Chapter 7

Mammalian In Vitro Translation Systems

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Abstract

Under cellular stress, tight and coordinated regulation of the gene expression allows to minimize cellular damage, maintains cellular homeostasis, and ensures cell survival. Among stress-induced cellular responses, alteration of translation rates represents one of the most effective and rapid regulatory mechanisms available for cells. Here we report on detailed protocols of mammalian in vitro translation systems. While most of the available in vitro translation methods are based on bacterial or yeast components, tailor-made and robust mammalian systems are sparse. Our protocols allow measuring global translation of the total mRNA pool as well as translation of one specific reporter mRNA. Furthermore, it provides access to measuring translational activity of isolated ribosomes combined with non-ribosomal cytosolic fractions using reduced amounts of biological starting material. The herein described method can be applied to (1) investigate the effects of stress-dependent soluble factors regulating translation (such as tRNA fragments or ribosome-associated ncRNAs), (2) compare translational activity and translational fidelity of different ribosomes supplemented with the same non-ribosomal fractions, and (3) to investigate protein biosynthesis in various mammalian cell lines as well as tissue samples.

Key words Protein synthesis, Ribosomes, Translation control, Stress response, In vitro translation

1 Introduction

Numerous studies show that mRNA and protein levels have poor correlation especially in mammalian cells, thus emphasizing the importance of posttranscriptional regulation of gene expression [1]. This regulation becomes particularly crucial when cells encounter stress and thus need to adapt their proteome accordingly. Cellular stress response includes radical reprograming of protein synthesis. Regulation of translation, as the final step of gene expression, allows immediate response to physiological changes that is essential for the stress adaptation and the cell survival [2]. Protein synthesis is one of the most energy-consuming processes in the cell, and it consumes about ~75% of the cellular energy [3]. Activation of the stress-induced pathways typically causes phosphorylation of several translational factors resulting in

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inhibition of global translation [4]. Translational slow-down allows conserving energy and re-directs it toward the cellular needs. Stress-induced reduction of overall protein translation is accompanied by the selective translation of specific mRNAs involved in cellular stress-response. Besides canonical translational factors, trans-acting factors like ncRNA have been shown to contribute to translation regulation in all domains of life [5]. In the recent past, tRNA-derived fragments (tRFs) have been uncovered as additional regulatory molecules in translation control and beyond [6]. A subclass of tRFs has been shown to directly bind to and regulate the ribosome. These so-called ribosome-associated ncRNAs (rancRNAs) represent an emerging group of ribo-regulators of protein biosynthesis [5, 7], and they also seem to be at work in mammalian cells [8]. Even though translation regulation during stress is a widespread phenomenon and found in all cells and organisms investigated, its molecular mechanisms are far from being understood in molecular terms.

A molecular understanding of the mechanism of translation and translational control benefits from the functional cell-free translational system. It provides a fast, inexpensive tool to study protein biosynthesis under controllable in vitro conditions. Here, we present a robust protocol to address global translation in vitro using mammalian cell lines and tissues (Fig. 1). Moreover, we present a reconstituted in vitro translation system that allows minimizing the required cellular material and monitoring the translational competence of either isolated ribosomes themselves or the soluble translation factors separately (Fig. 2). Furthermore, by combining isolated crude mammalian ribosomes with commercially available cytosolic extracts from rabbit reticulocytes allows the in vitro translation of single mRNA reporters (Fig. 3). We think the combinatory nature of the presented in vitro translation protocols represents a valuable tool for scientists interested in assessing the functional competence of mammalian ribosomes or studying the roles of soluble cytosolic regulators in translational control.

2 Materials

All solutions should be prepared using ultrapure water. If not otherwise indicated all steps should be performed on ice.

2.1 Translation with the Crude Cell Lysate

1. 10× translation buffer: 300 mM HEPES/KOH (pH 7.6), 1.5 M KOAc, 39 mM $MgOAc_2$. Prepare 10 ml, store at 4 °C (*see* **Note 1**).
2. Wash buffer: 30 mM HEPES/KOH (pH 7.6), 150 mM KOAc, 3.9 mM $MgOAc_2$, 4 mM DTT, 1 mM PMSF (*see* **Note 1**).

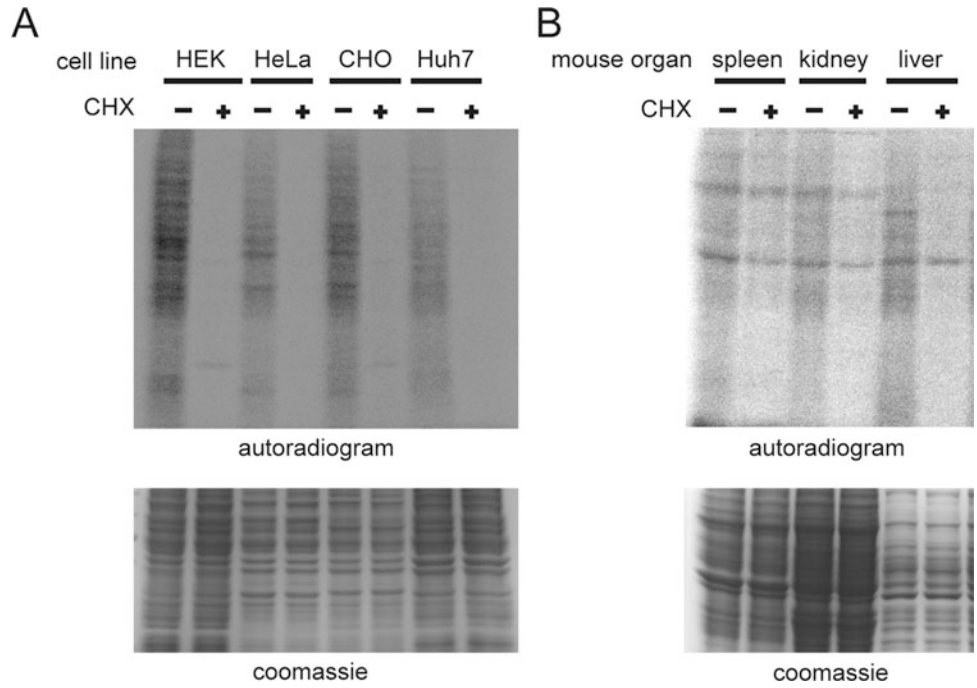

Fig. 1 In vitro translation with the crude cell lysates originating from mammalian cell lines (**a**) or mouse organs (**b**). Autoradiograms of dried SDS polyacrylamide gels represent ^{35}S-methionine-containing newly in vitro synthesized proteins. Cycloheximide (+CHX) at a final concentration of 7.5 mg/ml was used to inhibit in vitro protein synthesis and ensure that the radioactive signals obtained are indeed genuine in vitro translation products. The origins of immortalized mammalian cell lines (**a**) or mouse organs (**b**) are indicated. Coomassie-stained gels shown at the bottom visualize proteins of the cell lysate and serve as loading controls

3. Lysis buffer: 30 mM HEPES/KOH (pH 7.6), 150 mM KOAc, 3.9 mM $MgOAc_2$, 4 mM DTT, 1% Triton, protease inhibitor Tm complete (Roche), RNase inhibitor RNasin (Promega) (*see* **Note 1**). Prepare 1 ml aliquots, store it at −20 °C.
4. Pre-cooled minicentrifuge for cell debris removal.
5. Bradford assay reagent to measure protein concentration of the cell lysate.
6. Translational cocktail: 150 mM HEPES/KOH (pH 7.6), 750 mM KOAc, 19.5 mM $MgOAc_2$, 4 mM GTP, 17.5 mM ATP, 500 μM 19 amino acid mix except methionine (*see* **Note 1**).
7. 3 M creatine phosphate prepared in water, stored at −20 °C.
8. 20 mg/ml creatine phosphokinase prepared in water with 40% glycerol, stored at −20 °C.
9. ^{35}S-methionine 10 μCi/μl (Hartman Analytic).
10. 4× Laemmli buffer.

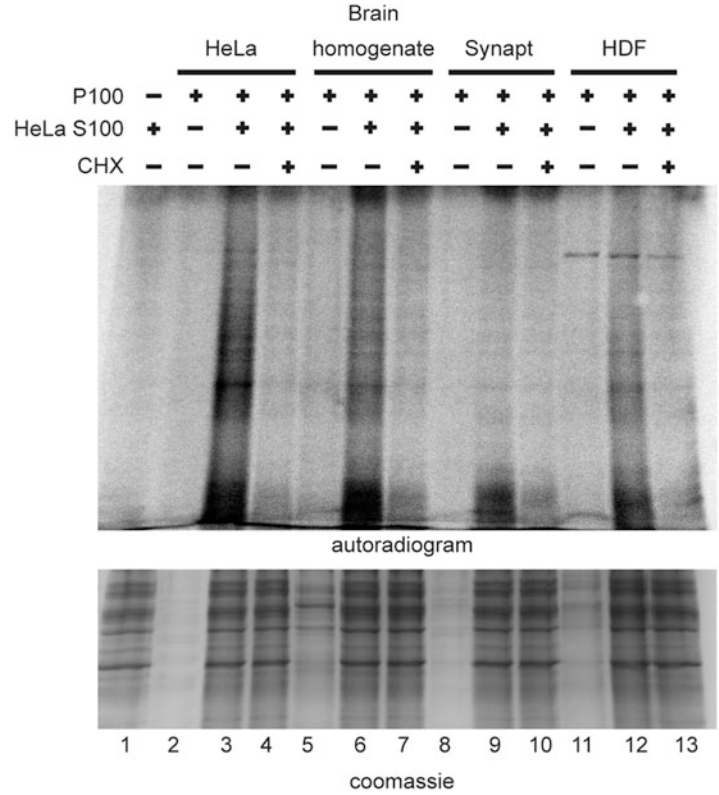

Fig. 2 Reconstituted in vitro translation system utilizing the endogenous mRNA pool. Crude ribosome pellets (P100) originating from HeLa cells, mouse brain homogenate, mouse synaptoneurosomes (Synapt), or from primary human cells Human Dermal Fibroblasts (HDF) were combined with the HeLa non-ribosome-containing cytosolic fraction (S100). Autoradiogram represents ^{35}S-methionine incorporated into newly synthesized proteins. Cycloheximide (CHX) at a final concentration of 4.5 mg/ml was used to inhibit in vitro protein synthesis (lanes 4, 7, 10, 13). Only upon combination of the P100 and S100 preparations, in vitro translation was apparent (lanes 3, 6, 9, 12). Reactions in the absence of P100 (lane 1) or S100 (lanes 2, 5, 8, 11) control for the purity of the preparations and show putative minimal background translational activities of the S100 or P100 preps alone. The Coomassie-stained gel shows proteins present in S100 and P100 preparations and serves as loading control

11. 10–12% SDS polyacrylamide gel and SDS-PAGE running buffer.
12. Destaining solution: 100 ml acetic acid, 400 ml methanol, water up to 1 l.
13. Coomassie staining solution: dissolve 8 g Coomassie blue in 1 l destaining solution.
14. Vacuum gel dryer.

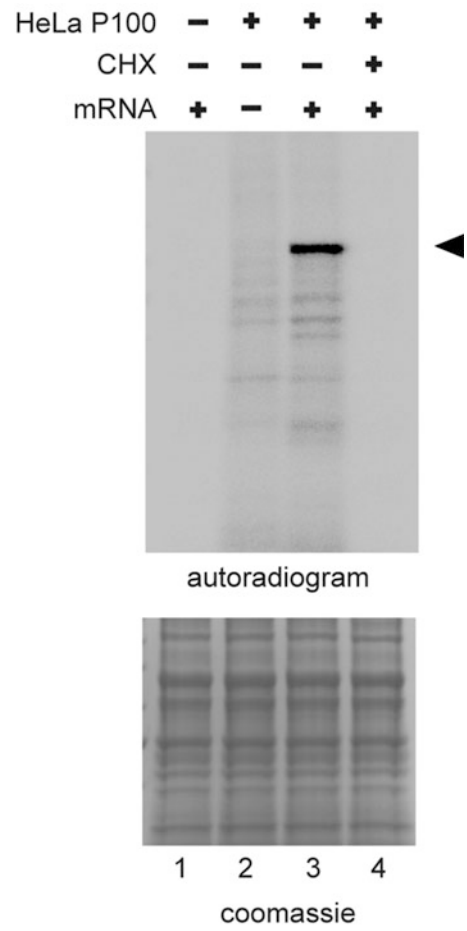

Fig. 3 Reconstituted in vitro translation system using a single reporter mRNA. Crude ribosome pellet (P100) originating from HeLa cells was combined with the non-ribosome-containing cytosolic fraction (S100) obtained from a commercial rabbit reticulocyte lysate and 100 ng of uncapped luciferase mRNA (lane 3). The 62 kDa reaction product is indicated by an arrow head. Smaller bands visible in the autoradiogram either represent products from minor amounts of endogenous mRNAs co-purified with the P100 preparation (lane 2) or products originating from luciferase mRNA fragments (lane 3). Cycloheximide (CHX) at a final concentration of 4.5 mg/ml was used to inhibit in vitro protein synthesis (lane 4). In the absence of P100, no in vitro translation reaction products are apparent (lane 1) thus controlling for the absence of active ribosomal particles in the S100 prep used. The Coomassie-stained gel shows proteins present in the S100 and P100 preparations and serves as loading control

2.2 Global In Vitro Translation with Isolated Ribosomes and Non-ribosome-Containing Cytosolic Fractions

1. Pre-cooled miniultracentrifuge with a fixed angle rotor S140AT (Beckman).
2. Sucrose solution: 1.1 M Sucrose, 30 mM HEPES/KOH (pH 7.6), 150 mM KOAc, 3.9 mM $MgOAc_2$, 4 mM DTT, 1 mM PMSF (*see* **Note 1**). Prepare 10 ml solution, store it at 4 °C for up to 2 days.

3. Ribosome storage buffer A: 20% glycerol, 30 mM HEPES/KOH (pH 7.6), 150 mM KOAc, 3.9 mM $MgOAc_2$, 4 mM DTT, protease inhibitor Tm complete (Roche), RNase inhibitor RNasin (Promega) (*see* **Note 1**). Prepare 1 ml aliquots, store it at −20 °C.
4. Resuspension buffer: 30 mM HEPES/KOH (pH 7.6), 150 mM KOAc, 3.9 mM $MgOAc_2$, 4 mM DTT, protease inhibitor Tm complete, RNase inhibitor RNasin (*see* **Note 1**). Prepare 1 ml aliquots, store it at −20 °C.

2.3 In Vitro Translation of One Specific Reporter mRNA with Isolated Ribosomes and Non-ribosome-Containing Fractions

1. Buffer C: 30 mM HEPES/KOH (pH 7.6), 2 mM $MgOAc_2$, 100 mM KOAc, 1 mM ATP, 0.2 mM GTP. Prepare 1 ml aliquots, store it at −20 °C.
2. Rabbit reticulocyte cell lysate (Promega).
3. Ribosome storage buffer C: 20% glycerol, 30 mM HEPES/KOH (pH 7.6), 2 mM $MgOAc_2$, 100 mM KOAc, 1 mM ATP, 0.2 mM GTP. Prepare 1 ml aliquots, store it at −20 °C.

3 Methods

3.1 In Vitro Translation with the Crude Cell Lysate

In vitro translation system with crude cell lysates is designed to monitor global translation using extracts of mammalian cells and organs (Fig. 1). This method allows to study effects of stress-induced translational factors and other soluble regulatory molecules, such as tRFs, on protein synthesis. The potential translational regulators of interest can be either depleted before cell harvesting (e.g., via RNAi or knock-out strategies) or can be added externally during the in vitro translation reaction. This method was successfully applied for studying tRFs regulating translation by directly binding to ribosomes (rancRNAs for ribosome-associated ncRNAs) in different immortalized mammalian cell lines (CHO, HeLa, HEK) [8, 9].

1. Mammalian cell lines: Harvest cells at 90–95% confluency. For a large-scale experiment, pellet cells from several 150 cm^2 plates. Alternatively, if the material is limited, pellet cells from one or two wells of a six-well plate.

   Mouse organs and tissues: after dissection rinse mouse organs with wash buffer twice.

   Optional: snap freeze cell pellet/organs and store at −80 °C.
2. Mammalian cell lines: Resuspend cells in lysis buffer. Use 400 μl of the lysis buffer for the cell pellet obtained from one 150-cm^2 plate, use 50 μl lysis buffer for the cell pellet obtained from one well of the six-well plate. To ensure sufficient cell opening pass the cell suspension 30 times through a 25G

needle. For the small-scale experiment, replace this step by intensive vortexing.

Mouse organs and tissues: Homogenize mouse organ in lysis buffer. Ideally add one volume lysis buffer to one volume of tissue/organ. In order not to dilute the sample too much, try to add as little lysis butter as possible.

3. Remove cell debris via centrifugation at 18,000 × *g* for 15 min at 4 °C in a pre-cooled centrifuge. For preparation of the mouse organ lysate, this step should be repeated several times until the lysate becomes clear.
4. Estimate protein concentration of the cell lysate using the Bradford assay. Dilute the lysate to 10–15 mg/ml with lysis buffer (*see* **Note 2**). Prepare small aliquots of the cell lysate to reduce the number of freezing–thawing cycles. Snap freeze aliquots and store at −80 °C.
5. To set up a translation reaction, pipette 6 μl of the cell lysate and add water up to 9.9 μl. To study the effect of the translational regulators, water can be replaced by the addition of the translational regulator (e.g., tRF, rancRNA) dissolved in water. As a control, translational inhibitors, such as harringtonine or cycloheximide, can be added at this step (*see* **Note 3**).
6. Pre-incubate the reaction at 37 °C for 10 min (*see* **Notes 4** and **5**).
7. During the pre-incubation time, prepare the translational master mix. For one reaction pipette 1.2 μl translation cocktail, 0.17 μl water, 0.08 μl 3 M Creatine phosphate, 0.06 μl 20 mg/ml creatine phosphokinase, 0.625 μl S^{35}-Methionine.
8. After the pre-incubation step, add 2.1 μl of the freshly prepared translational mix to each reaction.
9. Incubate reactions for 30 min at 37 °C (*see* **Note 6**).
10. Add 12 μl of 2× Laemmli buffer. Optional: store samples at −20 °C.
11. Boil samples for 5–10 min.
12. Run samples on a 10–12% SDS polyacrylamide gel.
13. Stain the gel for 10–15 min in the Coomassie staining solution. Coomassie staining visualizes mainly proteins originating from the cell lysate and serves as a loading control.
14. Distain the gel from a few hours to overnight.
15. Vacuum-dry the gel at 70 °C. For small (10 × 10 cm) gels, 45–60 min is sufficient.
16. Expose the dried gel to a phosphor imager screen from overnight up to several days.

3.2 Global In Vitro Translation with Isolated Ribosomes and the Non-ribosome-Containing Cytosolic Fraction

This method presents a reconstituted system consisting of the crude ribosomal pellet fraction after 100,000 × *g* centrifugation (P100) and the corresponding soluble fraction containing not-pelleted translation factors (S100) (Fig. 2). In this method, ribosomes (P100) isolated from mammalian cell lines, primary cells, or mouse organs are supplemented with the S100 fraction extracted from HEK, HeLa, or any other mammalian cell lines with an active translational status. This experimental setup is especially suited for studies focusing on the translational competence of the ribosome itself, e.g., after certain stress situations or for ribosomes isolated from different cell lines, or from tissues/organs at different developmental stages.

3.2.1 Isolation of P100 from Mammalian Cell Lines, Primary Cells, or Mouse Tissues

1. Prepare cell lysate as described in Subheading 3.1 (**steps 1–4**).
2. Pipette 0.5 ml of pre-cooled sucrose solution prepared in resuspension buffer in a pre-cooled 2 ml miniultracentrifuge tube.
3. Layer 200–500 μl cell lysate on top of the sucrose cushion.
4. Perform ultracentrifugation at 200,000 × *g* at 4 °C for 2 h in a fixed angel rotor S140AT.
5. Obtained pellet is P100. Resuspend pellet in ribosome storage buffer A.
6. Prepare 1:1000 or 1:100 dilution in water and measure OD_{260}. Estimate ribosome concentration using 1 OD_{260} unit equals to 18 pmol of crude 80S ribosomes.
7. Make 0.5 μM dilution with the ribosome storage buffer A, aliquot it, and snap freeze. Aliquots can be snap frozen and thawed several times.

3.2.2 Preparation of Concentrated S100

1. Prepare cell lysate from a HeLa or HEK cell pellet originating from two 150 cm^2 plates as described in Subheading 3.1 (**steps 1–4**).
2. Add the cell lysate to a miniultracentrifuge tube. Ensure that the volume of the lysate is large enough for the centrifugation step (usually should not be below 500 μl).
3. Perform ultracentrifugation at 200,000 × *g* at 4 °C for 2 h.
4. Collect the supernatant (S100). Aliquot it to avoid multiple freezing-thawing cycles. Snap freeze the aliquots and store it at −80 °C.

3.2.3 Setting up Translation Reaction

1. Pipette 0.5 pmol of P100 into a pre-cooled tube. As a control, translational inhibitors can be added at this step (*see* **Notes 3** and **7**).
2. Add 1× resuspension buffer to bring the volume up to 10 μl.

3. Pre-incubate the reaction at 37 °C for 10 min (*see* **Notes 4** and **5**).
4. During the pre-incubation time prepare the translational master mix. For one reaction pipette 3.75 µl of concentrated S100 (prepared in Subheading 3.2.2), 1.2 µl translation cocktail, 0.08 µl of 3 M Creatine phosphate, 0.06 µl of 1 mg/ml Creatine phosphokinase, 0.625 µl of ^{35}S-Methionine, resuspension buffer up to 10 µl.
5. After the pre-incubation step, add 10 µl of freshly prepared master mix to each reaction.
6. Incubate the reaction at 37 °C for 1 h (*see* **Note 6**).
7. Add 7 µl of 4× Laemmli buffer. Optional: store samples at −20 °C.
8. Follow **steps 11–16** in Subheading 3.1.

3.3 Translation of One Specific mRNA Reporter with Isolated Ribosomes and the Non-ribosome-Containing Cytosolic Fraction

In this system, mammalian crude 80S ribosomes (P100) isolated from cell lines (Fig. 3), primary cells, or mouse organs are supplemented with the S100 obtained from the commercial Rabbit Reticulocyte Lysate (RRL) system. This reconstitution enables to perform in vitro translation of one mRNA reporter with crude ribosomes isolated from the cells of interest.

3.3.1 Isolation of P100 from Mammalian Cell Lines, Primary Cells or Mouse Tissues

1. Isolate ribosomes from the cells of interest following the steps in Subheading 3.2.1.
2. Resuspend P100 pellet in ribosome storage buffer C.
3. Estimate ribosome concentration based on OD_{260} using 1 OD_{260} unit equals to 18 pmol of crude 80S (P100).
4. Make a 0.2 µM dilution of P100 with the ribosome storage buffer C, aliquot, and snap freeze. It can be snap frozen and thawed several times.

3.3.2 Prepare RR S100

1. Combine two aliquots of the RRL. Prepare S100 fraction from RRL following the steps in Subheading 3.2.2.

3.3.3 Set up in Vitro Translation Reaction

1. Pipette 1 µl of 0.2 µM P100. As a control, translational inhibitors can be added at this step (*see* **Notes 3** and **7**).
2. Prepare translational master mix. For one reaction, pipette 2.5 µl of RRL S100, 0.625 µl S^{35}-Methionine, RNase inhibitor RNasin (Promega), 7 µM Amino Acid Mixture Minus Methionine (Promega), 2 ng of capped mRNA or 100 ng of uncapped mRNA, buffer C up to 19 µl (*see* **Note 8**).
3. Add 19 µl of freshly prepared master mix to each reaction.
4. Incubate the reaction at 37 °C for 1 h (*see* **Note 6**).

5. Add 7 μl of 4× Laemmli buffer. Optional: store samples at −20 °C.
6. Follow **steps 11–16** in Subheading 3.1.

4 Notes

1. Prepare 10× translation buffer in 10 ml volume, store at 4 °C. Use it to prepare the wash buffer, lysis buffer, sucrose cushion buffer, and ribosome storage buffer A, resuspension buffer by making 1:10 dilution, and translational cocktail by making 1:2 dilution.
2. The measured protein concentration of the lysate does not necessarily directly correspond to its translational activity. Even different batches of the lysate deriving from the same kind of cells can have different translational activities. Titration of a new batch of a cell lysate can be informative to address the translation status of the lysate.
3. To include translation inhibition control, use cycloheximide or harringtonine dissolved in ethanol. First, pipette 0.9 μl of 100 mg/ml cycloheximide or 3 μl 10 mg/ml of harringtonine to the empty reaction tube. Evaporate ethanol by running a SpeedVac vacuum concentrator for 10 min at room temperature. Pipette all remaining components to set up the translation reaction.
4. During the pre-incubation, (1) translational inhibitor and translational regulators pre-bind the ribosome; (2) ribosomes that have already initiated on endogenous mRNAs present in the P100 preparation will finish their elongation.
5. To avoid formation of water condensates on the lid of the tube use a 37 °C incubator instead of water bath or heat block.
6. Depending on the translational activity of the cell lysate or P100/S100, the incubation time recommended in the protocol (30 or 60 min) can already correspond to the end point of the reaction. If translational activities of different cell lysates are compared or if the effects of translational factors or other soluble translation regulators are under investigation, time-course experiments should be performed.
7. To visualize the background signal originating from possible contaminations of the P100 and S100 fractions, set up one control reactions without P100 and one control reaction without S100 (Figs. 2 and 3). Substitute P100 with the ribosome storage buffer, S100—with the resuspension buffer in Subheading 3.2 and Buffer C in Subheading 3.3.

8. During P100 preparation, ribosomes are pelleted together with a small fraction of endogenous mRNAs that are also translated during the subsequent in vitro translation reaction. To ensure that the detected translational product is specific to the exogenously added mRNA, set up control reaction without the mRNA reporter (Fig. 3).

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