents to perform approximately 30 translation reactions per milliliter of lysate. Includes:

- 1ml Rabbit Reticulocyte Lysate, Nuclease Treated (5 × 200µl)
- 50µl Amino Acid Mixture Minus Methionine, 1mM
- 50µl Amino Acid Mixture Minus Leucine, 1mM
- 50µl Amino Acid Mixture Minus Cysteine, 1mM
- 10µl Luciferase Control RNA

PRODUCT	SIZE	CAT.#
Rabbit Reticulocyte Lysate System, Untreated	**1ml**	**L4151**

Bulk Rabbit Reticulocyte Lysate is available from Promega.

Storage/Stability: Store all components below –65°C. Product is sensitive to CO_2 (avoid prolonged exposure) and multiple freeze-thaw cycles, which may have an adverse effect on activity and performance.

Do not store lysate in the presence of dry ice. In addition, **do not** freeze-thaw the lysate more than 2 times.

3. General Considerations

In vitro translation systems are used to characterize plasmid clones, study mutations, examine translational signals and characterize mRNA populations rapidly. Two basic types of system are available: 1) translation systems for RNA templates, and 2) coupled transcription/translation systems for DNA templates. Many factors influence the choice of an in vitro translation system; some of these considerations are summarized in Section 10.B, Related Products.

The reaction conditions provided below are optimized for the Luciferase Control RNA supplied with the system and should be considered as a starting point for experiments. Many factors affect translation efficiency of specific mRNAs in the Rabbit Reticulocyte Lysate System and should be considered when designing in vitro translation experiments. The optimal mRNA concentration will vary for particular transcripts and should be determined empirically. In addition, the presence of certain nucleic acid sequence elements can have profound effects on initiation fidelity and translation efficiency. Poly(A)+ tails, 5´-caps, 5´-untranslated regions and the sequence context around the AUG start (or secondary AUGs in the sequence) (4) all may affect translation of a given mRNA.

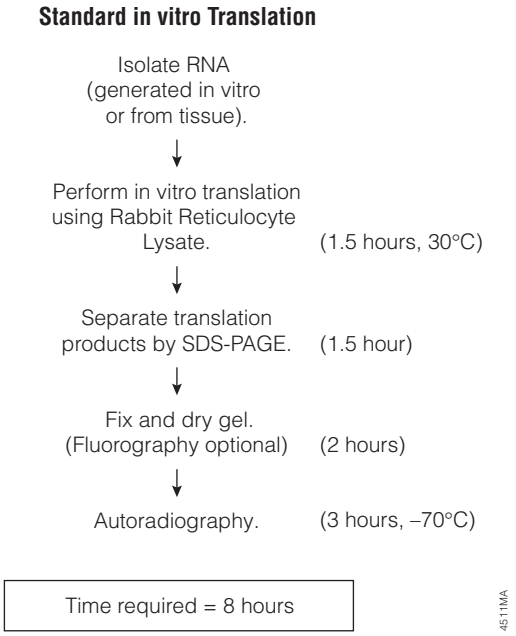

Figure 1. Flow chart of in vitro translation procedure using Rabbit Reticulocyte Lysate.

4. Rabbit Reticulocyte Lysate System Translation Procedure

4.A. Translation Procedure

The following is a general guideline for setting up a translation reaction. Examples are provided for standard reactions using [^{35}S]methionine (radioactive) or non-radioactive translation systems. With the Transcend™ Non-Radioactive Translation Detection System, biotinylated lysine residues are incorporated into nascent proteins during translation, eliminating the need for labeling with [^{35}S]methionine or other radioactive amino acids. This 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. For more information on the Transcend™ Systems, refer to Technical Bulletin #TB182. An alternative method for non-radioactive translation is the FluoroTect™ Green$_{Lys}$ in vitro Translation System, which allows fluorescent labeling of in vitro translated proteins. More information on this system is available in Technical Bulletin #TB285. All technical bulletins and manuals are available online at: **www.promega.com/protocols/**

4.A. Translation Procedure (continued)

Several measures should be taken to reduce the chance of RNase contamination. Wear gloves throughout the experiment. Use microcentrifuge tubes and pipette tips that have been exposed only to gloved handling. Adding 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 or RNasin® Plus RNase Inhibitor (Cat.# N2111, N2511 or N2611)
- Nuclease-Free Water (Cat.# P1193)
- appropriately labeled amino acid
- Transcend™ Colorimetric (Cat.# L5070) or Chemiluminescent (Cat.# L5080) Translation Detection System or FluoroTect™ $Green_{Lys}$ in vitro TranslationLabeling System (Cat.# L5001; for non-radioactive detection)
- Transcend™ tRNA (Cat.# L5061; for non-radioactive detection)

1. Remove the reagents from storage, and allow them to thaw slowly **on ice**.
2. **Optional:** 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 destroying local regions of secondary structure.
3. Assemble the reaction components, appropriate to the label being used, in a 0.5ml polypropylene microcentrifuge tube. Gently mix the lysate by stirring with a pipette tip after adding each component. If necessary, centrifuge briefly to return the sample to the bottom of the tube. **We recommend including a control reaction containing no mRNA.** This allows measurement of any background incorporation of labeled amino acids.

   Note: For control reactions using Luciferase Control RNA, please see Section 6.

Examples of Standard Reactions

Component	Standard Reaction Using [^{35}S]methionine	Standard Reaction Using Transcend™ tRNA
Rabbit Reticulocyte Lysate	35µl	35µl
Amino Acid Mixture Minus Leucine, 1mM	–	0.5µl
Amino Acid Mixture Minus Methionine, 1mM	1µl	0.5µl
[^{35}S]methionine (1,200Ci/mmol at 10mCi/ml) (see Notes 2 and 3)	2µl	–
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl	1µl
RNA substrate in water (1µg/µl) (see Notes 4 and 5)	2µl	2µl
Transcend™ Biotin-Lysyl-tRNA (see Note 8) or FluoroTect™ tRNA $Green_{Lys}$ tRNA	–	1–2µl

Note: Smaller scale translation reactions may be performed by reducing the recommended volumes proportionally.

Note: A lysate volume of 35µl (70% concentration) is optimal for translation of many RNAs. However, with certain RNAs, translation efficiency may increase by varying the lysate volume from 25µl (50% concentration) to 35µl (70% concentration). To achieve maximal expression in a reaction containing diluted lysate, you may need to optimize the Mg^{2+} and K^{+} concentrations.

4. Immediately incubate the translation reactions at 30°C for 90 minutes.

5. Analyze the results of translation. Procedures are provided for incorporation assays (Section 6.A), gel analysis of translation products (Section 6.B) and an assay for luciferase production in the control reactions (Section 8). For analysis of reactions using Transcend™ tRNA, refer to the *Transcend™ Non-Radioactive Detection Systems* Technical Bulletin #TB182. Protocols for using the FluoroTect™ $Green_{Lys}$ In Vitro Translation Labeling System are provided in Technical Bulletin #TB285.

Notes:

1. The addition of RNasin® Ribonuclease Inhibitor to the translation reaction is recommended but not required. RNasin® Ribonuclease Inhibitor acts to inhibit contaminating RNases.

2. We have found acceptable results using 1–4µl of [^{35}S]methionine (1,200Ci/mmol) at 10mCi/ml. Depending upon the translational efficiency of the experimental RNA and number of methionine residues present in the protein, the amount of [^{35}S]methionine can be adjusted to balance exposure time versus cost of label. Other radiolabeled amino acids can be used with the Rabbit Reticulocyte Lysate System. See Table 1 for recommendations.

Table 1. Recommended Volumes of Alternative Radiolabeled Amino Acids.

Amino Acid	Volume to Add to Reaction
[^{3}H]leucine (100–200Ci/mmol)	5µl
[^{14}C]leucine (300mCi/mmol)	5µl
[^{35}S]cysteine (1,200Ci/mmol)	5µl

3. We recommend using a grade of [^{35}S]methionine, PerkinElmer EasyTag™ L-[^{35}S]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).

4.A. Translation Procedure (continued)

4. An unfractionated total cytoplasmic RNA preparation is 90–95% rRNA, and as a result the mRNA translates poorly. Usually such preparations yield no better than 20–30% of the maximum incorporation attainable, and high final concentrations of 100–200µg/ml of RNA 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. For in vitro transcripts produced with the RiboMAX™ Large Scale RNA Production Systems (see Section 8.A), a final concentration of 5–80µg/ml in vitro transcript may be used for the translation. Using the RiboMAX™ Systems, milligram quantities of RNA can be produced. RNA from other standard transcription procedures may contain components at concentrations that inhibit translation. Therefore, a lower concentration of 5–20µg/ml in vitro transcript should be used.

   For preparation of DNA that will be used as a template for run-off RNA transcript production, the Wizard® SV DNA Purification Systems or Wizard® PCR Preps DNA Purification System are recommended. The SV Total RNA Isolation System is ideal for purifying total RNA free from DNA contamination. The PolyATtract® mRNA Isolation Systems, which are based on MagneSphere® technology, may be used for purifying run-off RNAs or isolating poly(A)+ mRNA, free from other nucleic acid contamination, in approximately 45 minutes.

5. Average preparations of mRNA give a stimulation over background of about 10- to 20-fold. If the translation efficiency of sample mRNA is low, refer to Section 9, Troubleshooting.

6. Hemin is added to Rabbit Reticulocyte Lysate because it suppresses an inhibitor of the initiation factor eIF2α. In the absence of hemin, protein synthesis in the Reticulocyte Lysate System will cease after a short period of incubation (5).

7. Adding polyamines, such as spermidine (0.1–0.5mM) and certain diamines (0.1–40mM), can stimulate translation (6). Adding these compounds results in reduction of the corresponding optimal Mg^{2+} concentration for a given RNA. Therefore, less Mg^{2+} is required for the translation reaction.

8. 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.

9. Except for the actual translation incubation, all Rabbit Reticulocyte Lysate System components should be kept on ice while in use. Unused Rabbit Reticulocyte Lysate should be frozen on a dry ice/ethanol bath and stored below –65°C. **Do not freeze-thaw the lysate more than two times.**

10. Each batch of Rabbit Reticulocyte Lysate contains 100–200mg/ml of endogenous protein (using BSA as a standard).

11. Use capped plastic vials or covered multiwell plates. This avoids changes in the reaction volume that may affect the concentration of important components.

12. 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 room temperature.

Table 2. Final Concentrations of Rabbit Reticulocyte Lysate Components Added to a 50µl Translation Reaction.

Component	Concentration
Creatine phosphate	10mM
Creatine phosphokinase	50µg/ml
DTT	2mM
Calf liver tRNA	50µg/ml
Potassium acetate	79mM
Magnesium acetate	0.5mM
Hemin	0.02mM

Table 3. Approximate Endogenous Amino Acid Pools.

Amino Acid	Concentration (µM)	Amino Acid	Concentration (µM)
Ala	157	Leu	5
Asn	51	Lys	51
Asp	1,093	Met	5
Arg	41	Phe	4
Cys	2	Pro	87
Gln	200	Ser	93
Glu	260	Thr	59
Gly	1,050	Trp	1
His	14	Tyr	3
Ile	9	Val	30

These amino acid concentrations should be used only as estimates. These values are not determined for individual lots of Rabbit Reticulocyte Lysate.

5. Post-Translational Analysis

Materials to Be Supplied by the User
(Solution compositions are provided in Section 10.A)

- 1M NaOH/2% H_2O_2
- 25% TCA/2% casamino acids (Difco® brand, Vitamin Assay Grade)
- 5% TCA
- Whatman® GF/A glass fiber filter (Whatman® Cat.# 1820021)
- acetone
- 30% acrylamide solution
- fixing solution
- separating gel 4X buffer
- stacking gel 4X buffer
- SDS polyacrylamide 10X running buffer
- **optional:** precast polyacrylamide gels
- fixing solution
- Whatman® 3MM filter paper

5.A. Determining Percent Incorporation of Radioactive Label

1. After the 50µl translation reaction is complete, remove 2µl from the reaction and add it to 98µl of 1M NaOH/2% H_2O_2.
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 with a small amount of cold 5% TCA. Collect the precipitate by vacuum filtering 250µl of the TCA reaction mix (Step 3). Rinse the filter three 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 ^{35}S incorporation, put the filter in 1–3ml of appropriate scintillation fluid, 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 described in Step 5. The measured counts per minute (cpm) are the cpm in 5µl of the TCA reaction mix.

7. To determine background counts, remove 2µl from a 50µl translation reaction containing no RNA 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 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

The most widely applicable and versatile method for analysis of cell-free translation products synthesized from mixtures of RNAs is SDS-PAGE analysis. A 10% acrylamide separating gel gives good separation of peptide mixtures between 20kDa and 100kDa, with peptides between 55kDa and 60kDa migrating halfway down the length of the gel.

Alternatively, precast polyacrylamide gels are available from a number of manufacturers. For protein analysis, Invitrogen 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 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 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 100°C for 2 minutes to denature the proteins.

   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).
3. Load a small aliquot (5–10µl) onto an SDS-PAGE 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 a stacking gel and 30mA in a 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. 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 10.A). 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 ^{35}S-, ^{14}C- and ^{3}H-labeled proteins. 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 (Cytiva), can be used for fluorographic enhancement of signal. Alternatively, the fixed gel can be exposed to a phosphor 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. 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 to help in discerning 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 (immobilize) the protein from the gel onto a nitrocellulose or PVDF membrane (7,8). 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 7, 9, 10 and 11. A general discussion of Western blotting with PVDF membranes is found in reference 12. PVDF membranes must be prewet in methanol or ethanol before equilibrating in transfer buffer. The blot then may be subjected to immunodetection analysis. For more information, refer to the *Protocols and Applications Guide*, Third Edition (13).

6. Positive Control Translation Reactions Using Luciferase

The assay for firefly luciferase activity is extremely sensitive, rapid and easy to perform. It is a good control for in vitro translations because only full-length luciferase is active. Additionally, luciferase is a monomeric protein (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.

6.A. Non-Radioactive Luciferase Control Reaction

Note: To analyze these reactions using a standard luciferase assay, Luciferase Assay Reagent is required. Both the Amino Acid Mixture Minus Leucine and the Amino Acid Mixture Minus Methionine are used in this reaction. By using both incomplete mixes, a sufficient concentration of all amino acids is obtained. As an alternative to assaying luciferase activity, this reaction can be performed using the Transcend™ tRNA and Non-Radioactive Detection Systems or the FluoroTect™ $Green_{Lys}$ in vitro Translation System.

1. Assemble the following reaction:

Rabbit Reticulocyte Lysate (see Section 4.A)	35µl
Amino Acid Mixture Minus Leucine, 1mM	0.5µl
Amino Acid Mixture Minus Methionine, 1mM	0.5µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
Luciferase Control RNA (1µg/µl)	2µl
Nuclease-Free Water	11µl
Final volume	**50µl**

2. Incubate the translation reaction at 30°C for 90 minutes.
3. Test for the synthesis of functional luciferase using either the standard luciferase assay (Section 7.A) or photographic luciferase assay (Section 7.B).
4. 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.

6.B. Radioactive Luciferase Control Reaction

This section provides information on how to perform a radioactive luciferase control reaction. For use of radiolabeled amino acids other than [^{35}S]methionine, see Section 4.A.

1. Assemble the following reaction:

Rabbit Reticulocyte Lysate (see Section 4.A)	35µl
Amino Acid Mixture Minus Methionine, 1mM	1µl
[^{35}S]methionine (1,200Ci/mmol) at 10mCi/ml (see Notes 2 and 3, Section 4.A)	2µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
Luciferase Control RNA (1µg/µl)	2µl
Nuclease-Free Water	9µl
Final volume	**50µl**

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

7. Positive Control Luciferase Assays

7.A. Standard Luciferase Assay (Luminometer or Scintillation Counter)

Scintillation counters should be used in the manual mode because light generated by the luminescent reaction decays slowly ($t_{1/2}$ is approximately 5 minutes). Samples should be introduced into the counting chamber of the instrument shortly after light production is initiated. Because the enzymatic reaction produces light at all wavelengths, samples to be quantitated in a scintillation counter should be measured with all channels open (“open window”). Measure light produced for a period of 10 seconds to 6 minutes. The light intensity of the reaction is nearly constant for about 20 seconds and then decays slowly.

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 be produced by calculating the square root of the measured cpm minus background cpm (i.e., [sample – background]$^{1/2}$). To measure background cpm, read a water blank. To measure activity in your sample, place the sample in a microcentrifuge tube, and then place the tube in the scintillation vial. Do not add scintillant; it will inactivate the luciferase.

It may be necessary to make a significant dilution of the sample (for a first check, dilute the sample 1:10,000 in water containing 1mg/ml BSA). If the photomultiplier tubes are saturated by too much light output, the scintillation counter may either produce no reading or identical readings from dilution to dilution.

7.A. Standard Luciferase Assay (Luminometer or Scintillation Counter; continued)

In many luminometers, the photomultiplier tube requires 1–2 seconds to stabilize after a sample is introduced. Therefore, allow an initial delay of 2–3 seconds, and then measure luminescence for 10 seconds to 5 minutes.

1. Add 50µl of **room-temperature** Luciferase Assay Reagent (LAR) to a luminometer tube (see Note below). It is important that the LAR be equilibrated **fully** to 25°C before beginning measurements. This can be accomplished by placing the tube containing the LAR into a container of room-temperature water for 30 minutes prior to use. The sample to be assayed should also be at room temperature.

   Note: Light intensity is a measure of the rate of catalysis by luciferase and thus is dependent upon temperature. For historical reasons, Promega performs the quality control assay for luciferase at 30°C using a water-heated luminometer. However, reproducible luciferase assay readings will result with assay temperatures of 20–25°C, which allows the assay to be performed without special equipment.

2. Add 2.5µl of the 50µl luciferase control translation reaction to the 50µl of LAR. Pipet quickly to mix, and place the reaction in a luminometer or scintillation counter (place the microcentrifuge tube inside the scintillation vial). Consult the appropriate operator's manual for operation of luminometers and scintillation counters.

7.B. 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, although individuals may differ in their ability to detect these low photon levels.

Note: One to two microliters of the control reaction can be added to 25µl LAR if desired.

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. An important consideration in 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 (14). If no alternative enzyme is available, 3´-overhangs can be removed to produce blunt ends using DNA Polymerase I Large (Klenow) Fragment prior to transcription.

The RiboMAX™ Large Scale RNA Production Systems are used to produce milligram amounts of RNA. These systems consistently produce 2–6mg/ml of RNA in a 1ml reaction, about 10- to 20-fold more RNA than is produced with a standard Riboprobe® System transcription reaction.

An additional advantage of the RiboMAX™ Systems is that the RNA synthesized using these systems is of higher quality for in vitro translation in Rabbit Reticulocyte Lysate Systems than RNA synthesized by standard methods (15). This is especially evident at high RNA concentrations, which normally inhibit in vitro translation (16). These systems are useful to researchers wishing to produce large amounts of RNA for in vitro translation. The reduction of components inhibitory to translation also may be advantageous for other applications requiring biologically active RNA.

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, which is available at: **www.promega.com/protocols/**

8.B. In vitro Synthesis of Capped RNA Transcript

Most eukaryotic mRNAs contain a $m^7G(5')ppp(5')G$ cap at the 5´-end, which is important for the 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 Rabbit Reticulocyte Lysate System. However, enhanced translation of certain capped transcripts has been observed (16).

Increasingly, uncapped messages are being used effectively in the Rabbit Reticulocyte Lysate Systems. We have found that comparable levels of protein synthesis can be achieved by simply increasing the amount of uncapped RNA added to the translation reaction (17).

For further information or if capping is desired, refer to the *RiboMAX™ Large Scale RNA Production Systems Technical Bulletin* #TB166. This Technical Bulletin includes a protocol for synthesis of capped RNA transcripts using the Ribo m^7G Cap Analog (Cat.# P1711 or P1712) and is available at: **www.promega.com/protocols/**

9. Troubleshooting

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

Symptoms	Causes and Comments
Control reaction produces no luciferase	Check the freshness of the reaction components. The lysate should not be used after more than two freeze-thaws. Do not use reagents after the expiration date.
	Ethanol or salt was present in the translation reaction. Ethanol or salt in the RNA preparation may inhibit translation.
The control reactions worked, but the sample reaction did not	Final concentration of RNA is outside of the appropriate range. The final concentration of RNA is important. For more information, see Section 4.A, Note 4.
Low translation efficiency of the sample mRNA	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** and then add the same **volume** of RNA to each reaction to ensure that other variables are kept constant.
	Potassium or magnesium concentration was not optimized. Optimum potassium concentration varies from 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 utilized (18). See Tables 2 and 3 (Section 4.A) for the concentrations of key components present in Rabbit Reticulocyte Lysate. For further optimization of salt concentrations, we recommend using the Flexi® Rabbit Reticulocyte Lysate (Cat.# L4540).

Symptoms	Causes and Comments
Low translation efficiency of the sample mRNA (continued)	Inhibitors were present in the translation reaction. To determine if inhibitors are present in the mRNA preparation, mix the mRNA with Luciferase Control RNA and determine if the 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 in Rabbit Reticulocyte Lysate (1).
	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 mRNA template.
	Ethanol was present in the translation reaction. Residual ethanol should be removed from mRNA preparations and labeled amino acids before they are added to the translation reaction.
Unexpected bands are present at higher molecular weights	Denaturing temperature was 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 α-macroglobulin, leupeptin or chymostatin.
	More than one peptide was translated from the RNA template. Leaky scanning for translation initiation can result in translation initiating at internal downstream methionines.
	^{35}S-labeled amino acid was beyond its expiration date. Older ^{35}S may dissociate from the amino acid and label other proteins in the lysate. Use fresh ^{35}S.
	The [^{35}S]methionine used was not of translational grade. There are reports of a 42kDa band with some grades of [^{35}S]methionine (5). We recommend EasyTag™ L-[^{35}S]methionine (PerkinElmer Cat.# NEG709A) to avoid this 42kDa band.

9. Troubleshooting (continued)

Symptoms	Causes and Comments
Unexpected bands are present on the gel (continued)	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
	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 was present or not enough SDS loading buffer was used. Use a loading buffer that contains 2% SDS and 100mM DTT.
Low protein yield	Mg^{2+} concentration was not optimal. Titrate the amount of Mg^{2+} used in the reaction (18).
	K^+ concentration was not optimal. Titrate the amount of K^+ used in the reaction (18).
	Amount of RNA used was not optimal. Titrate the amount of RNA added (18).
	Incubation of the reaction at 37°C can decrease protein synthesis. Incubate the translation reaction at 30°C.
	The addition of polyamines, such as spermidine and certain diamines, has been shown to stimulate translation (8). With the addition of spermidines and diamines, the optimal Mg^{2+} concentration will change. Therefore, these components must be co-optimized.
	Reaction time was not optimized. Increase reaction time to 90–120 minutes.
There is 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 on 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 RNA sample. Ethanol present in the RNA sample can cause smearing on the gel.

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10. Appendix

10.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.

calf liver tRNA

10mg/ml in RNase-free water. Store at –20°C.

creatine phosphate

0.2M in RNase-free water. Make up fresh for each use.

creatine phosphokinase

5mg/ml in 20mM Tris-HCl (pH 7.6), 50% glycerol. Store at –20°C.

dithiothreitol (DTT)

0.25M solution in RNase-free water. Store at –20°C.

fixing solution

50%	methanol
10%	glacial acetic acid
40%	water

hemin (1mM)

Add 65mg hemin hydrochloride to 2.5ml of 1M KOH. Add 5.5ml of RNase-free water. Add 1ml of 1M Tris-HCl (pH 7.9) and 89ml of ethylene glycol. Add 2.0ml of 1N HCl. Add RNase-free water to a final volume of 100ml. Store in a light-proof bottle at –20°C. The final pH of a 1:50 dilution is 6.8.

magnesium acetate

0.1M solution in RNase-free water. Filter-sterilize. Store at –20°C.

SDS sample buffer

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

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.

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.

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.

10.B. Related Products

Translation Systems

A number of cell-free translation systems have been developed for use with mRNA isolated from various sources. 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 generally is recommended when microsomal membranes are to be added for cotranslational processing of translation products. We recommend Flexi® Rabbit Reticulocyte Lysate for optimizing translation of particular RNAs through adjustments to salt and DTT concentrations. Wheat Germ Extract is recommended for translation of RNA preparations containing low concentrations of double-stranded RNA (dsRNA) or oxidized thiols, which are inhibitory to Rabbit Reticulocyte Lysate.

In vitro translation reactions may be directed by either mRNAs isolated in vivo or by RNA templates transcribed in vitro from the Riboprobe® and pGEM® Vectors. Procedures for the rapid isolation and poly(A)+ selection of cellular mRNAs are provided in the *Protocols and Applications Guide*, Third Edition (13). When using mRNA synthesized in vitro, the presence of a 5´ cap structure may enhance translational activity (19).

Coupled Transcription/Translation Systems

DNA sequences cloned in plasmid vectors also may be expressed directly using either the TnT® Coupled Reticulocyte Lysate Systems, TnT® Coupled Wheat Germ Extract Systems or *E. coli* S30 Coupled Transcription/Translation Systems. The TnT® Systems require plasmid constructs containing a phage RNA polymerase promoter (SP6, T3 or T7) for the initiation of transcription, but translation in this system is under eukaryotic control. Optimal translation will occur if the AUG initiation codon is in a "Kozak consensus" context (A/GCCAUGG) (4) in the absence of inhibiting secondary structure.

For further information on other in vitro translation systems and related products, refer to the *Wheat Germ Extract Technical Manual #TM230, TnT® T7 Quick Coupled Transcription/Translation System* and *TnT® SP6 Quick Coupled Transcription/Translation System Technical Manual* #TM045, *TnT® Coupled Reticulocyte Lysate System Technical Bulletin* #TB126, *Flexi® Rabbit Reticulocyte Lysate System Technical Bulletin* #TB127, E. coli *S30 Extract Systems for Circular DNA Templates Technical Bulletin* #TB092, or E. coli *S30 Extract Systems for Linear Templates Technical Bulletin* #TB102.

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10.B. Related Products (continued)

TnT® Coupled Wheat Germ Extract Systems

Product	Size	Cat.#
TnT® SP6 Coupled Wheat Germ Extract System	40 × 50µl reactions	L4130
TnT® T7 Coupled Wheat Germ Extract System	40 × 50µl reactions	L4140
TnT® T7/SP6 Coupled Wheat Germ Extract System	40 × 50µl reactions	L5030

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

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® T7 Quick for PCR DNA	40 × 50µl reactions	L5540

Other Translation Systems

Product	Size	Cat.#
Rabbit Reticulocyte Lysate/Wheat Germ Extract Combination System	12 reactions	L4330
Wheat Germ Extract	5 × 200µl	L4380
Flexi® Rabbit Reticulocyte Lysate System	5 × 200µl	L4540

Bulk Flexi® Rabbit Reticulocyte Lysate is available from Promega.

E. coli S30 Extract Systems

Product	Size	Cat.#
E. coli S30 Extract System for Circular DNA	30 × 50µl reactions	L1020
E. coli T7 S30 Extract System for Circular DNA	30 × 50µl reactions	L1130
E. coli S30 Extract System for Linear Templates	30 × 50µl reactions	L1030

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

Product	Size	Cat.#
Luciferase Assay System	100 assays	E1500
pGEM®-*luc* DNA	20µg	E1541

RNA Production Systems

Product	Size	Cat.#
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

RNA Purification Systems

Product	Size	Cat.#
RiboMAX™ Large Scale RNA Production System—SP6	50 reactions	P1280
RiboMAX™ Large Scale RNA Production System—T7	50 reactions	P1300

Potassium Chloride and Magnesium Acetate Solutions

Product	Size	Cat.#
Potassium Chloride, 2.5M	200µl	L4591
Magnesium Acetate, 25mM	100µl	L4581

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Non-Radioactive Translation Detection Systems

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

RNasin® Ribonuclease Inhibitor

Product	Size	Cat.#
RNasin® Ribonuclease Inhibitor	2,500u	N2111
	10,000u	N2115
Recombinant RNasin® Ribonuclease Inhibitor	2,500u	N2511
	10,000u	N2515
RNasin® Plus RNase Inhibitor	2,500u	N2611
	10,000u	N2615

Ribo m^7G Cap Analog

Product	Size	Cat.#
Ribo m^7G Cap Analog	10 A_{254} units	P1711
	25 A_{254} units	P1712

10.C. References

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11. Summary of Changes

The following changes were made to the 9/25 revision of this document:

1. Removed the discontinued Canine Pancreatic Microsomal Membranes, Cat. Y4041 from several sections.
2 Updated the cover image and fonts.
3. Updated storage temperature for Cat.# L4960 and Cat.# L4151 from –70°C to “below –65°C”.
4. Removed reference 3 and renumbered other references.
5. Removed an expired third party trademark.

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