

Determining Ribosome Translational Status by Ribo-ELISA

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[Abstract] The Ribo-ELISA was originally developed to elucidate the basis for the ribopuromycylation method (RPM)-based detection of ribosome bound nascent chains. The Ribo-ELISA enables characterization of the translational status of ribosomes, and can be applied to the discovery of super-ribosomal complexes with novel ribosome associated macromolecules that are isolated by physical fractionation in sucrose gradients or other methods.

Keywords: Ribosome, ELISA, Ribopuromycylation

[Background] Ribosomes are heterogeneous structures consisting of 40S and 60S subunits that are present in cells as monosomes and polysomes, when multiple ribosomes are bound to a single mRNA. Additionally, translating ribosomes can be associated with multiple molecular complexes that modulate translation. The ribosome ELISA (Enzyme-Linked ImmunoSorbent Assay) enables detection of translating ribosomes by *in vitro* puromycylation of ribosome associated nascent chains (David *et al.*, 2012). This chemical reaction proceeds spontaneously upon adding puromycin to ribosomes with bound nascent chains. This method quantitates the number of nascent chains present per ribosome and can be used to determine the translational status of monosomes relative to polysomes, and identify ribosomes bound to other macromolecules that alter its sedimentation rate or migration in sizing columns.

Materials and Reagents

A. Materials

1. 175 cm² flasks (Corning, catalog number: 431306)
2. Thinwall 13.2 ml polypropylene tube (Beckman Coulter, catalog number: 331372)
3. Parafilm (Bemis, catalog number: 701606)
4. Pipette tips (MultiGuard™ Tips 1-100 µl, 1-200 µl, 100-1,000 µl) (Sorenson BioScience, catalog numbers: 36060, 14220, 14200)
5. 1.5 ml Eppendorf tubes (Sigma-Aldrich, catalog number: Z666505)
Manufacturer: Eppendorf, catalog number: 022431081.
6. 96-well Millipore Multiscreen HTS PVDF plates (Merck, catalog number: MSIPS4W10)
7. Paper towels

B. Cell line(s)

Though this procedure was originally designed for HeLa cells, it can be easily adapted for any other cell lines. The described procedure and associated picture (Figure 1) uses HeLa cells (ATCC, catalog number: CCL-2.1)

C. Reagents

1. Trypsin-EDTA (Thermo Fisher Scientific, Gibco™, catalog number: 25300054)
2. StartingBlock™ blocking buffer (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 37538)
3. Hydrogen chloride (HCl) 1.6%
4. Potassium phosphate monobasic (KH₂PO₄)
5. Sodium chloride (NaCl)
6. Na₂HPO₄·7H₂O (Life Technologies)
7. Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Gibco™, catalog number: 41966029)
8. Penicillin/streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 10378016)
9. Fetal bovine serum (FBS) (Eurobio, catalog number: CVFSVF0101)
10. Tris-HCl pH 7.5 (Thermo Fisher Scientific, Invitrogen™, catalog number: 15567027)
11. Magnesium chloride hexahydrate (MgCl₂·6H₂O) (Sigma-Aldrich, catalog number: M2670)
12. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
13. Sucrose (Sigma-Aldrich, catalog number: 84097)
14. NP-40 10% (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 28324)
15. Complete Mini EDTA-free protease inhibitor tablets (Sigma-Aldrich, Roche Diagnostics, catalog number: 11836170001)
16. RNase Out (Life Technologies, catalog number: 100000840 or Thermo Fisher Scientific, Invitrogen™, catalog number: 10777019)
17. DEPC treated water (Thermo Fisher Scientific, Invitrogen™, catalog number: 750023)
18. 16% paraformaldehyde (Electron Microscopy Sciences, catalog number: 15710)
19. Glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 25030081)
20. 70% ethanol (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R40135)

D. Protein synthesis inhibitors

1. Cycloheximide (CHX) (Sigma-Aldrich, catalog number: C7698)
Stock solution (1,000x): 100 mg/ml (or 355 mM) in 50% ethanol
Store at -20 °C
2. Emetine dihydrochloride (Emetine) (Sigma-Aldrich, catalog number: E2375)
Stock solution (1,000x): 25 mg/ml (or 45 mM) in 50% ethanol
Store at -20 °C
3. Puromycin (PMY) (Sigma-Aldrich, catalog number: P7255)

Stock solution (1,000x): 50 mg/ml (or 91 mM) in 50% ethanol

Store at -20 °C

E. Antibodies

1. Primary antibodies

- a. Anti-puromycin mouse monoclonal antibodies: hybridoma clone 2A4 is available to the scientific community from the Developmental Studies Hybridoma Bank <http://dshb.biology.uiowa.edu/PMY-2A4>
- b. Anti-ribosomal P antibody: human polyclonal autoimmune antiserum (from lupus patients) which recognizes three proteins of the 60S ribosomal subunit, RPLP0, P1 and P2 (ImmunoVision, catalog number: HPO-0300)

2. Secondary antibodies

- a. Rabbit anti-mouse HRP conjugate (Cappel) (MP Biomedical, catalog number: 55564)
- b. Goat anti-human Ig-HRP (Jackson ImmunoResearch, catalog number: 109-035-003)

F. Solutions (see Recipes)

1. Phosphate buffered saline (PBS)
2. Growth medium
3. Homogenization buffer
4. Polysome buffer
5. 15% sucrose solution
6. 50% sucrose solution
7. 3% PFA

Equipment

1. PIPETMAN 1-20 µl (Sartorius, model: Proline® Plus, catalog number: 728030)
2. PIPETMAN 20-200 µl (Sartorius, model: Proline® Plus, catalog number: 728060)
3. PIPETMAN 100-1,000 µl (Sartorius, model: Proline® Plus, catalog number: 728070)
4. Water bath (JULABO, model: TW20)
5. Class II laminar flow hood (FASTER, model: SafeFAST Elite)
6. Cell Incubator (Heraeus, model: HERACell)
7. Centrifuge (Eppendorf, model: 5415 R)
8. Inverted microscope (Nikon Instruments, model: Eclipse TS100)
9. Microplate photometer (TECAN, model: Infinite M200)
10. Dounce homogenizer (DWK Life Sciences, Kimble, catalog number: 885300-0002)
11. Ceramic bead tubes Lysing matrix D (MP Biomedicals, catalog number: 116913050)
12. FastPrep-24 MP device (MP Biomedicals, catalog number: 116004500)
13. Ultracentrifuge (Beckman Coulter)

14. Rotor (Beckman Coulter, model: SW 41 Ti)
15. MultiScreenHTS Vacuum manifold (Merck, catalog number: MSVMHTS00)
16. Optional: Density gradient fractionator (Teledyne Isco)

Software

1. GraphPad Prism software

Procedure

The following procedure describes the first application of Ribo-ELISA (David *et al.*, 2012) which was used to detect puromycin association with ribosomes: at that time it was not clear whether puromycin binding was limited to translating ribosomes.

Either emetine or CHX can be used as elongation inhibitors to freeze polysome. **Unlike CHX, emetine irreversibly inhibits translation, and need not be maintained throughout the procedure.**

Day 1:

1. Pre-warm growth medium (see Recipes), PBS and trypsin-EDTA in 37 °C water bath. Trypsinize two 175 cm² flasks of cells at 70%-80% confluency. Remove the medium, wash with 10 ml of warm PBS, add 5 ml of trypsin-EDTA and leave for 5 min at 37 °C. Resuspend cells with 5 ml of growth medium.
2. Seed two 175 cm² flasks with 1 x 10⁷ HeLa cells per flask (Corning) containing 15 ml of warm growth medium each. Incubate for 30 h to allow HeLa cells to attach and proliferate.
3. **Sucrose gradients (see Video 1).** Prepare **15% and 50% sucrose solutions** (see Recipes). Add 5 ml of 50% sucrose solution in 13.2 ml Polypropylene centrifuge tube (Beckman Coulter). Tilt the tube (almost 90°) and carefully add 5 ml of 15% sucrose solution on the top (add slowly the solution flowing along the tube wall and slowly put the tube back to vertical). Seal the tube with Parafilm and gently put the tube in horizontal position on the bench. Wait 1-2 h and put it back to the vertical. The 15%-50% sucrose gradient is ready. Store it in the fridge O.N.

Note: Other methods can be used to prepare gradients. A convenient alternative is the freeze-thawing method which entails successive freezing (-80 °C) of 2 ml layered sucrose solutions starting from the highest sucrose concentration (50%, 40%, 30%, 20%, 10% w/v sucrose solutions) (Bastide et al., 2017).



Video 1. Sucrose gradients preparation. This video provides step by step guidelines for preparing 15% and 50% sucrose gradients.

Day 2:

1. Examine HeLa cells under an inverted microscope to ensure right confluence (between 60%-70%).
2. Incubate cells for 5 min with 10 ml of warm **growth medium** supplemented with 100 µg/ml CHX (or 20 µg/ml emetine) added directly to the medium.
3. Remove the medium, wash twice with 10 ml of warm PBS. Add 5 ml of trypsin-EDTA and put the cells back in the incubator for 5 min, then resuspend cells with 5 ml of growth medium, centrifuge at 400 x g for 5 min and wash the pellet twice with 10 ml of ice-cold PBS (400 x g, 5 min, 4 °C) (both solutions supplemented with 100 µg/ml CHX, see Recipes).

Note: From this step RNase free tips should be used.

4. Pellet cells by centrifugation (400 x g, 5 min, 4 °C). Resuspend cells in 1 ml ice-cold **homogenization buffer** (see Recipes).
5. Transfer the cell suspension to ceramic beads tubes (Lysing matrix D) on ice.

Note: Importantly, NP-40 in homogenization buffer is not essential when using ceramic beads to lyse cells: ribosomes are recovered with similar efficiency. Therefore, Ribosome ELISA can be performed in the absence of detergent.

6. 'Vortex' at RT for 20 sec (using the FastPrep-24 MP device).

*Note: In lieu of ceramic beads, cells can be stroked 10 times in ice cold **homogenization buffer** using Dounce homogenizer embedded in ice.*

7. Transfer supernatant to 1.5 ml Eppendorf tubes. Spin at 13,000 x g for 10 min at 4 °C.
8. Carefully collect the supernatant.

Note: If the supernatant is not perfectly clear, recentrifuge.

9. Slowly layer 1 ml clear lysate along the tube wall on the top of the cold 15-50% sucrose gradient. Balance the rotor with another sample or a tube filled with water. Spin at 151,263 x g (35,000 rpm) (Beckman Coulter, rotor SW41.Ti) for 2.5 h at 4 °C.

10. Fractionate (1 ml fractions) and measure absorbance continuously at 254 nm using a gradient system automate (Teledyne Isco). You will get 11 fractions.

Note: If you lack access to an automated gradient collector, fractions can be collected in the old-fashioned way; manually from the top. Use a PIPETMAN 100-1,000 µl with a 1 ml tip. Push the piston to the 1 ml max, and place the tip in contact with the meniscus. Carefully, collect 1 ml sucrose by releasing the pipette piston slowly. Always keep the tip in contact with the meniscus. Put each fraction in a clean 1.5 ml tube (11 fractions).

11. Add 100 µl of each fraction to wells of a 96-well Millipore Multiscreen HTS PVDF plate (11 wells, lane 1). Repeat this operation five times in following lanes to get technical replicates (3 wells per fraction) for two experimental conditions (anti-puromycin staining and anti-P protein staining). Load an identical plate as control ('no puromycin labeling'). Incubate plates for 10 min on ice, and then vacuum the liquid through using the MultiScreenHTS Vacuum manifold.
12. Add 200 µl/well of polysome buffer (see Recipes) and vacuum again.
13. **Puromycin labeling.** Add 100 µl of PMY (91 µM) containing **polysome buffer** to the experimental plate and 100 µl of **polysome buffer** to the control plate. Incubate for 10 min on ice. In parallel, skip this step in order to generate the 'no puromycin labeling' control plate.
14. **Wash.** Flick plate out into sink, briefly drain inverted on paper towels. Add 200 µl of polysome buffer, flick plate out again. Repeat this operation twice.
15. **Fixation.** Add 100 µl of 3% paraformaldehyde (PFA) (see Recipes) inside the hood. Incubate for 10 min at room temperature (RT). Dispose of the PFA waste properly.
Note: We used this fixation step in the original protocol to mimic the RPM procedure on cells. However, this step is not necessary to detect puromycylated nascent chains.
16. **Wash.** Wash wells with 200 µl PBS 3 times.
17. **Membrane blocking.** Incubate with 100 µl of StartingBlock™ blocking buffer for 30 min at RT. Vacuum.
Note: In the original procedure, StartingBlock™ gave very low background Ab binding. Using different Abs, other blocking buffers might be superior.
18. **Primary antibody.** Incubate for 1 h with 50 µl of either 2.5 µg/ml anti-PMY mAb (in StartingBlock™) at RT or anti-Ribosomal P antibodies (1/5,000, in StartingBlock™)
19. **Wash.** Wash wells with 200 µl PBS 3 times.
20. **Secondary antibody.** Incubate with 50 µl of secondary antibody HRP conjugate, rabbit anti-mouse for PMY staining and goat anti-human for ribosomal P staining (1/100 in StartingBlock™) for 1 h at RT.
21. **Wash.** Wash wells with 200 µl PBS 3 times.
22. **Signal Generation.** Add 100 µl of TMB substrate (KPL) to each well. When some blue color becomes visible in the well, stop the reaction using 50 µl of 1.6% HCl. Transfer the liquid in each well to a plastic plate with a transparent bottom. Then, measure absorbance at 450 nm.

23. **Quantification.** Determine the level of background staining by using the average of unlabeled wells from the control plate and subtract it from test values. In the illustrated example, data were graphed using GraphPad Prism software (Figure 1).

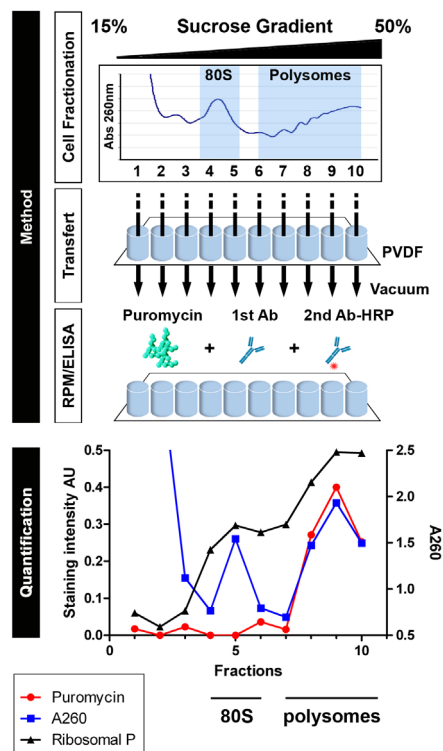


Figure 1. Schematic representation of Ribosome ELISA applied to detect PMY association with translating ribosomes (from David *et al.*, 2012). HeLa cells or HeLa cells incubated with emetine for 15 min were lysed and fractionated on 15-50% sucrose gradients. Fractions were bound to PVDF 96-well plates and incubated with PMY, which results in ribosome-catalyzed puromycylation. Ribosomes were detected by A_{260} of fractions or by ELISA for the ribosomal P proteins (here resolved into the 3 known species) as detected by human autoimmune Abs, which establishes that 60S subunit and monosome (80S) were bound to PVDF (fractions 4 and 5). Puromycylation was detected by ELISA for PMY using 12-D10, and clearly demonstrates that monosomes and free 60S subunits do not stably associate with PMY, which requires nascent chains.

Data analysis

For data analysis, we previously used GraphPad Prism software. An example is presented in the following paper: David *et al.*, 2012.

Notes

Polysome profile can vary dramatically depending on the cell line and translation rate.

Recipes

1. Phosphate buffered saline (PBS)
 - 210.0 mg/L KH_2PO_4
 - 9,000 mg/L NaCl
 - 726.0 mg/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (Life Technologies)
2. Growth medium
 - Dulbecco's modified Eagle's medium with glutamine (Thermo Fisher Scientific)
 - 1% penicillin/streptomycin
 - 7.5% fetal bovine serum (FBS)
3. Homogenization buffer
 - 50 mM Tris-HCl pH 7.5
 - 5 mM MgCl_2
 - 25 mM KCl
 - 0.2 M sucrose (Sigma-Aldrich)
 - 0.5% NP-40 (Thermo Fisher Scientific)
 - 100 $\mu\text{g/ml}$ CHX (Sigma-Aldrich)
 - EDTA-free protease inhibitors (Roche)
 - 10 U/ml RNase Out (Life technologies)
 - DEPC water (Thermo Fisher Scientific)
4. Polysome buffer
 - 50 mM Tris-HCl pH 7.5
 - 5 mM MgCl_2
 - 25 mM KCl
 - 0.2 M sucrose (Sigma-Aldrich)
 - 100 $\mu\text{g/ml}$ CHX (Sigma-Aldrich)
 - EDTA-free protease inhibitors (Roche)
 - 10 U/ml RNase Out (Life technologies)
 - DEPC water (Thermo Fisher Scientific)
5. 15% sucrose solution
 - 15% sucrose (m/v)
 - 50 mM Tris-HCl pH 7.5
 - 5 mM MgCl_2
 - 25 mM KCl
 - 100 $\mu\text{g/ml}$ CHX (Sigma-Aldrich)

- EDTA-free protease inhibitors (Roche)
- 10 U/ml RNase Out (Life technologies)
- DEPC water (Thermo Fisher Scientific)
- 6. 50% sucrose solution
 - 50% sucrose (m/v)
 - 50 mM Tris-HCl pH 7.5
 - 5 mM MgCl₂
 - 25 mM KCl
 - 100 µg/ml CHX (Sigma-Aldrich)
 - EDTA-free protease inhibitors (Roche)
 - 10 U/ml RNase Out (Life technologies)
 - DEPC water (Thermo Fisher Scientific)
- 7. 3% PFA
 - Dilute stock solution (16%) in PBS 1x

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