Ribopuromycylation in Coronavirus-Infected Cells

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Abstract

Ribopuromycylation enables the visualization and quantitation of translation on a cellular level by immunofluorescence or in total using standard western blotting. This technique uses ribosome catalyzed puromycylation of nascent chains followed by immobilization on the ribosome by antibiotic chain elongation inhibitor emetine. Detection of puromycylated ribosome-bound nascent chains can then be achieved using a puromycin-specific antibody.

Key words Ribopuromycylation, Translation, Puromycin, Emetine, Puromycylation

1 Introduction

Ribopuromycylation (RPM) is a method that allows visualization and quantitation of global or cellular translation via the addition of puromycin to ribosome bound nascent polypeptide chains [1]. Puromycin (PMY) is an aminonucleoside tyrosine tRNA mimic that enters the A site of prokaryotic and eukaryotic ribosomes and is covalently incorporated by ribosome-catalyzed reaction into the nascent chain C terminus, resulting in termination of translation [2]. Eggers et al. first used puromycin as a tag for nascent proteins using polyclonal PMY antibodies for immunoblotting and immunoprecipitation of nascent proteins [3]. Following this work, Schmidt et al. produced monoclonal PMY antibodies to overcome nonspecific interactions of polyclonal antibodies for use by flow cytometry to measure relative translation rates in cells exposed to PMY [4]. David et al. improved on their initial RPM protocol by the addition of emetine. Emetine is a translation elongation inhibitor that irreversibly binds the 40S ribosomal subunit. When compared to cycloheximide, another translation elongation inhibitor, emetine was shown to increase RPM signal [5]. RPM is now a widely used technique for the study of cellular translation [6–9]. RPM is a useful technique to compare the level of translation when using translation inhibitors, viral infections, stressors,
different cell lines, or disease models. The technique can be utilized for both immunofluorescence and western blot detection. The western blot method allows total protein translation to be visualized, similar to $^{35}$S Met labeling, and is useful to visualize an overall change or differences at the population level. Importantly, the immunofluorescence method allows for translation to be visualized on a single-cell level in which individual cells, for example, infected and uninfected, can be compared.

2 Materials

2.1 RPM to Tag Nascent Polypeptides

1. Coverslips.
2. Tweezers.
3. 6- and 24-well plates.
4. Vero cells.
5. EMEM: Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal calf serum 2 mM l-glutamine and 2.2 g/l sodium bicarbonate.
6. IBV strain BeauR [10].
7. Incubator.
8. BES medium (IBV infection media): 1× Eagle’s minimum essential medium (EMEM), 0.3% tryptose phosphate broth, 0.2% bovine serum albumin (BSA), 20 mM N,N-bis 2-hydroxyethyl-2-aminoethanesulfonic acid (BES), 0.21% sodium bicarbonate, 2 mM l-glutamine, 250 U/ml nystatin, 100 U/ml penicillin and streptomycin (see Note 1).
9. 50 mM sodium arsenite.
10. 21.2 mM puromycin.
11. 180 nM emetine.

2.2 Detection of Tagged Nascent Polypeptides by Immuno-fluorescence (IF)

1. Phosphate-buffered saline (PBS).
2. 4% paraformaldehyde in PBS.
3. 0.1% Triton ×100 in PBS.
4. Monoclonal anti-puromycin antibody (see Note 2).
5. 0.5% BSA in PBS.
6. Fluorescently conjugated secondary antibody, e.g., anti-mouse Alexa Fluor 488.
7. 20 mg/ml 4′,6-diamidino-2-phenylindole (DAPI).
8. Mounting media.
9. Nail varnish.
10. Microscope slides.
11. Rocking platform shaker.
12. Confocal microscope.

### 2.3 Detection of Tagged Nascent Polypeptides by Western Blot (WB)

1. 4× Laemmli sample buffer (sample buffer): 277.8 mM Tris–HCl pH 6.8, 44.4% glycerol, 4.4% sodium dodecyl sulfate (SDS), 0.02% bromophenol blue, 10% β-mercaptoethanol.
2. Sonicator.
3. SDS-PAGE gel equipment, e.g., Biorad mini protean IV or other suitable electrophoresis equipment.
4. Western blotting equipment, e.g., Biorad Turboblot or other suitable western blotting equipment.
5. Nitrocellulose membrane.
6. Rocking platform shaker.
7. Phosphate-buffered saline (PBS).
8. PBST: 0.1% Tween-20 in PBS.
9. 5% milk in PBST (Marvel or equivalent).
10. Monoclonal anti-puromycin antibody (see Note 2).
11. Fluorescently/HRP-conjugated secondary antibody suitable for western blot.
12. Western blot imaging system.

## 3 Methods

### 3.1 RPM and Detection of Tagged Nascent Polypeptides by IF

1. Seed cells in a 24-well plate or equivalent on sterilized coverslips aiming for 60–80% confluency (see Note 3) at the time of puromycin addition [5] (see Note 4).
2. Wash cells with PBS.
3. Infect cells with 150 μl virus at the desired MOI or mock infect using 1x BES and incubate for 1 h at 37 °C (see Note 5).
4. Add 850 μl 1x BES and incubate at 37 °C for a further 23 h (see Note 6).
5. One hour prior to RPM, add 10 μl sodium arsenite to the existing media to give a final concentration of 500 μM and incubate at 37 °C (see Note 7).
6. At the time of RPM, add 8.7 μl puromycin to the existing media to give a final concentration of 18.4 μM and incubate for 30 s at room temperature (RT) (see Note 8).
7. Add 11.5 μl emetine to the existing media to give a final concentration of 208 μM and incubate for 1 min at RT (see Note 9).
8. Wash cells three times using RT 1x BES.
9. Fix cells using 4% paraformaldehyde for 15 min at RT (see Note 10).

10. All subsequent incubation steps should be performed at RT on a rocking platform shaker. Permeabilize cells using 0.1% Triton X-100 for 10 min.

11. Label cells with monoclonal anti-puromycin antibody (see Note 2) and anti-virus antibody for 1 h diluted as appropriate in 0.5% BSA in PBS.

12. Wash cells three times with PBS with no incubation between washes. After the third wash, incubate for 5 min.

13. Wash cells two further times including a 5-min incubation.

14. Label cells with fluorophore-conjugated secondary antibodies complimentary to the primary antibodies, diluted as appropriate in 0.5% BSA in PBS. Incubate for 1 h protected from light.

15. Wash cells three times with PBS with no incubation between washes. After the third wash, incubate for 5 min protected from light.

16. Wash cells two further times including incubation for 5 min protected from light.

17. Stain nuclei using DAPI diluted 1:20,000 for 4 min.

18. Replace nuclear stain with water.

19. Mount coverslips cell side down on a glass slide using mounting media and blot excess using paper towel (see Note 11).

20. Seal around coverslip using nail varnish.

21. Image on confocal microscope (see Note 12).

### 3.2 Immuno-fluorescence Quantification

1. Individual cellular puromycin signal can be quantified using image analysis software, e.g., ImageJ (Fiji) [11].

2. Puromycin signal for infected cells can be normalized using surrounding uninfected cells if using a suitably low MOI or using a mock infected well.

3. 50–100 cells for each treatment should be quantified.

4. Data can be plotted by scatter plot to represent individual values.

### 3.3 RPM and Detection of Tagged Nascent Polypeptides by WB

1. Seed cells in a six-well plate or equivalent aiming for 60–80% confluency (see Note 3) at the time of puromycin addition [5] (see Note 4).

2. Infect cells with 500 μl virus at the desired MOI or with BES medium for mock infected cells and incubate for 1 h at 37 °C (see Note 5).

3. Add 2.5 ml BES medium and incubate cells at 37 °C for a further 23 h (see Note 6).
4. One hour prior to RPM, add 30 μl of sodium arsenite to the existing media to give a final concentration of 500 μM and incubate at 37 °C (see Note 7).

5. At the time of RPM, add 26.1 μl of puromycin to the existing media to give a final concentration of 18.4 μM and incubate for 30 s at RT (see Note 8).

6. Add 34.5 μl of emetine to the existing media to give a final concentration of 208 μM and incubate for 1 min at RT (see Note 9).

7. Wash cells three times with PBS with no incubation between washes.

8. Add 150 μl sample buffer, scrape cells using a pipette tip and collect cells with a pipette into a microfuge tube.

9. Boil samples for 3 min (see Note 13).

10. Sonicate samples for 2 min at 70% amplitude or equivalent.

11. Boil samples again for 3 min.

12. Load samples onto an SDS-PAGE gel and run at 120 V until the loading dye has reached the bottom of the gel.

13. Transfer onto nitrocellulose using western blot transfer equipment.

14. All the subsequent incubation steps should be performed on a rocking platform shaker at room temperature. Block the membrane using 5% milk in PBST for 1 h.

15. Incubate the membrane with monoclonal anti-puromycin antibody diluted in 5% milk in PBST for 1 h (or overnight at 4 °C). It is also useful to label a housekeeping gene to allow signal normalization (e.g., GAPDH) and a viral marker to confirm infection on either separate or reused membranes. Multiple antibodies may be used on the same membrane where the western blot imaging system and primary antibody species allow.

16. Wash the membrane three times with PBST with no incubation between washes. After the third wash, incubate for 5 min.

17. Wash the membrane two further times including a 5-min incubation.

18. Incubate the membrane with a reporter-conjugated secondary antibody complimentary to primary antibody and compatible with the western blot imaging system.

19. Wash the membrane three times with PBST with no incubation between washes. After the third wash, incubate for 5 min.

20. Wash the membrane two further times including a 5-min incubation.

21. Image the membrane using western blot imaging system.
22. ImageJ (Fiji) or other analysis software can be used to quantify the band intensities for relative translation levels. At least three puromycin-stained bands should be quantified per lane for both viral and non-viral proteins if possible. Puromycin signal for non-viral (Y-axis) proteins and viral proteins (X-axis) can be plotted and the respective trendline and correlation equation can be compared between experiments to determine the level of shut off and the relative viral translation in response to the change in cellular translation.

4 Notes

1. Use infection media appropriate to the virus of interest.
2. A monoclonal puromycin antibody is suggested to prevent unwanted off-target binding.
3. It should be noted that precise puromycin and emetine addition timings will be difficult to maintain with high number of samples/wells. Therefore, we recommend a maximum of 4–6 samples at a time. If additional samples are needed; these should the processed independently.
4. Cells should be 60–80% confluent at the time of puromycin addition as when cells reach 90–100% confluent, cellular translation will decrease due to competition, lack of space, and cell stress. Therefore, ensuring cells are 60–80% confluent will provide a more accurate representation of cellular translation activity. During the experiment, the incubator door should be kept closed or opened as little as possible to reduce temperature fluctuation, which will cause cell stress and may reduce translation, especially in the time prior to RPM treatment.
5. Alter infection conditions to suit individual virus infection standard operating practice.
6. Virus infection duration can be adjusted to relevant timings specific to virus being used and to answer specific questions.
7. Translational inhibition via sodium arsenite treatment may need optimization for individual cell lines. A concentration range of 250–1000 μM is suggested. Incubation time can also be altered (e.g., 2 h).
8. Puromycin and emetine concentrations may need optimization for individual cells lines; suggested ranges include: puromycin 8–20 μM and emetine 80–210 μM.
9. Ensure puromycin and emetine volume is large enough to ensure rapid and even mixing to prevent variation in the puromycin signal throughout the well.
10. The protocol can be modified to look at localized sites of translation. This can be achieved by subjecting the cells to a coextraction/fixation procedure to remove free puromycin as detailed by Bastide et al. and V’Kovski et al. This addition allows the reduction of background puromycin signal and therefore increases the translation resolution [12, 13].

11. Use a pipette tip to carefully lever coverslip out of well and pick up using fine sharp or curved tweezers. Practice may be required to consistently remove coverslips from the wells as they are very easily broken.

12. Ensure confocal laser settings are kept constant throughout the experiment to allow accurate puromycin signal quantification and comparison across samples.

13. Samples can be frozen at −20 °C and further processed at a later stage after thawing.

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