ProductInformation

***in vitro* TRANSLATION SYSTEM**
Using Reticulocyte Lysate from Rabbit

Product No. **RLT-1**
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TECHNICAL BULLETIN

Product Description
Sigma's *in vitro* Translation System accurately and efficiently translates message from a variety of sources. Successful translations can be obtained from total RNA, mRNA isolates and both capped and uncapped RNA isolated from yeast, plants or eukaryotic sources. The lysate is prepared from rabbit reticulocytes using a procedure which results in a very active translation system with minimal background. The endogenous RNA in the reticulocytes is degraded to make the system dependent on exogenous RNA. An ATP regenerating system, hemin, and calf liver tRNA are added to optimize translation. It is not necessary to add a 5N terminal 7-methyl guanosine cap to the mRNAs during or after transcription for efficient translation. To minimize loss of activity by freeze-thaw cycles, the lysate is supplied in five aliquots.

The kit is supplied with four different labeling mixes. Two of the mixes are used to label capped mRNA with either methionine or leucine. The other two mixes are for labeling uncapped mRNA with the same amino acids. The labeling mixes for uncapped RNA translate up to 70% as efficiently as those used for capped transcripts. This has been accomplished by reducing the amount of potassium acetate in the mix. The reticulocyte lysates demonstrate strong fidelity for the appropriate AUG initiator codon.

Reagents
Reagents sufficient for 60 reactions

- Reticulocyte Lysate, from rabbit , 5 x 220 µl
  Product No. R5768

- Minus Methionine Labeling Mix, 100 µl
  Product No. M0169
  200 mM creatine phosphate, 3 M potassium acetate, 10 mM magnesium chloride, and an amino acid mixture (2 mM each, excluding methionine).

- Minus Leucine Labeling Mix, 100 µl
  Product No. L6531
  200 mM creatine phosphate, 3 M potassium acetate, 10 mM magnesium chloride, and an amino acid mixture (2 mM each, excluding leucine)

- Minus Methionine Uncapped Labeling Mix, 100 µl
  Product No. M9918
  200 mM creatine phosphate, 0.5 M potassium acetate, 10 mM magnesium chloride, and an amino acid mixture (2 mM each, excluding methionine)

- Minus Leucine Uncapped Labeling Mix, 100 µl
  Product No. L6406
  200 mM creatine phosphate, 0.5 M potassium acetate, 10 mM magnesium chloride, and an amino acid mixture (2 mM each, excluding leucine)

- Control RNA, 0.1 mg/ml 50 µl
  Product No. R5893
  An 1800 base RNA transcript that encodes *Xenopus* elongationon factor1-a[1]. When translated, it yields a protein of approximately M_r 50,246.

- Water, Nuclease-free, 2 x 1 ml
  Product No. W4003

Reagents and Equipment Required but Not Provided
(Sigma product numbers are given where appropriate)
Dye for SDS-PAGE analysis
25% TCA, Product No. T4040 diluted 4-fold in water
10% TCA, Product No. T4040 diluted 10-fold in water
RNase A, Product No. R6513
Diluent for RNase A (10 mM Tris-HCl, pH 7.5, 15 mM NaCl)
10% SDS PAGE gel
Glass fiber filters, Product No. F5019
Radiolabeled amino acid
95% Ethanol, Product No. E7148

Precautions and Disclaimer
Sigma's *in vitro* Translation Kit is for laboratory use only; not for drug, household or other uses. Kit contains components which are hazardous. Warning statements are included on the label or in the components section of this bulletin where applicable. In addition, standard procedures for safely handling radioactive materials should be followed.

Storage/Stability
Store at -70°C
The kit can be stored for six months at -70°C with no detectable loss in activity. The system can be completely inactivated when stored at -20°C for several hours. During preparation of the reaction mixtures for incubation, the lysate can be kept on ice for up to 30 minutes with minimal loss of activity. Unused lysate can be refrozen at -70°C for future use. A small amount of activity is lost with each refreezing. For best activity, the lysate should be added to the labeling reaction immediately after thawing.

Procedure

I. Preliminary
Sigma recommends using reaction volumes of 25 µl or larger. For optimal translation, we suggest the reaction mix consist of 65% lysate, 5% labeling mix with the balance made up of labeled amino acid, mRNA and water. The amounts added can be varied, depending on the particular requirements. Translation is possible when only 50% of the reaction mix is lysate.

There are several preliminary steps that should be taken before beginning the *in vitro* translation reaction.

1. It is imperative that the translation reactions be kept free of RNase. Use RNase-free microcentrifuge tubes, pipette tips, water and reagents. Gloves should be worn to prevent nuclease contamination from fingers. Steps should be taken to ensure that mRNA isolates are free of RNase.

2. The mRNA isolate should be dissolved in water or 0.1 mM EDTA (Product No. E7889 diluted 5,000-fold in water) because the lysate has been optimized for a specific salt concentration.

3. Pure mRNA saturates the translation lysate at a concentration of approximately 10-20 µg/ml. Total RNA saturates the lysate at a concentration of 300-600 µg/ml. For best results, adjust the RNA concentration to 50-200 µg/ml for mRNA and 1.5-3.0 µg/ml for total RNA.

4. If necessary, the RNA can be concentrated by lyophilization.

5. Heat the RNA to 75-80°C for two minutes. Remove from heat and immediately chill on ice before translation. This will break up aggregates and any secondary structure for improved translational efficiency.

II. Reaction for Capped mRNA:

1. Thaw the standard Minus Methionine or Minus Leucine Labeling Mix, labeled amino acid (^{35}S-methionine or ^{3}H-leucine, respectively), control mRNA and sample mRNA and place on ice.

2. To three 1.5 ml microcentrifuge tubes add the following: To tube 1 (negative control) add 5 µl of water, to tube 2 (positive control) add 5 µl control RNA, to tube 3 (sample) add 5 µl of sample mRNA. Place all three tubes on ice.

3. Quickly thaw the lysate by warming with hands and immediately place on ice.

4. Prepare translation reaction mixture sufficient for 12 reactions as shown below. If fewer than 12 reactions are required, scale back components proportionally.

200µl	Lysate
15 µl	Appropriate labeling mix
15 µl	Labeled amino acid (^{35}S-methionine approx. 1200 Ci/mM or ^{3}H-leucine approx. 169 Ci/mM)
10 µl	Water, nuclease-free
240 µl	Total volume

Note: Synthesis of non-radioactive protein is easily accomplished by adding equal volumes of the two mixes (Minus Met and Minus Leu). When equal volumes are mixed, the leucine and methionine will be 1 mM, which is sufficient for translation.

5. Add 20 µl of this mix to the each of the three tubes prepared in Step 2 for a final volume of 25 µl. Mix thoroughly but gently using the pipet tip.

6. Cap the tubes and incubate for 60 minutes at 30°C.

7. To evaluate the end product, proceed to the desired analytical procedure below. See Section IV for gel analysis by SDS-PAGE and Section V for determining incorporation of amino acids.

III. Reaction for Uncapped mRNA

Uncapped transcripts can be translated quite efficiently by using the master mixes for uncapped transcripts. Cap dependency is reduced under conditions of low ionic strength. Uncapped globin transcripts are translated more efficiently at 25 mM K^+ and poorly at 150 mM K^+ (concentration of master mixes for capped mRNA). At 25 mM K^+, uncapped globin transcripts are translated up to 70% as efficiently as capped globin transcripts.

IV. Gel Analysis by SDS-PAGE

1. Prepare Reducing Protein Dye: In a 15 ml conical flask, add the following components:
   - 1.25 ml of 1 M Tris-HCl, pH 6.8
   - 4.0 ml of 10% SDS
   - 2.0 ml of 100% glycerol
   - 30 mg (0.3%) of bromophenol blue
   - 2.75 ml of water

2. Add 1/10 volume (2.5 µl) of a 1 mg/ml RNase A solution (diluted in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl) to the translation reaction and incubate for ten minutes at 30°C. This treatment removes a background band believed to be aminoacyl tRNA.

3. Add 12.5 µl of reducing protein dye. Heat the samples for two minutes at 95°C.

4. Samples are now ready to load onto a SDS-PAGE gel.

Note: If the samples are not to be analyzed by SDS-PAGE, treatment with RNase A and addition of reducing dye is unnecessary.

V. Determination of Label Incorporation into Protein

1. Prepare decolorizing solution (1 N NaOH, 1 mM DL-methionine and 1.5% H_2O_2). To a 50 ml sterile conical flask, add:

   - 4 ml 10 N NaOH
   - 400 µl 100 mM DL-methionine
   - 20 ml 3% hydrogen peroxide

   QS to 40 ml with water

2. Remove 2 ml of translation reaction and pipet into two 12 X 75 mm tubes, each containing 0.5 ml of water. Triturate the sample a few times with the water in the tubes. This serves to stop the reaction by dilution.

3. Add 0.5 ml of decolorizing solution and incubate for ten minutes at room temperature. This deacylates charged tRNA and bleaches out the red color in the reticulate lysates.

4. Add 1 ml of 25% TCA solution to the tube and incubate for five minutes on ice.

5. Collect the precipitates by vacuum filtration through a glass fiber filter.

6. Rinse the tubes and filters two times with 1 ml of 10% TCA.

7. Rinse the filters with 95% ethanol.

8. Dry the filters under a heat lamp.

9. Place the filters in separate scintillation vials. Add 5 ml of scintillation fluid and count. Count using a window with a lower limit of 0 and an upper limit of 670. A difference of 10- to 20-fold should be observed for the translated protein and the negative control.

Results

Troubleshooting Tips

1. Presence of as little as 0.1% dsRNA can inhibit protein synthesis. Addition of 5 mM cAMP or the addition of higher concentrations of dsRNA (20-50 mg/ml) can prevent, or even reverse the inhibition[2].

2. Contaminating polysaccharides also can inhibit protein synthesis. Polysaccharides are sometimes co-purified when the RNA is isolated by phenol extraction. Precipitation of RNA using 2 M LiCl or 3 M sodium acetate[3] is a more efficient method for removing contaminating polysaccharides. If the RNA concentration is less than 0.5-1 mg/ml, recovery is poor. To completely remove the contaminating polysaccharides, several rounds of precipitation may be necessary.

3. The amount of each amino acid in the lysate varies. Radiolabeled methionine is most frequently used because the amount of methionine in reticulocytes is very low (5 mM). The average methionine content of proteins is approximately 2%; however, there are proteins where the methionine content is very low. In these cases, it is necessary to label with another radioactive amino acid.

4. High incorporation (60-80%) of added label into protein implies the amount of the labeled amino acid may be limiting. This will cause translation to be terminated before completion. The addition of more labeled amino acid or cold amino acid (if adding more label is impractical) midway through the reaction will allow synthesis to continue to completion.

5. Calf liver tRNA is added to our lysate so a wide variety of proteins can be efficiently translated. There are, however, some mRNAs which contain high proportions of a single amino acid and may be translated with low efficiency because the tRNA is limiting.

References

1. Kreig, P.A., *et al.,* Dev. Biol. **133**, 93-100 (1989)
2. Farrell, P.J., *et al*., Cell **11**, 187-200 (1977)
3. Palmiter, R., Biochem. **13**, 3606-3615 (1974)