Retic Lysate IVT™ Kit

(Part Number AM1200)

Protocol

I. Introduction 1

A. Product Description

B. Materials Provided with the Kit and Storage Conditions

C. Materials not Provided with the Kit

D. Related Products Available from Applied Biosystems

II. Retic Lysate IVT Procedure 6

A. Input RNA

B. Reaction Assembly and Incubation

C. (Optional) Optimization of Translation Mix

III. Analysis of Translation Products 12

A. Translation Product Analysis Overview

B. Analysis of Translation Products by SDS-PAGE

C. Measurement of Radiolabel Incorporation by TCA Precipitation

D. Determining the Efficiency and Yield of Translation

IV. Troubleshooting 18

A. Positive Control Reaction

B. Low Protein Yield

C. Multiple Bands Below the Full-Length Product

V. Appendix 23

A. Phenol/Chloroform Extraction

B. SDS-Polyacrylamide Gel Solutions

C. References

D. Quality Control

E. Safety Information

P/N 1200M Revision C **Revision Date: November 3, 2008**

For research use only. Not for use in diagnostic procedures.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the Retic Lysate IVT™ Kit.

Warranty and Liability: Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in "Limited Warranty for Consumables," which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption "EXCLUSIONS, CONDITIONS, EXCEPTIONS, AND LIMITATIONS" in the full warranty statement. Please contact Applied Biosystems if you have any questions about our warranties or would like information about post-warranty support.

I. Introduction

A. Product Description

The Retic Lysate IVT™ Kit is optimized for a high level of protein synthesis and for appropriate protein folding. When radiolabeled amino acid is added to the translation reaction, the system will generate a large amount of high specific activity protein from both capped and uncapped mRNAs. The Ambion® Retic Lysate Kit has been optimized for high yield of products and maximal biological activity for in vitro translated protein.

The synthesized proteins can be analyzed by several methods including TCA precipitation, SDS-polyacrylamide gel electrophoresis and autoradiography (or phosphorimaging), western blotting using specific antibodies, or functional analysis using detection by colorimetric, fluorescent, or luminescent methods.

Reticulocyte lysate

The Reticulocyte Lysate in the Ambion Retic Lysate IVT Kit can efficiently translate exogenous RNAs from organisms ranging from yeast to higher plants and mammals. It is prepared from reticulocytes isolated from rabbits treated with acetylphenylhydrazine. The reticulocytes are isolated and lysed using a procedure that results in a very active translation system. In order to make the lysate dependent on exogenous RNA, the endogenous mRNA is degraded with calcium dependent micrococcal nuclease (Pelham et al. 1976). The nuclease is then inactivated by chelation of calcium with EGTA. The lysate has been optimized for translation by the addition of an ATP regenerating system, hemin, yeast tRNA, and calf liver tRNA. For user convenience the lysate is supplied in 5 separate 220 µL aliquots. This minimizes refreezing of unused portions, which may cause loss of activity.

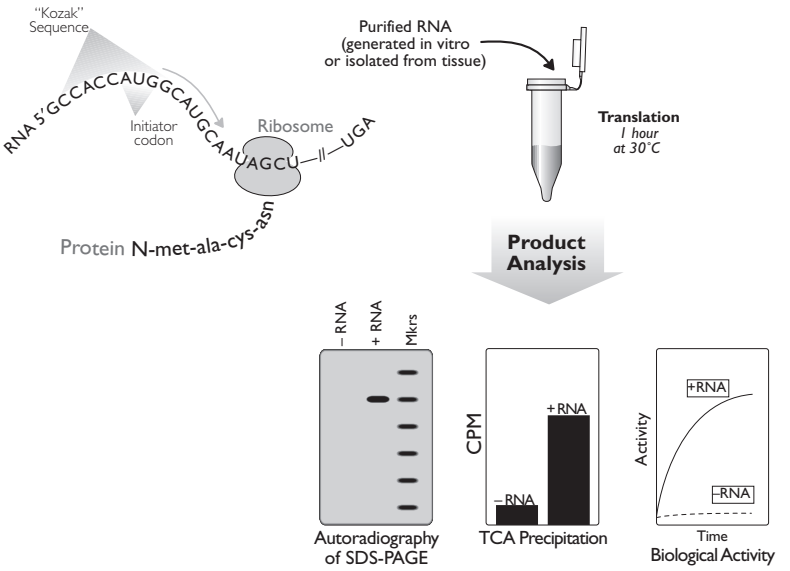
The translation mixes

Four Translation Mixes are supplied with the Retic Lysate IVT Kit so that either radiolabeled methionine or leucine can be used, and to provide the means to maximize protein yield when optimization is necessary. The 20X Translation Mix (either minus-methionine or minus-leucine) will support the efficient translation of most capped and uncapped mRNAs. There are 2 additional master mixes supplied for optimization of translation; these are called High Salt Mix and Low Salt Mix. With some messages, optimizing the translation mix using these reagents may boost protein yield approximately 20–30%. The High Salt Mix and Low Salt Mix are supplied minus-methionine.

Cotranslational and post-translational modifications

Co- and post-translational modifications can occur in vitro using the Retic Lysate IVT Kit; phosphorylaton, lipidation, acetylation, and isoprenylation have all been seen. Processing events, such as signal peptide cleavage and core glycosylation can be analyzed after addition of microsomal membranes to a standard translation reaction (microsomal membranes are not included with the kit). The types of modification that occur, as well as the degree and fidelity of these modifications, vary with the RNA template.

Figure 1. Retic Lysate IVT Procedure



B. Materials Provided with the Kit and Storage Conditions

The Retic Lysate IVT Kit should be stored below –70°C. If your freezer is opened frequently, however, it may not actually remain at a constant temperature. In vitro translation systems lose activity very rapidly at higher temperatures; storage at –20°C for several hours can completely inactivate them.

CAUTION

Do not store cell-free translation systems in the liquid phase of a liquid nitrogen storage system. Liquid nitrogen may gradually seep into the tubes containing the lysate and can cause them to explode violently when they are thawed.

Amount	Component	Storage
5 x 200 µL	Retic Lysate	–80°C
100 µL	20X Translation Mix minus-Met	below –70°C
100 µL	20X Translation Mix minus-Leu	below –70°C
100 µL	High Salt Mix minus-Met	below –70°C
100 µL	Low Salt Mix minus-Met	below –70°C
50 µL	Capped Xef-1 RNA (50 µg/mL)	below –70°C
2 x 1.75 mL	Nuclease-Free Water	any temp*

* Store Nuclease-free Water at –20°C, 4°C or room temp.

C. Materials not Provided with the Kit

Radioactive amino acid

We recommend using [^{35}S]methionine to radiolabel in vitro translation products. If the RNA does not encode any methionine residues, use [^{14}C]leucine instead.

- [^{35}S]methionine, >1200 Ci/mmol, 10 mCi/mL; the products listed below contain a stabilizer that increases their stability over conventional radiolabeled amino acids:
  –– PerkinElmer™ [^{35}S]methionine Cat #NEG709A
  –– Amersham Biosciences [^{35}S]methionine Cat #AG 1094
- [^{14}C]leucine 300 mCi/mmol
  –– PerkinElmer™ [^{14}C]leucine Cat #NEC279

Constant temperature water bath (30°C)

Protein yield is highest when Retic Lysate IVT reactions are done in water baths, but other 30°C incubators can be used.

Materials for quantitation of incorporation of labeled amino acid into protein:

- Decolorizing Solution:
  1 N NaOH
  1 mM D-methionine or L-methionine
  1.5% hydrogen peroxide
- Trichloroacetic Acid: 25% and 5–10% (w/v) in water
- Ethanol (either absolute or 95%)
- Glass fiber filters (for example Whatman GFC)
- Filtration manifold
- Scintillation Fluid

Reagents and equipment for SDS-PAGE analysis, including:

- (Optional) RNase A (1 mg/mL) or RNase Cocktail (P/N AM2286)
- Reagents and apparatus for SDS-PAGE (gel recipes in section V.B on page 24)
- Gel Dryer
- Autoradiography cassette and x-ray film.

D. Related Products Available from Applied Biosystems

mMESSAGE mMACHINE® P/N AM1340, AM1344, AM1348	High yield transcription kits for production of large amounts of capped RNA. These kits employ Ambion's novel, patented MEGAscript® technology, and include cap analog. Kits are available with T7, SP6, and/or T3 RNA polymerase.
mMESSAGE mMACHINE® T7 Ultra Kit P/N AM1345	The mMESSAGE mMACHINE T7 Ultra Kit incorporates Anti-Reverse Cap Analog (ARCA) into Ambion's patented high yield transcription technology to generate RNA transcripts that yield much higher amounts of protein when translated in vitro or in vivo, than messages with traditional cap analog. The increased translation efficiency provided by ARCA is further enhanced by the addition of a poly(A) tail to the transcripts. Experiments comparing ARCA and ARCA/poly(A) tailed transcripts to cap analog and cap analog/poly(A) tailed transcripts show significantly higher levels of protein synthesis with ARCA capped RNA.
MEGAscript® Kits P/N AM1330–AM1338	High yield transcription kits for production of large amounts of RNA. By employing Ambion's novel, patented MEGAscript® technology, these kits use concentrations of nucleotides that would normally inhibit the RNA polymerases, resulting in ultra high-yield transcription reactions. Kits are available with T7, SP6, and/or T3 RNA polymerase.
Cap Analog & Variants See web or print catalog for P/Ns	Cap analog, $m^7G(5')ppp(5')G$, is used for the synthesis of 5' capped RNA by in vitro transcription. Cap analog is also used as a highly specific inhibitor of the initiation step of protein synthesis. Applied Biosystems also offers cap analog variants. All of the Cap Analog products are tested in vitro transcription, and are certified nuclease-free.

Poly(A) Tailing Kit P/N AM1350	The Ambion Poly(A) Tailing Kit uses *E. coli* Poly(A) Polymerase to polyadenylate the 3' termini of in vitro transcribed RNA. Polyadenylation plays an important role in the stabilization of RNA in eukaryotes and enhances the efficiency of translation initiation.
RNase Cocktail™ P/N AM2286, AM2288	RNase Cocktail contains RNase A and RNase T1 in the same proportions as found in RNase A/RNase T1 Mix included in the RPA III™ Kit, but at twice the concentration. A 1:2 dilution of RNase cocktail is equivalent to RNase A/RNase T1 Mix.
Electrophoresis Reagents See web or print catalog for P/Ns	Applied Biosystems offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our web site for a complete listing as this product line is always growing.

II. Retic Lysate IVT Procedure

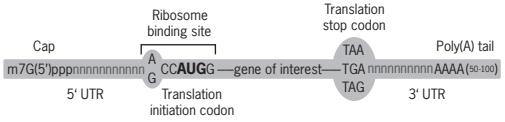
A. Input RNA

RNA sequence requirements

Typically in vitro transcripts are used as template for in vitro translation reactions, but complex total or poly(A) RNA isolated from biological sources can also be used.

In nature RNA that will be translated into protein contains several translation signals; these are a 5' 7-methyl guanosine cap, a ribosome binding site containing an AUG translation start codon (known as a Kozak consensus sequence), a translation stop codon and a poly(A) tail. RNA containing these translation signals is optimal, but RNA lacking some of them [cap or poly(A) tail] can also be translated in this system. RNA that lacks translation signals will typically be translated at a slower rate, and with reduced fidelity of translation initiation compared to RNA that has them. Optimal protein synthesis will occur if the Kozak sequence lies in a region that is free of inhibitory secondary structure; short 5' UTRs typically lack secondary structure. On the other hand, capped or uncapped RNAs with a moderately long 5' UTR lacking stable secondary structure may be efficiently translated even without the Kozak consensus sequence in the Retic Lysate IVT Kit.

Figure 2. Translation Signals



RNA should be in water or 0.1 mM EDTA

Ideally, RNA should be in high quality, RNase-free water or 0.1 mM EDTA. We do not recommend using water that has been treated with 0.01% diethylpyrocarbonate (DEPC) to suspend RNA for in vitro translation, because even after autoclaving, DEPC-treated water contains a mild inhibitor of translation.

NOTE

Small volumes of in vitro transcription reactions can be used directly in Retic Lysate IVT, see below for specific information.

RNA purity and concentration

RNA purity is important for in vitro translation, the purer the RNA, the higher the yield of protein will be. The recommended amount of input RNA varies with the source, and type of RNA. With a previously untested RNA we suggest trying at least 2 amounts of input RNA to see

which yields more protein; the highest amount of input RNA doesn't necessarily yield the most protein. ***The maximum volume of RNA is 5.25 µL.***

RNA from in vitro transcription reactions

Type of in vitro transcribed RNA	Amount
capped (or capped and tailed) single transcript	0.1–0.5 µg
uncapped single transcript	0.5–2.5 µg

During in vitro transcription, a variety of metabolites are produced that can reduce or inhibit subsequent in vitro translations. For this reason no more than 1–2 µL of an in vitro transcription reaction should be added directly to a Retic Lysate IVT reaction. If more RNA is needed, first purify the RNA by one of the following methods, they are listed in order of highest RNA purity yield to lowest RNA purity:

- Phenol/chloroform extraction followed by ethanol precipitation (see section V.A on page 23).
- Purification using a glass fiber binding RNA clean-up (e.g. the Ambion MEGAclear Kit P/N AM1908) or isolation product (e.g. the Ambion RNAqueous Kit P/N AM1912).
- Lithium chloride precipitation (removes unincorporated NTPs)
- Spin column purification

RNA isolated from biological sources

Type of RNA	Amount
total RNA	2.5–7.5 µg
poly(A) RNA	0.1–0.5 µg

Which Translation Mix should I use?

For initial experiments with a given RNA template, use the 20X Translation Mix. It is formulated to translate most RNA with high efficiency. To maximize protein yield, especially from uncapped transcripts, it may be beneficial to optimize the salt concentration of the translation mix using the Low And High Salt Mixes; this is discussed in section *II.C. (Optional) Optimization of Translation Mix* starting on page 9.

B. Reaction Assembly and Incubation

1. Thaw the reaction components

All reagents should be microfuged briefly before opening to prevent loss and/or contamination of material. Keep all reagents on ice before and during the assembly of the reaction.

The Retic Lysate should be thawed as quickly as possible, either by holding it in the palm of your hand or by placing it in a water bath. Once the Retic Lysate is thawed, immediately place it on ice. Multiple freeze-thaws cycles should be avoided, because they will inactivate the lysate.

2. Assemble Retic Lysate IVT reactions

Add the following amounts of the indicated reagents in the order shown to a clean 0.5 mL tube on ice. Be sure to include a minus-RNA control.

1 rxn	10 rxn	Component
to 25 µL	to 250 µL	Nuclease-free Water (final volume)
1.25 µL	12.5 µL	20X Translation Mix (–met or –leu)
1 µL	10 µL	Radiolabeled amino acid
17 µL	170 µL	Retic Lysate
0.1–7.5 µg	1–75 µg	RNA template*

* See section *II.A. RNA purity and concentration* on page 6 for recommendations on the amount of RNA to use.

NOTE

To make unlabeled protein using Ambion's Retic Lysate IVT Kit, omit the radiolabeled methionine, and add unlabeled methionine to a final concentration of 50 µM to the reaction mixture.

3. Mix well

Vortex each tube gently, then centrifuge briefly to collect the reaction at the bottom of the tube.

4. Incubate at 30°C for 60–90 min in a water bath

A cabinet incubator can alternatively be used for this incubation, however, at Ambion we have seen lower yields when reactions are incubated in cabinet incubators.

Synthesis of high molecular weight proteins

If the input RNA codes for high molecular weight proteins (≥100 kDa), extend the incubation time to 90 min to 2 hr. It takes ribosomes approximately 30 min to translate a 250 kDa protein, so after 1 hr, a substantial number of the growing polypeptide chains would be incomplete. The Retic lysate in this kit continues to initiate new peptides for longer than 1 hr.

5. (optional) Add 2.5 µL RNase, and incubate at 30°C for 10 min

If you are planning to analyze the translation products by SDS-PAGE you will probably want to include this RNase treatment. It removes a group of bands that represent radiolabeled aminoacyl tRNAs with an apparent mass of 25–30 kD resulting from the translation of fragments of globin RNA remaining after treatment with Micrococcal (Staphylococcal) nuclease. Do the RNase digestion as follows:

a. Add 2.5 µL of 1 mg/mL RNase A or Ambion RNase Cocktail (P/N AM2286).

b. Incubate at 30°C for 10 min.

6. Place the reactions on ice for 5 min

This will stop the reactions. Samples may be stored on ice for immediate analysis or stored at –20°C for analysis later.

C. (Optional) Optimization of Translation Mix

Traditionally it has been necessary to cap mRNAs synthesized in vitro for the RNA to be translated efficiently. Although it is true that capped and tailed RNA generally yields more protein than the same RNA without a cap, uncapped RNA templates can be translated with high efficiency using this kit.

Capped or uncapped RNA and salt concentration in the translation mix

In most cases, cap dependency is reduced under conditions of low ionic strength, most likely due to reduced secondary structure of the RNA. For instance, uncapped globin transcripts are translated most efficiently in a translation reaction containing 1X Low Salt Mix (25 mM final concentration of potassium), but very poorly in a reaction with 1X High Salt Mix (150 mM potassium), this is shown in Figure 3a. Capped globin transcripts, on the other hand, are translated most efficiently in a reaction using 1X High Salt Mix. The effect of salt on translation efficiency of capped and uncapped mRNA is very dependant on the RNA (see Figure 3b and 3c). Each individual mRNA has unique ionic requirements for optimum translation which cannot be predicted in advance, but the majority of uncapped RNA transcripts will translate more efficiently under relatively low salt conditions.

Using the Low and High Salt Mixes

The Low and High Salt Mixes are provided to facilitate optimization of the salt concentration in translation. They are supplied at 20X, and should be mixed in different ratios for optimization experiments (for example the following ratios could be tested 1:4, 2:3, 3:2, and 4:1). The compositions of the Translation Mixes supplied with the Retic Lysate IVT Kit are listed in Table 1 below, and the final concentrations of potassium acetate in the translation mixes both alone and mixed in the suggested ratios are shown in Table 2.

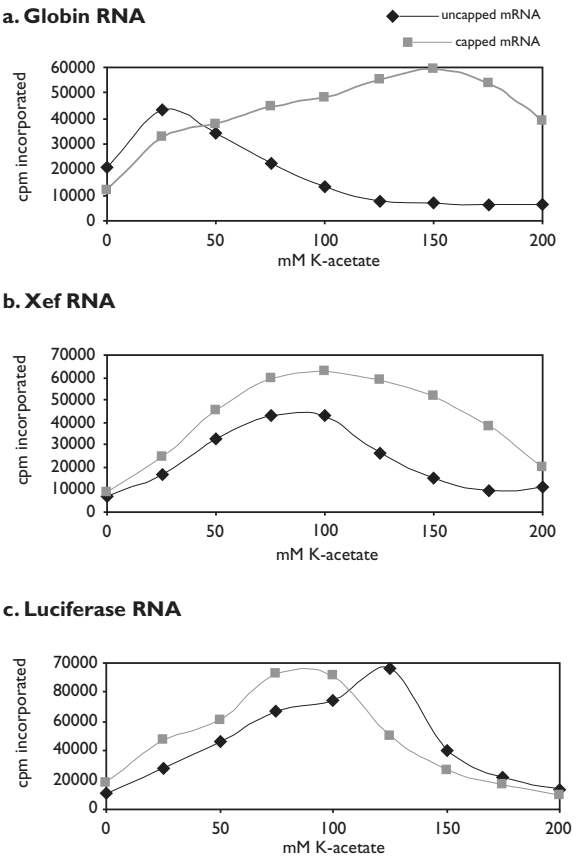


Figure 3. Comparison of Protein Synthesis from Capped and Uncapped RNA

Capped and uncapped globin, Xef, and luciferase RNAs were translated using the 20X Translation Mix (80 mM final concentration of potassium acetate), the Low Salt Mix (25 mM potassium acetate), the High Salt Mix (150 mM potassium acetate), and mixtures of the Low and High Salt Mixes in the presence of radiolabeled methionine. The reactions were TCA precipitated to determine the number of counts incorporated into protein, and the data were plotted against the final concentration of potassium acetate in the reactions.

Table 1. Translation Mix Components

20X TranslationMix		Optimization Translation Mixes	
		Low Salt Mix	High Salt Mix
1.6 M	potassium acetate	0.5 M	3 M
10 mM	magnesium acetate	10 mM	10 mM
200 mM	creatine phosphate	200 mM	200 mM
1 mM	amino acids (–met or –leu)	1	1 mM

Table 2. Potassium Acetate Concentration of the Translation Mixes

Translation Mix	Potassium acetate concentration	
	20X solution (supplied)	Concentration in a 25 µL translation rxn
20X Translation Mix	1.6 M	80 mM
Low Salt Mix	500 mM	25 mM
High Salt Mix	3 M	150 mM
1:4 Low Salt:High Salt Mix*		125 mM
2:3 Low Salt:High Salt Mix*		100 mM
3:2 Low Salt:High Salt Mix*		75 mM
4:1 Low Salt:High Salt Mix*		50 mM

* These Low Salt:High Salt translation mixes are made by mixing the supplied Low Salt and High Salt Mixes in the ratios shown (e.g 1:4). Then use them in the translation reaction in place of the 20X Translation Mix.

III. Analysis of Translation Products

A. Translation Product Analysis Overview

There are several ways to analyze Retic Lysate IVT reaction products:

- SDS gel electrophoresis followed by autoradiography (see section III.B below)
- TCA precipitation and scintillation counting (see section III.C starting on page 14)
- Functional testing using detection by fluorescent/luminescent methods
- Western blot and antibody recognition

Separation of the products on an SDS gel gives information about the size of the products as well as their purity (single full-length protein or incomplete products). Additionally, the band of interest can be excised from the gel, and scintillation counted to determine the yield of full length protein.

TCA precipitation gives important information about the stimulation of translation over the control sample (minus-RNA). It also facilitates verification of incorporation of the labeled amino acid and rough estimation of the amount of protein synthesized.

Fluorescent and luminescent proteins can often be analyzed based on these properties in an appropriate imaging device. If antibodies are available to the protein products, they can be used to characterize the reaction products.

B. Analysis of Translation Products by SDS-PAGE

We recommend using relatively thin (0.5–1 mm) 10–15% SDS polyacrylamide gels. See section V.B on page 24 for gel recipes.

1. Take 5–10 µL of the completed reaction, and add an equal volume of 2X SDS sample buffer

Generally 5 µL of the Retic Lysate IVT reaction is enough to produce a visible signal on film after an overnight exposure to x-ray film, or a phosphorimager screen. See page 24 for the 2X SDS sample buffer composition.

2. Incubate 3–5 min at 95°C

Spin tubes briefly in a microfuge to collect the samples at the bottom of the tube after the heat treatment. Allow samples to cool to room temperature.

3. Load samples on gel and run

Rinse the wells of the gel, and load the samples. Typically, electrophoresis is carried out at a constant current of 15 mA (~90 Volts) in the stacking gel and 30 mA (~200 Volts) in the separating gel. Electrophoresis is usually continued until the bromophenol blue dye is near the bottom of the gel (within ~1 cm). If radiolabled reactions were run on

the gel, be aware that the dye front contains the free radiolabeled amino acids, so you may want to cut the dye front from the gel and discard it in a radioactive waste receptacle.

4. Fix, stain and dry gel

After electrophoresis, fix the proteins by immersing the gel in 45% methanol, 10% acetic acid for 5 minutes with gentle agitation. If desired stain the gel with Coomassie Blue to ensure that the gel has run properly, or dry it directly without staining. Fluorography can be used at this step to reduce exposure times.

5. Expose dried gel to x-ray film or to a phosphorimager screen

Use an intensifying screen for x-ray film and expose it at –70°C overnight.

6. (optional) Excise gel fragment to determine the protein yield

If the number of methionines in the protein is known, then the yield of in vitro synthesized full-length product can be determined as follows: Identify the location of the full length protein on the gel after autoradiography and mark it. Excise the gel fragment corresponding to the full-length protein and put it into a 500 µL microfuge tube. Add 100 µL of 30% hydrogen peroxide, vortex to mix and incubate overnight at 60°C to hydrolyze the acrylamide. Transfer all of the hydrolyzed material into a counting tube with scintillation fluid and measure the radioactivity by scintillation counting. The protein yield can then be determined based on the specific activity of the labeled methionine, the molecular weight of the protein, and the number of methionines in the protein (see section III.D.3 on page 11).

C. Measurement of Radiolabel Incorporation by TCA Precipitation

After the translation, remove 2 aliquots from each translation reaction (including the minus-RNA control and the Capped Xef RNA if it was translated). One aliquot will be diluted, decolorized, and incubated with TCA. The TCA will precipitate any proteins present in the sample, but not unincorporated radiolabeled amino acids. Precipitated protein will then be collected on a glass fiber filter and counted in scintillation fluid. The second aliquot will be scintillation counted directly to determine the total number of cpm that were present in the translation reaction.

1. Mix a 2–5 µL sample from each reaction with 0.5 mL water

At the end of the reaction, or at the desired time intervals, remove 2–5 µL from each translation reaction (including the minus-RNA control and the Capped Xef RNA if it was translated) and add it to 0.5 mL of water in a glass test tube (e.g. 12 x 75 mm).

2. Add 0.5 mL decolorizing solution, incubate 10 min at 30°C

Conc.	Component
1 N	NaOH
1 mM	D-methionine or L-methionine
1.5 %	hydrogen peroxide

Vortex mixture briefly before incubation.

Treatment with NaOH deacylates the charged tRNA and ensures that the only precipitable counts are coming from label incorporated into protein. The hydrogen peroxide bleaches out the red color in the reticulocyte lysate, which can cause quenching during scintillation counting.

3. Add 1 mL cold 25% TCA (w/v), mix and incubate on ice 5 min

Add the cold trichloroacetic acid (TCA) and vortex briefly. Allow the tubes to sit on ice for at least 5 minutes to efficiently precipitate all the protein in the sample.

4. Collect the precipitates by vacuum filtration

We recommend filtration through Whatman GFC glass fiber filters or their equivalent. Filters should be pre-wetted with 5% or 10% TCA before filtering samples.

Rinse the tubes 3 times with cold 5% or 10% TCA, and apply these washes to the filters also.

(Optional) Wash the filters with 95% ethanol or acetone after rinsing tubes to facilitate water removal from the filters.

5. Allow the filters to dry and count the precipitated protein in scintillation fluid

If the filters are not completely dry, some quenching may occur. Filters should become transparent when immersed in the scintillation fluid. Measure cpm using the appropriate scintillation counter setting for the isotope used in the translation reaction.

The cpm counted at this step represents the number of cpm incorporated into (TCA precipitable) protein. Note that most truncated protein fragments will be large enough to precipitate with TCA.

6. Determine the total counts in the reaction

Remove another 2–5 µL aliquot (same volume as taken for TCA precipitation) and dilute it 10 fold in decolorizing solution. Vortex briefly and incubate 10 min at 30°C. Spot the same volume of this mixture directly onto a dry glass fiber filter. Count with the TCA precipitated samples.

For example, if 5 µL was removed for TCA precipitation, add another 5 µL to 45 µL decolorizing solution, after incubation at 30°C. Then spot 5 µL of dilution onto the filter.

Total cpm in the reaction is equal to the cpm x 10 (dilution factor).

D. Determining the Efficiency and Yield of Translation

1. Fold stimulation

Fold stimulation is defined as:

$$\frac{\text{cpm incorporated into protein (determined in step 5 on page 15)}}{\text{cpm incorporated in minus-RNA control reaction (determined in step 5 on page 15)}}$$

Most RNA templates give a stimulation of 20 fold over the minus-RNA control, while some may give as high as 50 fold stimulation. The amount of stimulation is dependent on the amino acid composition of the protein and on the quality of the RNA template. Proteins with a high methionine content will give larger apparent stimulations when radiolabeled methionine is used in the translation reaction.

If little or no stimulation is seen by TCA precipitation, it is still worthwhile to analyze the samples on a gel because a specific product may be visible even though the cpm incorporated suggests no appreciable synthesis.

2. Percent Incorporation

Percent incorporation is defined as:

$$\frac{\text{cpm incorporated into protein (determined in step 5 on page 15)}}{\text{total cpm in the reaction (determined in step 6 on page 15)}} \times 100$$

3. Amount of protein synthesized: rough estimate

The amount of protein synthesized can be estimated *roughly* knowing the amount of labeled and unlabeled amino acid added to the reaction (specific activity of radioactive amino acid used), and the amino acid content and molecular weight of the protein encoded by the input DNA. This estimation cannot differentiate between TCA precipitable counts that have been incorporated into full length protein and counts that are present in prematurely terminated polypeptides.

To determine the amount of synthesized product, TCA precipitable counts should be divided by the specific activity of the radiolabeled amino acid (in dpm/mol) and then divided by the number of methionines in the full-length protein. This will convert cpms to pmoles of product. The molecular weight of the protein can then be used to convert pmoles of product to picograms of protein.

a. Determination of specific activity

Estimation of the amount of protein synthesized first requires the determination of the specific activity of the methionine in the reaction. There are 3 sources of methionine in the reaction: the lysate itself, the radiolabeled methionine and the unlabeled methionine (if added).

Specific activity = Ci/mol.

First, determine the radioactivity added to the reaction:

Using [^{35}S]Methionine (1200 Ci/mmol, 10 µCi/µL) and adding the recommended 1 µL: 10 µCi/µL x 1 µL = 10 µCi.

Then determine the number of moles of methionine added:

The molarity of the label is:

$$\frac{10\ \text{Ci}}{\text{L}} \times \frac{\text{mol}}{1{,}200{,}000\ \text{Ci}} = 8.3\ \mu\text{M} = \frac{8.3\ \text{pmol}}{\mu\text{L}}$$

The lysate itself has an endogenous pool of methionine of approximately 5 µM. For each reaction, 17 µL of lysate is added.

Thus 5 pmol/µL x 17 µL = 85 pmol

The specific activity of the methionine is:

$$\frac{10\ \mu\text{Ci}}{8.3 + 85\ \text{pmol}} = \frac{10 \times 10^{-6}\ \text{Ci}}{93.3 \times 10^{-12}\ \text{mol}} = \frac{107{,}181\ \text{Ci}}{\text{mol}}$$

b. (Optional) Convert cpm to dpm

Newer scintillation counters approach 95–100% counting efficiency, thus it is reasonable to assume that cpm are equal to dpm. If this is not the case with your machine, the following calculation should be made.

$$\text{dpm of sample} = \frac{(\text{cpm of sample} - \text{cpm of scintillation fluid alone})}{\text{counting efficiency of scintillation counter}}$$

c. (Optional) Convert dpm to pmol methionine incorporated

The specific activity of the methionine is 107,181 Ci/mol.

Since 1 Ci = 2.22 x 10^{12} dpm, then 1 pmol of methionine corresponds to 235,798 dpm.

$$\frac{1\ \text{pCi}}{2.2\ \text{dpm}} \times \frac{1\ \text{pmol Met}}{107{,}181\ \text{pCi}} = \frac{1\ \text{pmol Met}}{235{,}798\ \text{dpm}}$$

$$\text{dpm of sample} \times \frac{1\ \text{pmol Met}}{235{,}798\ \text{dpm}} = \text{pmol Methionine incorporated}$$

d. Convert pmol met incorporated to pg protein synthesized

From the pmol of methionine incorporated, divide by the number of methionines (n) in the full-length protein and multiply by its molecular weight (M).

$$\text{pmol met incorp.} \times \frac{\text{1 pmol protein}}{\text{n met}} \times \frac{\text{M pg}}{\text{1 pmol protein}} = \text{pg protein}$$

The amount of protein calculated above is present in the sample of the reaction taken for TCA precipitation. If 5 µL was taken for TCA precipitation, multiply the pg protein above by 5 to obtain the total amount of protein synthesized in the 25 µL reaction.

IV. Troubleshooting

A. Positive Control Reaction

The Capped Xef RNA is included as a positive control RNA; it can be used as template for in vitro translation to verify that the kit is working properly. The Capped Xef RNA is an in vitro synthesized capped RNA which codes for Xenopus elongation factor 1α (Krieg 1989). The 1800 base RNA will yield a protein of approximately 50,246 Da when translated with the Retic Lysate IVT Kit. The protein contains 12 methionines, 25 leucines and 6 cysteines.

Positive control reaction setup

Use 5 µL of the Capped Xef RNA in a translation reaction following the procedure in section *II.B. Reaction Assembly and Incubation* starting on page 8. Be sure to include a minus-RNA control in the experiment.

After the in vitro translation, assess the reaction products by PAGE (see section III.B on page 12) and/or by TCA precipitation (see section III.C on page 14).

Expected result of the positive control reaction

Typical radiolabel incorporation is approximately 20,000 to 40,000 cpm/µL lysate when using [^{35}S]methionine. Note that the 50,246 Da Xef protein runs aberrantly fast and will comigrate with a 42–45 kDa marker.

The minus-RNA control measures the background level of incorporation, and translation of the Capped Xef RNA demonstrates that the system is functioning properly. Including both of these control reactions in experiments is the best way to determine if the failure to obtain significant translation of an unknown mRNA preparation is due to the mRNA itself or another variable.

B. Low Protein Yield

If an RNA template yields less than ~10 fold stimulation over the corresponding minus-RNA control, then consider doing the time course experiment described below to determine whether there are inhibitors of translation in the RNA or if there is a problem with the RNA template itself. Other factors that can cause or mimic low protein yield include the following:

Use of a suboptimal translation mix (see section II.C on page 9),

The protein encodes few or no methionines.
The average methionine content of proteins is approximately 2%, which results in good radiolabeling with [^{35}S]methionine in vitro. Since the methionine pool in the Reticulocyte Lysate is low (5 µM), and the [^{35}S]methionine is labeled to high specific activity, even proteins with

just 1 or 2 methionines can be radiolabeled well with this kit. Proteins with no methionines, however, should be labeled with [^{14}C]leucine, or with a radiolabled mixture of all the amino acids (Amersham Biosciences Cat #AGQ0080). Alternatively, consider using another method (besides radiolabeling) to assess protein synthesis (see section III.A on page 12).

There was too much or too little RNA in the reaction.
To test this possibility, make dilutions of the RNA, and then use the same volume of RNA containing different amounts of RNA in the translation.

1. Track radiolabel incorporation over time

Set up 3 separate translation reactions with the following RNA templates:

i. Capped Xef RNA (2.5 µL)

ii. experimental RNA (Use 2.5 µL or less RNA; see section *II.A. RNA purity and concentration* on page 6 for recommendations on the appropriate amount of template RNA.)

iii. a mixture of 2.5 µL of the Capped Xef RNA and the experimental RNA (same amount as was used in ii)

a. Carry out the translation reactions as described in section II.B starting on page 8, but remove 2 µL aliquots of the reactions to ice every ~15 min during the translation incubation (step 4).

b. At the end of the 60–90 min incubation, take 2 aliquots from each reaction and count 1 of them directly in a scintillation counter to determine the total number of counts in the reaction (step 6 on page 15). TCA precipitate the protein from the second aliquot and from the aliquots taken at each timepoint following the instructions in section III.C on page 14.

c. You can also run an aliquot of the translation reaction on an SDS polyacrylamide gel (instructions in section III.B on page 12).

d. Using the data from TCA precipitations, plot a time course of the percent radiolabel incorporation (cpm precipitated/cpm in the reaction X 100) against time; this will show the ***rate*** of protein synthesis.

Expected results:
If the rate of radiolabel incorporation is low throughout the time course compared to the rate seen with the Capped Xef RNA, it indicates that translation is hindered by stable RNA secondary structure or impurities in the template RNA. Stable RNA secondary structure in the experimental RNA will inhibit its own translation, but it will not

strongly affect translation from the Capped Xef RNA in the mixed sample (iii). Whereas impurities in the experimental RNA should decrease translation of both RNAs. (See below for suggestions.)

If the rate of radiolabel incorporation is high during the first 15 min and then drops dramatically, the problem may be due to the presence of double stranded RNA (dsRNA). dsRNA in the experimental template should not influence translation of the Capped Xef RNA in the mixed sample (iii). See the suggestions in sections 2 and 4 below.

2. Stable RNA secondary structure

It may be possible to overcome stable RNA secondary structure (which can inhibit translation by the same mechanism as dsRNA) by heating the RNA to 75°C for 3–5 min immediately before adding it to the translation reaction (do not place in ice).

3. Clean up impure RNA

There are many relatively common possible RNA contaminants that can interfere with translation, including the following:

- Calcium: It could reactivate the micrococcal nuclease that is used to remove endogenous RNA from the Retic Lysate. Calcium can be removed as described below.
- Ethanol: relatively easy to remove by ethanol precipitation (see step V.A.5 on page 23)
- Polysaccharides: for example heparin, or other polysaccharides copurified with the RNA
- Salt: for example chloride or phosphates

Phenol/chloroform extraction and ethanol precipitation is the most rigorous way to remove contaminants from RNA (described in section V.A on page 23). Other methods include subjecting the RNA to a glass fiber-type RNA isolation procedure or cleanup procedure (e.g. the Ambion RNAqueous® Kit, P/N AM1912 or MEGAclear™ Kit, P/N AM1908) lithium chloride precipitation or spin column chromatography (e.g. Ambion NucAway™ Spin Columns, P/N AM10070).

4. Double stranded RNA

Double stranded RNA (dsRNA) is a potent inhibitor of translation, it inhibits by causing the inactivation of translation factor eIF-2. If this is a problem try heating the RNA for 3–5 min at 75°C, then add it directly to translation (do not place it on ice). If the problem persists, you may need to switch to an invitro translation system that is not susceptible to translation inhibition by dsRNA.

C. Multiple Bands Below the Full-Length Product

1. Limiting amino acid concentration

The appearance of several distinct bands below the product of expected size might be due to an insufficient amount of the limiting amino acid. [^{35}S]Methionine is most frequently used since the methionine pool in the Retic Lysate is low, and radiolabeled methionine is available at high specific activity. The average methionine content of proteins is approximately 2%, but may be higher for some, especially larger proteins. If the amount of methionine is severely limiting, translation may cease before the polypeptide is complete. Increasing the pool of available methionine by adding 25 µM unlabeled methionine to the reaction mixture will reduce the number of incomplete polypeptide chains and increase the yield of full-length product.

2. Insufficient tRNA in the lysate

If using less RNA in a Retic Lysate IVT reaction increases the yield of full-length products, it may indicate that the tRNA population is not optimal for the translation of the protein.

The lysate in the Ambion Retic Lysate IVT kit has been supplemented with both bovine liver and yeast tRNA so that a wide variety of proteins can be translated efficiently. mRNAs which contain high proportions of a single amino acid, however, may be poorly translated because the tRNA for that amino acid becomes limiting. An example of this is the silkworm protein fibroin where glycine, alanine and serine together account for 85% of the amino acid content (Lizardi, et al. 1979). If truncated products are seen, and a single amino acid is present in a high concentration, it may be help translation to add more of a specific purified tRNA to the translation reaction.

3. Nonstandard codon usage

There are also cases where rare codon usage will affect translation. The RNA of some lower organisms, for example, *Tetrahymena*, can not be translated in reticulocyte lysates. This is due to non-standard codon usage. This problem can be circumvented by adding purified *Tetrahymena* tRNA to the lysate.

4. Protein has an abnormal amino acid composition

If the protein you want to translate contains a highly abnormal amino acid composition, it might be necessary to obtain tRNA from the same source as the mRNA to obtain efficient translation.

5. Improper initiation and/or termination of translation

If a single mRNA species produces 2 or more distinct products, polypeptides might initiate at the authentic initiation site but terminate prematurely, or polypeptides might initiate at inappropriate internal sites and terminate at the correct site. As more genes are sequenced and expressed in reticulocyte lysates, more examples of these phenomena are being seen (Kozak, 1986).

The ability of a lysate to initiate at the proper AUG appears to depend on the 5' untranslated region of the mRNA and on how the lysate was prepared. Kozak has shown that a number of commercial lysates do not discriminate efficiently between AUGs (Kozak, 1990). Coding sequences that include a eukaryotic ribosomal binding site (Kozak consensus sequence) a short distance upstream of the start AUG ensure initiation at the proper site. Transcripts lacking the Kozak consensus sequence may also be efficiently translated if they contain a relatively long 5' untranslated region that is free of irreversible secondary structure.

V. Appendix

A. Phenol/Chloroform Extraction

Phenol extraction is a method commonly used for deproteinization of nucleic acids. Most proteins are more soluble in phenol than in aqueous solutions. Conversely, nucleic acids are more soluble in aqueous solutions than in phenol. Centrifugation of the mixture will separate the organic from the aqueous phase; the lower phase is the organic phase and will contain the protein, usually as a white flocculent material at the interface. The upper aqueous phase will contain nucleic acids. Chloroform is mixed with phenol to enhance protein denaturation and phase separation. Chloroform in the phenol also improves its ability to remove lipids; isoamyl alcohol is added to prevent foaming.

The most rigorous way to perform a phenol/chloroform extraction is to first extract with buffer saturated phenol (Ambion P/N AM9710, AM9712), followed with a phenol:chloroform:isoamyl alcohol (Ambion P/N AM9720, AM9722) extraction, and finally, to extract the sample with chloroform:isoamyl alcohol. Instructions to prepare these reagents can be found in *Current Protocols in Molecular Biology* (Ausubel et al., eds.). Each of these extractions is done as follows:

1. Adjust the aqueous volume of the sample to between 100 and 200 µL with nuclease-free water or TE.
2. Add an equal volume of organic solvent solution, vortex for 2 min to mix thoroughly.
3. Spin at top speed in a room temperature microcentrifuge for 2 min.
4. Recover the aqueous phase by removing it to a new tube.
5. After the organic extractions are complete, the RNA can be precipitated with 0.5M ammonium acetate and 2.5 to 3 volumes of 95–100% ethanol at –20°C for ≥15 min. Centrifuge at ≥10,000 x g for 15–20 min to recover the RNA.

B. SDS-Polyacrylamide Gel Solutions

1. Resolving gel

(minigel: 0.75 mm thick and 10 x 7 cm)

Acrylamide concentration			
10%	12.5%	15%	Component
4.9 mL	4.2 mL	3.3 mL	water
2.5 mL	2.5 mL	2.5 mL	1.5M Tris-HCl, pH 8.8
2.5 mL	3.1 mL	3.8 mL	acrylamide/bis 37.5:1, 40%
100 µL	100 µL	100 µL	10% SDS
50 µL	50 µL	50 µL	10% ammonium persulfate
20 µL	20 µL	20 µL	TEMED*

* adding TEMED will polymerize the gel

2. Stacking gel

(6.7% Acrylamide)

Amount	Component
980 µL	water
440 µL	1.5M Tris-HCl, pH 6.8
300 µL	acrylamide/bis 37.5:1, 40%
18 µL	10% SDS
10 µL	10% ammonium persulfate
10 µL	TEMED*

* adding TEMED will polymerize the gel

3. 2X SDS sample buffer

Amount	Component
2 mL	glycerol
4 mL	10% SDS
0.25 mg	bromophenol blue
2.5 mL	0.5 M Tris-HCl. pH 6.8
to 10 mL	water

Store at room temperature. Just before use, remove an aliquot of sample buffer, and add either 10% β-mercaptoethanol, or DTT to 200 mM. After the experiment, discard any unused sample buffer, containing the reducing agent, as it cannot be stored.

4. 10X gel running buffer

Amount	Component
30 g	Tris Base
188 g	glycine
100 mL	10% SDS
to 1 L	water

C. References

Anderson CW, Baum PR and Gestelend RF (1973) Processing of adenovirus 2-induced proteins. *J Virol* **12**:241–252.

Kozak M (1986) Bifunctional messenger RNAs in eukaryotes. *Cell* **47**:481–483.

Kozak M (1990) Evaluation of the fidelity of initiation of translation in reticulocyte lysates from commercial sources. *Nuc Acid Res* **18**:2828.

Kreig PA et al. (1989) *Dev Biol* **133**:93–100.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **227**:680–685.

Lizardi PM, Mahdavi V, Shields D and Candelas G (1979) Discontinuous translation of silk fibroin in a reticulocyte cell-free system and in intact silk gland cells. *Proc Natl Acad Sci USA* **76**:6211–6215.

Pelham HRB and Jackson RJ (1976) An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur J Biochem* **67**:247–256.

D. Quality Control

Functional Testing

The positive control reaction is done following the procedure in section IV.A on page 18. The reaction products are assessed by TCA precipitation.

E. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.

- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.