Chapter 19

Analyzing mRNA Localization to the Endoplasmic Reticulum via Cell Fractionation

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Abstract

The partitioning of secretory and membrane protein-encoding mRNAs to the endoplasmic reticulum (ER), and their translation on ER-associated ribosomes, governs access to the secretory/exocytic pathways of the cell. As mRNAs encoding secretory and membrane proteins comprise approximately 30% of the transcriptome, the localization of mRNAs to the ER represents an extraordinarily prominent, ubiquitous, and yet poorly understood RNA localization phenomenon.

The partitioning of mRNAs to the ER is generally thought to be achieved by the signal recognition particle (SRP) pathway. In this pathway, mRNA localization to the ER is determined by the translation product – translation yields an N-terminal signal sequence or a topogenic signal that is recognized by the SRP and the resulting mRNA–ribosome–SRP complex is then recruited to the ER membrane. Recent studies have demonstrated that mRNAs can be localized to the ER via a signal sequence and/or translation-independent pathway(s) and that discrete sets of cytosolic protein-encoding mRNAs are enriched on the ER membrane, though they lack an encoded signal sequence. These key findings reopen investigations into the mechanism(s) that govern mRNA localization to the ER.

In this contribution, we describe two independent methods that can be utilized to study this important and poorly understood aspect of eukaryotic cell biology. These methods comprise two independent means of fractionating tissue culture cells to yield free/cytosolic polyribosomes and ER membrane-bound polyribosomes. Detailed methods for the fractionation and characterization of the two polyribosome pools are provided.

Key words: mRNA localization, Endoplasmic reticulum, Cytosol, Polyribosome, rRNA, mRNA

1. Introduction

The endoplasmic reticulum (ER) is the site of synthesis of secretory and membrane proteins, which comprise ca. 30% of the cell’s proteome (1). A fundamental question in cell biology concerns the cellular mechanisms that compartmentalize the synthesis of secretory and membrane proteins to the ER. Pioneering in vitro
studies in the 1980s by Blobel and colleagues established the signal recognition particle (SRP) pathway as (a) the mechanism of mRNA partitioning to the ER (2–5). In this model, all newly exported mRNAs initiate translation on cytosolic ribosomes. In the case of mRNAs encoding secretory or membrane proteins, translation yields the synthesis of an N-terminal signal sequence or a transmembrane domain which is recognized by the SRP, resulting in a suppression of protein synthesis. The ribosome–nascent polypeptide–SRP complex is then recruited to the ER via binding interactions with the ER-resident SRP-receptor. Upon binding of the ribosome–nascent polypeptide–SRP complex to the ER, SRP is released, translation resumes, and the growing peptide is co-translationally translocated into the ER for further processing.

While the SRP pathway has been widely accepted to be the mechanism by which mRNA are partitioned between the ER and the cytosol, several experimental observations indicate that SRP-independent mechanisms contribute to mRNA partitioning in the cell. For example, several groups have observed significant overlap in the composition of cytoplasmic and membrane-associated mRNAs (6–8). Though the Signal Hypothesis predicts that signal sequence-encoding mRNAs would be present, perhaps at low enrichment, in cytosolic polysomes, this model does not provide a mechanism for how mRNAs lacking encoded signal sequences would be partitioned to the ER. In addition, genetic ablation of components of the SRP pathway in yeast does not affect the viability of the organism (9). Instead, disabling of the SRP pathway function is compensated by an expansion of ER, indicating that the cells are able to adapt to the absence of the SRP pathway by activating or expanding compensatory pathways (9). Consistent with these findings, depletion of SRP54 (the signal peptide binding SRP subunit) by RNA interference in trypanosome is not lethal, and loss of SRP54 does not affect the processing of signal peptide containing proteins (10). In HeLa cells, RNAi knockdown of SRP54 does not have any effect on the growth or viability of the cells. Further, loss of SRP54 affects the expression of the membrane receptor DR4 but not DR5, suggesting that cells have multiple pathways to bring about mRNA partitioning between the cytosol and ER (11). In support of the existence of an alternative, SRP-independent mRNA partitioning mechanism, our group recently reported that deletion of the encoded signal sequence of Grp94, an ER-localized mRNA, or mutational loss of its translation function did not disrupt mRNA localization to the ER (12). In fact, specific subsets of mRNAs encoding cytoplasmic and nucleoplasmic proteins have been consistently observed to be enriched on the ER, even though they do not encode a signal peptide (6–8, 13). A very recent report from the Walter lab suggests a mechanism by which such noncanonical mRNA partitioning can occur; in yeast, the localization of HAC1 mRNA to the
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ER is mediated by a conserved element in the 3' UTR (14). However, it is not known whether such direct mRNA localization is an exception to the common rule of SRP-dependent partitioning or if it represents a broader, primary mechanism by which all mRNAs are sorted between the cytosol and the ER. In order to study this phenomenon, it is essential to systematically analyze individual mRNAs and assess their partitioning between the cytosol and the ER.

This paper describes two methods for analyzing mRNA partitioning between the ER and the cytosol. The first method, sequential detergent extraction, takes advantage of the difference in the lipid composition of the plasma membrane and the ER membrane. Digitonin, a β-sterol binding detergent that selectively solubilizes the cholesterol-rich plasma membrane and leaves the ER and nuclear membrane intact, is used to release cytosolic polysomes. Then, the permeabilized cells are washed and the ER-bound polyribosomes are solubilized by any of a variety of detergents, including dodecylmaltoside, NP40, or an admixture of Nonidet P-40 (NP40) and sodium deoxycholate (DOC). The second method, mechanical homogenization followed by differential centrifugation, is a variation of the classical differential centrifugation method for cellular fractionation that was developed by Claude in the 1940s and later perfected by Palade and others (15, 16).

Over the past decades, the field of mRNA localization has been niched to studies in *Drosophila* embryos and budding yeast (17, 18). There is a critical need for more studies on the mechanisms of subcellular mRNA localization in higher eukaryotic cells to enable a broader understanding of this important biological phenomenon. The methods outlined in this paper provide tools needed to study mRNA partitioning between the cytosol and the ER, a ubiquitous mRNA sorting process that, conservatively, directs the subcellular localization of >30% of the transcriptome.

2. Materials

As compared to DNA, RNA is very susceptible to degradation due both to nonspecific cleavage in the presence of divalent cations, and more importantly, the near ubiquitous presence of RNase activity. Thus, great care should be taken to avoid RNase contamination at every step of the following experiments. The most common sources of RNase are human skin and microbial growth in stock solutions. Hence, gloves should always be worn while handling RNA and stock solutions should be stored in small aliquots and discarded at frequent intervals.

To reduce/eliminate RNase contamination, buffers and solutions can be treated with 0.1% (v/v) diethyl pyrocarbonate.
(DEPC) (see Note 1) overnight at 37°C and then autoclaved for 15 min to remove unreacted DEPC. Buffers containing free amine groups (TRIS, HEPES, etc.) and solutions/buffers that cannot be autoclaved (sucrose, MOPS, etc.) cannot be DEPC-treated. Such solutions/buffers should be made up using molecular biology grade, RNAse-free reagents and DEPC-treated water. All glassware should be baked at 400°C for 4 h to inactivate RNAses. Sterile disposable tips and tubes are generally RNAse-free. Non-disposable plasticware (such as ultracentrifuge tubes) can be treated twice with RNAZap (Ambion) or 0.2% SDS, and rinsed thoroughly in DEPC-treated water.

2.1. Cell Culture

1. HEK293 cell line (ATCC).
2. Cell-culture medium: Dulbecco’s modified Eagle’s medium (DMEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; Invitrogen).
3. Trypsin 0.05% in 0.53 mM ethylenediaminetetraacetate (EDTA) (Invitrogen).
4. 1× Phosphate buffered saline (PBS): 10 mM sodium phosphate dibasic (Na2HPO4), 2 mM potassium phosphate monobasic (KH2PO4), 2.7 mM potassium chloride (KCl), 137 mM sodium chloride (NaCl), 0.5 mM magnesium chloride (MgCl2), 1 mM calcium chloride (CaCl2). (Mediatech; Cat. no. 21-030-CV).
5. 1% (w/v) digitonin (Calbiochem; Cat. no. 300410) in DMSO (freeze in 100 µl aliquots; see Note 2).
6. RNaseOut™ Recombinant Ribonuclease Inhibitor: 40 U/µl stock. Store at −20°C (Invitrogen; Cat. no. 17-0969-01).
7. Complete™ Protease Inhibitor Cocktail: Complete™ EDTA-free (Roche Molecular Biochemicals; Cat. no. 1-873-580). Make a 100× stock in DMSO and store at −20°C. Use at a final concentration of 1×.
8. Diethyl pyrocarbonate (DEPC)-treated water. Prepare as a 0.1% (v/v) solution and incubate at 37°C overnight. Autoclave for 15 min to destroy unreacted DEPC.
9. Stock solutions: 4 M potassium acetate (KOAc); 1 M potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (K-HEPES); 1 M magnesium acetate [Mg(OAc)2]; 0.2 M ethyleneglycol bis (2-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA) at pH 8.0; 10% (v/v) Nonidet P-40 (NP-40); 10% (w/v) sodium deoxycholate (DOC); 20% (w/v) n-dodecyl-β-D-maltoside (DDM).
10. Permeabilization buffer: 110 mM KOAc, 25 mM K-HEPES, pH 7.2, 2.5 mM Mg(OAc)2, 1 mM EGTA, 0.03% digitonin, 1 mM DTT, 50 µg/ml cycloheximide (CHX), 1× Complete
Protease Inhibitor Cocktail, and 40 U/mL RNaseOUT™. Digitonin, DTT, CHX, Complete Protease Inhibitor Cocktail, and RNaseOUT™ must be added fresh.

7. Wash buffer: 110 mM KOAc, 25 mM K-HEPES at pH 7.2, 2.5 mM Mg(OAc)₂, 1 mM EGTA, 0.004% digitonin, 1 mM DTT, and 50 μg/ml CHX. Digitonin, DTT and CHX must be added fresh.

8. Lysis buffer: 400 mM KOAc, 25 mM K-HEPES at pH 7.2, 15 mM Mg(OAc)₂, 1 mM DTT, 50 μg/ml CHX, 1× Complete Protease Inhibitor Cocktail, and 40 U/mL RNase Out with either 2% (w/v) DDM or 1% (v/v) NP-40 and 0.5% (w/v). DTT, CHX, Complete Protease Inhibitor Cocktail and RNaseOUT™ must be added fresh.

9. Sucrose cushion: 0.5 M sucrose in lysis buffer.

2.3. Fractionation by Differential Centrifugation


2. Cell homogenizer (Isobiotec), obtained from Isobiotec, Ortenauer Strasse 13, 69126 Heidelberg, Germany.

3. 3 ml Luer-Lok™ syringes (BD Pharmingen).

4. Stock solutions: 200 mM KCl, 150 mM MgCl₂, 1 M Tris–HCl at pH 7.4, 2 M sucrose, 100 mM dithiothreitol (DTT; freeze in 100 μl aliquots), 10 mg/ml CHX (freeze in 100 μl aliquots).

5. Hypotonic lysis buffer (HLB): 10 mM KCl, 7.5 mM MgCl₂, 50 mM Tris–HCl, pH 7.4, 1 mM DTT, 50 μg/ml CHX, 1× Complete Protease Inhibitor Cocktail, 40 U/ml RNAseOUT™. Add CHX, DTT, Complete Protease Inhibitor Cocktail, and RNAseOUT™ just prior to using the solution.

6. Polycarbonate ultracentrifuge tubes, 11×34 mm (Beckman; Cat. No. 343778).

2.4. Immunofluorescence Microscopy

1. Coverslips.

2. Fixation buffer: 4% paraformaldehyde in 1× PBS.

3. Acetone.

4. Blocking solution: 1% (w/v) BSA and 0.2% (v/v) Triton X-100 in 1× PBS.

5. Primary antibody diluted in 1% (w/v) BSA and 0.05% (v/v) Triton X-100 in 1× PBS.

6. Fluor-conjugated secondary antibody in 1% (w/v) BSA and 0.05% (v/v) Triton X-100 in 1× PBS.

7. DAPI (DNA stain) diluted in 1% (w/v) BSA and 0.05% (v/v) Triton X-100 in 1× PBS.

8. Antifade mounting medium.

2.5. Polysome Analysis

1. 15% sucrose in lysis buffer.
2. 40% sucrose in lysis buffer.
3. Polyallomer centrifuge tubes, 14 × 89 mm (Beckman; cat # 331372).
4. Teledyne/Isco gradient fractionator with a continuous UV flow cell.

2.6. RNA and Protein Extraction

1. TRIzol® Reagent (Invitrogen; see Note 3).
2. Chloroform.
3. Isopropanol.
4. 0.3 M guanidine chloride in 95% isopropanol.
5. Ethanol.
6. 75% (v/v) ethanol.
7. Nuclease-free water (to resuspend RNA).
8. Protein sample buffer: 0.5 M unbuffered Tris and 5% SDS.

2.7. Denaturing Formaldehyde Agarose Gel Electrophoresis and Northern Blotting

1. 10× MOPS: 0.2 M 3-(N-morpholino)propanesulfonic acid (MOPS), 80 mM sodium acetate (NaOAc), 10 mM EDTA at pH 7.4. This buffer is light sensitive and should be stored in an amber bottle. The color of this solution slowly changes to orange with time. This does not affect its activity.
2. Agarose (electrophoresis grade).
3. 37.5% (w/v) formaldehyde.
4. Formamide (deionized).
5. RNA tracking dye (Ambion).
6. SYBR safe RNA dye (Invitrogen) (optional).
7. DNA/RNA gel electrophoresis apparatus.
8. 20× SSC: 3 M NaCl and 0.3 M NaOAc at pH 7.0.
9. 10 N Sodium hydroxide (NaOH).
10. Northern transfer buffer: 5× SSC and 10 mM NaOH.
11. Methylene blue stain: 0.02% (w/v) methylene blue in 0.2 M NaOAc at pH 5.2.
13. Whatman 3MM filter paper or equivalent.
15. Stratalinker UV Crosslinker (Stratagene).
17. 100 μM oligonucleotide directed against the RNA sequence to be detected.
18. γ-[32P]-dATP at 6,000 Ci/mmol; end-labeling grade (MP Biomedicals).
19. Sephadex G-25 quick spin column or equivalent (Roche).
20. Scintillation fluid, scintillation vials, and liquid scintillation spectrometer.
21. ExpressHyb hybridization solution (Clontech).
22. Hybridization oven and glass tubes.
23. Low-stringency wash buffer: 0.5× SSC and 0.1% (w/v) SDS in deionized water.
24. High-stringency wash buffer: 0.1× SSC and 0.1% (w/v) SDS in deionized water.

1. 12.5% denaturing polyacrylamide gel, 0.75 mm thick.
2. Gel electrophoresis system (Bio-Rad).
3. 5× gel loading dye: 0.2 M Tris–HCl at pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, 0.05% (w/v) bromophenol blue, 10 mM β-mercaptoethanol (BME). Add β-BME to the sample buffer just prior to use.
4. 5× SDS running buffer: 250 mM Tris–HCl, 2 M glycine, and 1% (w/v) SDS.
5. CAPS transfer buffer: 50 mM 3-[cyclohexylamino]-1-propane sulfonic acid (Sigma C2632) at pH 11, 0.075% SDS, and 20% (v/v) methanol.
7. Ponceau stain: 0.1% (w/v) Ponceau S and 5% (v/v) acetic acid.
8. 1× PBS-T: 1× PBS and 0.2% Tween-20.
9. 5% milk in 1× PBS-T.
10. Primary and secondary antibodies.
11. Enhanced chemiluminescence (ECL) reagents (Denville Scientific).
12. X-ray film and cassette (Denville Scientific).

3. Methods

3.1. Methods for Cell Fractionation

Cell fractionation has been used for several decades to analyze the molecular composition and functionalities of the organelles/metabolic compartments of eukaryotic cells. Here we describe cell fractionation methods developed for the study of the molecular mechanism(s) that govern mRNA partitioning between the cytosol and the ER. The experimental objective of these methods
is to separate, to high enrichment, cytosolic polysomes and membrane-bound polysomes. This chapter describes two independent methods of cellular fractionation namely, (1) sequential detergent extraction, and (2) mechanical fractionation followed by differential centrifugation.

3.1.1. Fractionation by Sequential Detergent Extraction

This method takes advantage of the relatively high cholesterol content of the plasma membrane, as compared to other cellular membranes. Digitonin is a β-sterol binding detergent that selectively solubilizes the plasma membrane, leaving the ER and nuclear membranes intact. Hence, sequential treatment with digitonin followed by a more lytic detergent, such as an NP-40/DOC cocktail, yields cytosolic- and membrane-bound polysome fractions, respectively (schematically illustrated in Fig. 1a). The various steps of the sequential detergent extraction procedure have been validated in HEK293 cells by immunofluorescence microscopy, where it can be seen that disruption of the plasma membrane with digitonin results in the release of (depolymerized) tubulin, without affecting the ER, the actin cytoskeleton, or the intermediate filament network (Fig. 1b). Following addition of the ER lysis buffer, the ER fraction is recovered in a soluble fraction and the nuclei, actin cytoskeleton, and intermediate filament network remain (Fig. 1b). Companion immunoblot analyses of marker protein distributions show that the cytosolic proteins GAPDH and tubulin are present in the cytosolic fraction, as expected, and the ER-membrane proteins, TRAPα and ER-lumenal protein, GRP94 are present in the ER fraction (Fig. 2a). The detergent-insoluble material consists primarily of nuclear and cytoskeletal elements, as evidenced by the marker proteins histone H3 and actin, respectively (Fig. 2a). Similarly, Northern blot analysis of the mRNA composition of the cytosol and membrane fractions show that the cytosol fraction is enriched for mRNAs encoding histone (H3F3A) and GAPDH, whereas the membrane fraction is enriched in mRNAs encoding ER resident proteins, such as GRP94 and calreticulin (Fig. 2b).

The method described below is for cells grown in monolayer. However, the protocol can be easily adapted for non-adherent cells by performing permeabilization, wash and lysis in suspension, and pelleting cells at 3,000 × g for 5 min between the different steps. The volumes of reagents mentioned in the following protocol are scaled to extract polysomes from ten million cells.

1. Seed HEK293T cells in a T75 flask to be 80–90% confluent on the day of the experiment.
2. Aspirate the media and wash the cells once with 10 ml of 1× PBS (room temperature).
3. Treat the cells with 10 ml of ice-cold PBS (1×) containing 50 µg/ml CHX for 10 min on ice (see Notes 4 and 5). Perform all remaining steps on ice using ice-cold reagents.
4. Add 1 ml of permeabilization buffer to the cells, taking care not to dislodge the cells (see Notes 6 and 7) and incubate for 5 min. Tilt the flask to drain the soluble material (cytosol fraction) and collect the cytosol in a pre-cooled microcentrifuge tube.
Fig. 2. Validation of the detergent fractionation method via Western and Northern blot analysis. (a) Protein from the total (T), cytosol (C), membrane-bound (M), and insoluble (IN) fractions of HEK293 cells were extracted using the TRizol® Reagent and equivalent amounts of protein were resolved on a 10% SDS polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and analyzed for the presence of ER resident (TRAPα and GRP94), cytosolic (tubulin and GAPDH), and nuclear (Histone H3F3A) proteins by western blotting. Primary antibodies were used at a dilution of 1:3,000 and HRP-conjugated secondary antibodies were used at 1:5,000. (b) RNA from the T, C, M, and IN fractions derived from the sequential detergent fractionation procedure were resolved on a denaturing agarose gel and transferred to a nylon membrane. The ribosomal RNA were stained using methylene blue and the profile was documented. The membrane was then probed for membrane-bound mRNAs (GRP94 and calreticulin) and cytosolic mRNAs (GAPDH and Histone H3F3A) using \( \gamma^{-}[32P]\) labeled antisense oligonucleotides. Following overnight exposure on phosphorimager plates, images were collected using Typhoon 9400 and image size/contrast adjusted using Adobe Photoshop v7.0. (c) Cytosol and membrane-bound (endoplasmic reticulum) polysomes were resolved on 15–50% sucrose gradients. The position of the ribosomes in the gradient was assessed by UV spectrometry (A_{254} nm). (d) RNA extracted from the sucrose gradient fractions were assessed by denaturing agarose gel electrophoresis followed by Northern blot for GRP94 and GAPDH. The RNA integrity is demonstrated by methylene blue staining of rRNA.
5. Wash cells gently with 1 ml of wash buffer and combine the wash with the cytosol fraction.

6. Treat the cells with 1 ml of lysis buffer for 5 min. Drain and collect the soluble material (membrane fraction).

7. Clarify both the cytosolic and membrane fractions at $7,500 \times g$ for 10 min to remove cell debris. Transfer the supernatants to clean, prechilled microcentrifuge tubes.

8. The various steps of this process can be visualized by immunofluorescence microscopy by staining for TRAPα (ER), tubulin (cytosol), vimentin (intermediate filaments), and actin (cytoskeleton) (see Subheading 3.2; Fig. 1b).

9. To analyze the RNA/protein content in the cytosol and membrane fractions (Fig. 2b), directly extract using 1 ml TRIzol® Reagent per 0.25 ml of sample (see Subheading 3.4). Samples in TRIzol® can be frozen at $-70^\circ\text{C}$ for storage prior to processing.

10. Alternately, the polysome profiles of the cytosolic and membrane-bound fractions can be analyzed by layering 1 ml of the cytosolic and membrane fractions on 15–40% linear sucrose gradients and subjecting them to velocity sedimentation (see Subheading 3.3). The ribosome composition in the gradient fractions can be analyzed by generating an $A_{254}$ nm trace manually or by using an automated gradient analyzer (see Subheading 3.3; Fig. 2c). The gradient fractions can then be extracted using TRIzol® reagent and assessed for RNA quality and composition by denaturing formaldehyde agarose gel and Northern blotting (see Subheading 3.5; Fig. 2d).

11. If the polyribosomes in the cytosol and membrane fractions need to be recovered for downstream applications, layer the clarified lysate over 1/3 volume of 0.5 M sucrose in the same buffer as that of the sample. Centrifuge at $100,000 \times g$ for 40 min in a Beckman TLA 100.2 rotor at 4°C. Ribosome pellets will appear clear and glassy.

A cell cracker, or more technically, a ball-bearing homogenizer, is a precision device that efficiently and reproducibly disrupts cell structure while maintaining organelle integrity. The following protocol (schematically outlined in Fig. 3a) allows for efficient recovery of membrane-bound and cytosolic polysomes. By immunoblot analysis of marker protein distribution, we identified conditions that allowed separation of the nucleus from the ER (as evidenced by the relative absence of the nuclear marker histone H3 in the ER fraction; Fig. 3b, lane 2) and the ER-bound polysomes from free-polysomes (Fig. 3b, lanes 2–4). Using this method, ER membranes (indicated by the ER membrane marker, TRAPα) were recovered in a $15,000 \times g$ spin
(Fig. 3a) as were, appropriately, ER-bound polysomes (Fig. 3b). The 44,000 × g spin, which should theoretically sediment free polysomes (based on K-factor calculations) yields a fraction enriched for cytosolic polysomes (Fig. 3b). The supernatant from the 100,000 × g spin (which theoretically sediments monosomes and individual ribosomes) contains the cytosolic proteins such as histone H3F3A and GAPDH, as expected, and also
GRP94 (ER lumen protein), which is partially released during cell homogenization.

1. Aspirate media from cells and wash the cells once with 10 ml PBS (1×) at room temperature.

2. Add 6 ml of ice-cold 1× PBS to each well and scrape cells with a cell scraper. All subsequent steps were performed on ice, using ice-cold solutions.

3. Pellet cells at 1,000 \( \times g \) for 4 min at 4°C. (Extract cell pellets from two wells of the 6-well plate with 1 ml of the TRIzol\textsuperscript{®} Reagent; see Note 8).

4. Resuspend pellets from the remaining four wells in 4 ml of ice-cold HLB.

5. Let cells swell on ice for 10 min.

6. Rinse the cell cracker (precooled and kept on ice) with cold HLB using 5-ml Luer-Lok\textsuperscript{™} syringes (see Note 9). Fill a precooled 5-ml Luer-Lok\textsuperscript{™} syringe with the cell suspension, avoiding air bubbles. Pass the cell suspension through precooled Cell Cracker with the 12 \( \mu \)m clearance ball bearing 12 times (six passes on each syringe). Pool the lysates and adjust the volume to 4 ml if needed.

7. Adjust homogenate to 250 mM sucrose and 4 mM MgCl\(_2\) using 2 M sucrose and 150 mM MgCl\(_2\).

8. Centrifuge at 1,000 \( \times g \) for 5 min at 4°C to pellet unbroken cells and nuclei (solubilize the pellets in TRIzol\textsuperscript{®} Reagent; see Note 8).

9. Centrifuge the supernatant from step 8 at 15,000 \( \times g \) for 15 min at 4°C (solubilize the pellets in TRIzol\textsuperscript{®} Reagent).

10. Centrifuge the supernatant from step 9 at 44,000 \( \times g \) for 15 min at 4°C (solubilize the pellets in TRIzol\textsuperscript{®} Reagent).

11. Centrifuge the supernatant from step 10 at 100,000 \( \times g \) for 1 h at 4°C (solubilize the pellets in TRIzol\textsuperscript{®} Reagent).

12. Add 1 ml TRIzol\textsuperscript{®} Reagent to 250 \( \mu \)l of the supernatant from 100,000 \( \times g \) spin (see Note 10).

13. Proceed to TRIzol\textsuperscript{®} extraction (Subheading 3.5) or freeze the samples at −80°C until RNA/Protein extraction.

### 3.2. Immunofluorescence Microscopy

1. Plate cells onto glass coverslips 12–24 h prior to analysis.

2. Rinse the cells in cold 1× PBS.

3. Fix the cells in ice-cold 4% paraformaldehyde in 1× PBS for 15 min, on ice.

4. Rinse thrice with cold 1× PBS for 5 min each.

5. Permeabilize the fixed cells in either cold PBS (1×) with 1% (v/v) Triton X-100 for 10 min on ice, or cold acetone, with incubation at −20°C for 10 min.
6. Rinse thrice with cold 1× PBS for 5 min each.
7. Block using the blocking solution for 1 h at room temperature (RT), or overnight at 4°C.
8. Add primary antibody solution (sufficient to cover the cell layer) and incubate at RT for 1 h. Alternatively, invert the coverslip onto a 50 µl drop of the primary antibody solution, on parafilm, and incubate for 1 h at RT.
9. Rinse thrice with 1× PBS for 5 min each at RT.
10. Repeat step 8 with secondary antibody solution and DAPI solution, and incubate for 1 h at RT, in the dark.
11. Rinse thrice with 1× PBS for 5 min each at RT.
12. Mount the cover slip on a slide using antifade mounting medium.
13. Seal the slides with nail polish.

**3.3. Polysome Gradient Analysis**

Pour 15–40% linear sucrose gradient as follows:

1. Add 5 ml of 15% sucrose in a Beckman centrifuge tube (SW40).
2. Underlay 5 ml of 40% sucrose solution slowly so that the interface is not disturbed (see Note 11).
3. Cover the tube with parafilm and slowly tip the tube so that it is laying on its side. Support the tube on both sides with microcentrifuge tube racks to prevent rolling.
4. Let the gradient form over 2 h. A gradient maker such as SG 15 Gradient Maker (GE Healthcare) can also be used to generate a linear gradient.
5. Carefully tip the tube back up and place it on ice for at least 30 min. If needed, the gradient can be stably stored on ice overnight.
6. Overlay the sample on the sucrose gradient and centrifuge at 45,000 × g for 3 h.
7. Fractionate the gradient, either via an automated gradient fractionator (i.e., Teledyne/ISCO) or manually; in the absence of a gradient fractionator the centrifuge tubes can be manually punctured, fractions collected, and the UV absorbance (254 nm) measured for each fraction to generate the polyribosome trace.
8. The gradient fractions can further be extracted using TRIzol® reagent and the RNA and protein composition can be assessed by Northern and Western blotting, respectively.

**3.4. RNA and Protein Extraction Using the TRIzol® Reagent**

TRIzol® Reagent can be used to sequentially extract RNA and protein from the same biological sample.

1. Incubate the TRIzol®-treated samples at room temperature for 10 min to allow dissociation of nucleoprotein complexes (see Note 8).
2. Spin the TRIzol®-treated samples at 10,000 × g for 10 min to remove insoluble material (optional).

3. Add 200 μl of chloroform per ml of TRIzol® and vortex for 15 s.

4. Incubate at room temperature for 3 min to allow phase separation.

5. Spin at maximum speed for 15 min on a table-top centrifuge at 4°C.

6. RNA isolation: Carefully transfer 0.6 ml of the aqueous phase (containing the RNA) to a clean tube (see Note 12) and add 0.5 ml of isopropanol. Mix well and incubate at room temperature for 10 min to precipitate RNA (see Note 13). Spin at 13,000 × g for 10 min to pellet the RNA. Wash the RNA pellet with 1 ml of 75% ethanol. Air dry the pellet for 2–3 min (do not allow the pellet to over dry) and re-suspend the RNA in appropriate volume of DEPC-treated water (see Note 14). The RNA sample can be stored in nuclease-free water at −80°C for 1–2 years, or in 70% ethanol at −80°C, indefinitely.

7. Protein isolation: Remove any remaining aqueous phase and as much of the interphase as possible without removing the organic phase which contains the protein. Add 0.3 ml of ethanol to 0.6 ml of the organic phase to precipitate any DNA. Incubate for 5 min at room temperature and spin at 3,000 × g for 5 min to remove the DNA. The DNA pellet will be barely visible and very soft. Take care to not disturb it while removing the supernatant. To precipitate the protein, add 0.75 ml of isopropanol per 0.4 ml of the organic phase–ethanol mixture and incubate for 10 min at room temperature to precipitate the protein. Spin at 13,000 × g for 10 min to pellet the protein. Wash the pellet for 20 min with 1 ml of 0.4 M guanidine hydrochloride in 95% isopropanol at room temperature. Repeat this step for a total of three washes. Wash using 1 ml of ethanol to remove the salt and air-dry the pellet for 2–3 min. Resuspend the protein pellet in protein sample buffer. If the pellet does not go into solution upon incubation at room temperature for 15–20 min, heating the sample at 65°C for 15 min with occasional vortexing will assist solubilization (see Note 15).

8. RNA and protein quantification: Measure the RNA concentration by UV absorption at 260 nm. The yield of RNA from ten million cells is typically about 50–100 µg, and can be used in a variety of downstream applications including, but not limited to Northern blotting, RT-PCR, and cDNA microarrays (see Note 16). The protein concentration is best measured by standard assays such as the BCA assay (Pierce). Absorption at 280 nm can be used to get a rough estimate of the protein...
concentration, but these measurements are often dubious due to possible nucleic acid contributions to the absorbance readings.

1. Rinse the gel tray, comb, and the electrophoresis apparatus with 0.2% SDS and DEPC-treated water.

2. Formaldehyde agarose gel: Combine 1 g of agarose in 82 ml of DEPC-treated water and dissolve by heating. Add 10 ml of 10× MOPS buffer to the melted agarose. When the solution cools to about 60°C, add 8 ml of formaldehyde in a fume hood (see Notes 17 and 18).

3. Formamide/formaldehyde sample buffer: Mix 200 μl of formamide, 70 μl of formaldehyde, 30 μl of 10× MOPS, and 27 μl tracking dye (Ambion). Add 24 μl of sample buffer to 8 μl of RNA sample and heat the samples for 10 min at 65°C. Cool the samples to room temperature and load.

4. Run the gel in 1× MOPS buffer at 120 V for 2 h (see Note 19).

5. Set up the Northern transfer as follows: Cut one piece of Hybond™ membrane and six pieces of Whatman 3MM filter paper to the size of the gel. Soak the gel and the membrane in Northern transfer buffer for 5–10 min. Assemble the Northern transfer by sandwiching the gel and the membrane between six filter paper squares (three on each side) and place this assembly on a stack of paper towels. Gently roll a glass pipette over the assembled transfer stack to eliminate any trapped air. Wet two long pieces of the filter paper precut to the width of the gel and place one end on top of the transfer stack and dip the other end in transfer buffer. This will serve as a wick to facilitate downward capillary transfer (see Note 20). Place a plastic tray on top of the transfer stack to limit buffer evaporation. Transfer for 2–12 h.

6. Crosslink RNA to the membrane using the auto crosslink option setting on a Stratagene Stratalinker. After this point, the reagents do not need to be RNase-free.

7. Stain the membrane with methylene blue stain and record the rRNA banding pattern.

8. Destain the membrane in water and prehybridize the membrane using ExpressHyb™ hybridization solution for 30 min. The hybridization should be carried out at 42°C when using oligo probes, and at 50°C when using random probes (see Note 21).

9. Radiolabeled probes: oligonucleotide probes can be end-labeled with γ-[32P] ATP using T4 polynucleotide kinase
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Alternately, probes can be made by random priming of a fragment of the target gene using α-[\textsuperscript{32}P] CTP, with a first strand cDNA synthesis kit (Ambion). Unincorporated α-[\textsuperscript{32}P] ATP is removed using a G-25 Sephadex quick spin column.

10. Quantify the radiolabeled probe using a liquid scintillation counter and add $1 \times 10^7$ cpm $[^{32}\text{P}]$-labeled DNA probe to the prehybridized membrane. It is important to denature the probes generated by random priming by boiling at 95°C and rapidly cooling on ice before adding to the blot.

11. Hybridize the probe for 8 h to overnight.

12. Rinse the membrane in low-stringency wash buffer for 30 min, followed by high-stringency wash buffer for 30 min.

13. Place the blot on prewet filter paper, cover with Saran Wrap and expose to a phosphorimager screen for 3 h or longer and scan (see Note 22).

14. Radiolabeled probes can be stripped off a membrane by washing in 95°C 0.5% SDS solution (see Note 23).

15. The membrane can be reprobed using a different probe by repeating steps 8–14.

1. Dilute the protein samples 1:4 in 4× sample buffer. Boil the samples at 95°C for 5 min. Cool and load 20 μg of protein per lane on a 10% SDS polyacrylamide gel.

2. Run the gel at a constant voltage of 120 V until the dye front reaches the end of the gel, in 1× SDS running buffer.

3. Soak the gel in CAPS transfer buffer.

4. Cut nitrocellulose membrane to the dimensions of the gel and soak in CAPS buffer.

5. Semi-dry Western transfer: assemble the transfer by sandwiching the membrane and the gel between four pieces of prewet Whatman 3MM filter paper (cut to the dimensions of the gel), two pieces per side.

6. Perform Western transfer at a constant current of 100 mA for 30 min.

7. Ponceau stain the membrane to check for efficient transfer.

8. Destain the membrane in water and block in 5% milk in 1× PBS-T for 1 h at room temperature (see Note 24).

9. Wash the membrane in 1× PBS-T once for 15 min and twice for 5 min.

10. Add the primary antibody diluted to the appropriate final concentration in 2% milk in 1× PBS-T to the blot and incubate for 1 h at room temperature (see Note 25).

11. Repeat step 10.
12. Add the HRP-conjugated secondary antibody in 2% milk in 1× PBS-T and incubate for 1 h at room temperature (see Notes 24 and 25).

13. Repeat step 10.

14. Develop the blot using ECL reagents.

4. Notes

1. DEPC is a suspected carcinogen. Avoid inhalation and skin contact and always handle in a fume hood. However, after autoclaving, DEPC-containing solutions are no longer reactive or hazardous, though they have a slightly sweet odor.

2. Digitonin stock solutions are unstable to long-term storage. We recommend making up small volumes of stock solution and discarding at monthly intervals.

3. TRIzol® Reagent contains phenol and should be used with caution. Standard safety procedures should be followed to dispose phenol-containing solutions.

4. HEK293T cells are weakly adherent. Care should be taken to avoid dislodging them, e.g. pipetting reagents gently on to the sides of the tissue culture vessel. Coating the surface of tissue culture plasticware or coverslips with poly-d-lysine or collagen will enhance cell attachment. For fractionation experiments, collagen coating is preferred.

5. The 10-min incubation of cells on ice at this point is important to enable microtubule depolymerization. If cells are not incubated on ice for a sufficiently long period, tubulin will be primarily present in the insoluble fraction, rather than the cytosol.

6. This procedure can be easily scaled up or down by correspondingly changing the volume of reagents used. The main factor to consider is that the amount of the reagents used should be sufficient to cover the entire surface of the cell monolayer.

7. For adherent cells such as HEK293T, it is preferred to perform the permeabilization/cytosol release step on the monolayer. When lifted by trypsinization, the recovery of the cytosol fraction from adherent cells can be variable and should be thoroughly evaluated prior to experimentation.

8. It may be necessary to pass the TRIzol®-ized sample through 27½ gauge needle to completely shear the DNA. If shearing the DNA by this method, extreme care should be taken to avoid direct contact with TRIzol® Reagent.
9. Luer-Lok™ syringes are necessary to avoid sample loss while using the cell cracker; standard syringes tend to detach due to the high pressure generated when cells pass through.

10. Alternately, precipitate the protein from the supernatant fraction using trichloroacetic acid method and RNA using lithium chloride method. Please refer to Maniatis et al. (19) for detailed protocols.

11. Keep the tip of the needle only slightly under the interface so that when the needle is retracted, it does not leave a track of 40% sucrose through the 15% layer.

12. Be careful to not touch the interphase with the micropipette tip as this reduces the quality of the RNA preparation. It would be best to try not to recover the last 5% of the aqueous phase.

13. If working with small quantities of RNA, precipitate at −20°C with and include yeast tRNA or glycogen as a carrier to aid precipitation.

14. Even trace amounts of DEPC can inactivate most polymerases and other enzymes. Hence, use commercial nuclease-free water if RNA will be used for molecular biology applications downstream.

15. While there is anecdotal suggestions that the protein pellet from TRIzol® extraction is difficult to get into solution, we have found that our sample buffer efficiently solubilizes the pellet and works well in the standard Laemmli buffer system. We have confirmed by SDS-PAGE that the profile of proteins from HEK293T extracted by TRIzol®, or directly extracted in sample buffer, are identical.

16. For applications that require the RNA sample to be strictly free of DNA, it may be necessary to include a step of DNase-treatment using RNase-free DNase such as Turbo-DNase from Ambion.

17. Formaldehyde is highly toxic. All waste generated (including the gel) should be disposed following standard safety procedures.

18. If required, 2 µl of SyBr safe dye can be added to the gel solution to enable visualization of the RNA after electrophoresis.

19. The 1× MOPS buffer can be reused several times unless RNAse contamination is suspected.

20. Make certain that there is no buffer short circuit between the wick and the paper towels. If required, place pieces of parafilm around the edge of the gel to prevent the wick from touching the paper towels. This is to ensure that the buffer transfer occurs only through the gel.
21. This is only a general rule of thumb and works in our hands for most of the probes we use in the lab. The stringency of the hybridization depends on the temperature of hybridization. Hence it may be required to identify an optimal hybridization temperature for a given probe depending on its length, Tm, etc. to ensure specificity and sensitivity.

22. Drying of the membrane hybridized with the probe fixes the probe to the membrane and make it very difficult to strip. Hence care should be taken to avoid membrane drying if it is to be reprobed. In the event of the membrane drying, it could be placed in −20°C for an extended period, to allow the radiolabel to decay before reprobing for another RNA.

23. Efficient stripping may require multiple washes in 95°C 0.5% SDS. Stripping of the probes may need to be checked by exposing the blot overnight to a phosphorimager screen.

24. This step can be done at 4°C overnight.

25. If you reuse antibody solutions, omit sodium azide in the HRP-conjugated secondary antibody stock solution. Sodium azide inhibits the activity of the HRP enzyme.

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