3.4 Reagents

A. RRL prepared as indicated above

B. E Mix (5X)

This provides the energy for protein synthesis and a high energy phosphate reservoir (creatine phosphate, CP) to allow rephosphorylation of nucleotide diphosphates generated from consumption of triphosphates. The enzyme to do this is creatine kinase which is added at the master mix stage).

E mix also contains the amino acids (e.g. 19 -Met) lacking the one which is added in the form of radioisotope (e.g. 35S met) in order that newly synthesized proteins can be detected by autoradiography. We generally use 35S Met as the radiolabel, from a stock of 2.5 mCi/mL. Other labelled amino acids can be used with the appropriate amino acid mix.

For 200 uL E mix:

ATP	0.1M 10uL
GTP	0.1M 10uL
CP	0.4M 25uL
19 aa -met	40 uL
35S met	100uL
2 M Tris base	3uL (check pH and titrate to approx. 7)
H2O	up to 12 uL
TOTAL	200uL

Store at -80øC.

C. CB 20 X SP6 linked, RRL

This is a 20-fold concentrate of ions, compensated for the contribution from transcript at 10% concentration, for optimal translation. Since the RRL is desalted in H2O or low Tris buffer only, it can be ignored in the compensation. However, if you ever change the % of transcript in your reaction you have to make a new CB 20 X.

Our standard SP6 transcription final concentrations are:

- 80 mM HEPES pH 7.5
- 15 mM MgCl2
- 2 mM Spermidine
- 10 mM DTT.

The final translation conditions we desire are:

- 10 mM Tris Ac pH 7.5
- 100 mM KAc OR 50 mM KCl
- 2.5 mM MgAc2
- 1 mM DTT.

So, for 10% transcript in translation, CB20X is:

(1) for a KAc buffer:

200mM Tris Ac pH 7.5	1mL from a 2M stock
2M KAc	5mL from a 4M stock
20mM MgCl2	200uL from a 1M stock
add H2O	3.8mL
TOTAL	10mL

(2) for a KCl buffer:

200mM Tris Ac pH 7.5	1mL from a 2M stock
1M KCl	2.5mL from a 4M stock
20mM MgAc2	200uL from a 1M stock
add H2O	6.3mL
TOTAL	10mL

NOTE: The standard buffer to use is usually the KAc buffer, but I have found that for the most part a KCl buffer results in increased levels of translation. Therefore, if you are concerned about the levels of your translations you may want to consider a KCl buffer instead.

D. tRNA 10 mg/ml (calf liver)

Since rabbit reticulocytes are geared up for globin synthesis, the worry is that such a lysate may not contain optimal distribution/levels of the various tRNA's for synthesis of other proteins that might have a very different amino acid composition from globin. Hence the supplementation with calf liver tRNA. In any case, it does indeed stimulate translation.

E. PIN (200X)

Protease inhibitor mix, to protect your translation product from any proteases that may be in

your reagents. The concentrations used will not interfere with the translation reaction. This component is optional, but is useful if further processing of the translation product is required, especially with membranes.

The PIN 200X contains 20 ug/mL each of chymostatin, antipain, leupeptin and pepstatin, and 40 ug/mL of aprotinin (Trasylol). I have also used a supplemented PIN-7 mix containing in addition 100 ug/mL of bestatin and 20 ug/mL E-64. Leupeptin, pepstatin and aprotinin and soluble in water, chymostatin and antipain (?) in DMSO, bestatin in methanol, and E-64 in 50% ethanol/50% water. Note: 1 mg aprotinin = 14 TIU (trypsin inhibitor units) = 5,900 KIU (kallikrein inhibitor units). PMSF will inhibit translation.

F. Creatine kinase

As already described, it transfers high energy phosphates from creatine phosphate to ADP and GDP to generate ATP and GTP. Use at 4 mg/ml in 50% glycerol:10 mM Tris AC pH 7.5.

G. RNAsin

As described for transcription, this is human placental RNAse inhibitor and is useful for protection of your mRNA.

H. Amino acids stock solutions (20 mM each) and mix of 19 amino acids - methionine (1 mM each)

Prepare stock solutions of each amino acid at 20 mM in .01 N HCl for Trp, Val, Ile, Asn, Phe, Asp, Glu and Lys and in 0.1 N HCl for Tyr, (Cys must be pH'd to >10 with KOH in order to dissolve); the rest can be made in nH2O. Easiest way is to weigh out approximately what you need, say, to make up 10 ml of 20 mM, jotting down the exact amount weighed out, and putting the powder into the labelled 15 ml tube. Then calculate how much water/HCl solution to add to each to bring them to 20 mM and add to each. Label both the tubes and caps to avoid cross contamination of amino acids. To make the 19 amino acid mix (1 mM each) simply mix 0.5 ml of each of the 19 with 0.5 ml H2O. Final concentration of each cold amino acid in the translation reaction should be 20 to 40 mM.

I. CB 20X for RRL, unlinked

Translations can be performed using ethanol precipated RNA dissolved in water or low concentration buffer. Ion contributions from the SP6 transcription reaction are not compensated for, so we use an unlinked CB 20X. This buffer is also used for further processing of translation products under constant conditions - gel exclusion columns, sucrose gradients etc.

	CB 20X	**FINAL**
KAc	2M	100mM
Tris Ac pH 7.5	200mM	10mM
MgCl2	50mM	2.5mM

DTT	20mM	1mM

J. Translation inhibitors - Stored at -80øC.

i) initiation inhibitors:

- ATA - aurintricarboxylic acid, free acid. 0.1 mM final, 2 mM stock.
- 7mG-5'p - 7-methyl guanosine 5' phosphate. 4 mM final, 80 mM stock. We typically use these two inhibitors in conjunction.

ii) elongation inhibitors:

- cycloheximide - 1 mM final, 20 mM stock. Will terminate elongation without dissociating ribosomal subunits.
- puromycin - 1 mM final, 20 mM stock in 10 mM Tris-Ac pH 7.5. Will terminate elongation and dissociate 30S and 60S ribosomal subunits.