Radioactive Pulse-Chase Analysis and Immunoprecipitation

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[Abstract] Labeling of newly-synthesized polypeptides with radioactive amino acids followed by immunoprecipitation allows quantitative analysis of the fate of a given protein in a time-dependent manner. This biochemical approach is usually used to study a variety of processes, such as protein folding, co-translational modifications, intracellular transport, and even its rate of degradation. Here, I describe step by step a simple technique to both label newly-synthesized influenza A virus (IAV) hemagglutinin (HA) with [^{35}S]-methionine and then follow its maturation and transport through the secretory pathway by SDS-PAGE and fluorography (Magadan *et al.*, 2013).

Materials and Reagents

1. ~5 x 10^6 MDCK cells (ATCC, catalog number: CCL-34)
2. 0.05% 1x Trypsin-EDTA (phenol red) (Life Technologies, catalog number: 25300054)
3. DPBS (Life Technologies, catalog number: 14190-250)
4. Anti-HA antibodies (mostly home-made)
5. nProtein A Sepharose 4 Fast Flow (GE, catalog number: 17-5280-02)
6. 4x NuPAGE LDS sample buffer (Life Technologies, catalog number: NP0007)
7. NuPAGE Novex 4-12% Bis-Tris protein gels (Life Technologies, catalog number: NP0321PK2)
8. NuPAGE MES SDS running buffer (Life Technologies, catalog number: NP000202)
9. DMEM media without methionine and cysteine (Life Technologies, catalog number: 21013024)
10. EDTA-free protease inhibitor cocktail (Roche Diagnostics, catalog number: 04693159001)
11. 10% Triton X-100 surfact-amps detergent solution (Thermo Fisher Scientific, catalog number: 28314)
12. UltraPure 1 M Tris-HCl Buffer (pH 7.5) (Life Technologies, catalog number: 15567-027)
13. UltraPure 5 M NaCl (Life Technologies, catalog number: 24740-011)
14. UltraPure 0.5 M EDTA (pH 8.0) (Life Technologies, catalog number: 15575-020)

15. Methanol (Sigma-Aldrich, catalog number: 322415)
16. Acetic acid (Sigma-Aldrich, catalog number: 320099)
17. DMEM media (Life Technologies, catalog number: 10569-010)
18. DMEM media with 7.5% fetal bovine serum (see Recipes)
19. Pulse medium (see Recipes)
20. Chase medium (see Recipes)
21. Non-denaturing lysis buffer (see Recipes)
22. Fixation solution (see Recipes)
23. L-cysteine (Sigma-Aldrich, catalog number: W326305) (see Recipes)
24. [^{35}S]-methionine (PerkinElmer, catalog number: NEG-709-A005MC) (see Recipes)
25. L-methionine (Sigma-Aldrich, catalog number: M9625) (see Recipes)

Equipment

Note: Designated for working with radioactive materials.

1. Appropriate containers to dispose liquid and solid [^{35}S]-waste following local radiation safety guidelines
2. T-75 tissue culture flask (Thermo Fisher Scientific)
3. 50 ml BD Falcon tube (BD Biosciences, Falcon®)
4. A water bath set at 37 °C
5. A refrigerated micro-centrifuge
6. A rotator for 1.5 ml micro-centrifuge tubes settled at 4 °C
7. 1.5 ml micro-centrifuge tubes
8. A chamber to run mini-gels [I routinely use the XCell SureLock Mini Cell electrophoresis system (Life Technologies, catalog number: EI0001)]
9. Carestream Kodak BioMax MR films (Sigma-Aldrich, catalog number: Z350400)
10. Kodak X-OMAT 2000A processor
11. A gel-dryer
12. A cassette for autoradiography

Procedure

A. Radioactive Pulse-Chase

1. Detach MDCK cells [grown in a T-75 tissue culture flask and infected with 10 infectious doses of influenza A/Puerto Rico/8/34 (PR8) virus per cell for 5 h at 37 °C (Magadan *et al.*, 2013)] by incubating them with 5 ml Trypsin-EDTA for ~15 min at 37 °C.

2. Transfer cells to a 50 ml BD Falcon tube and wash them twice with 10 ml pre-warmed DPBS by centrifugation for 1 min at 2,500 *x g*, room temperature.
3. Resuspend cells in 1 ml DPBS and transfer them to a 1.5 ml micro-centrifuge tube.
4. Centrifuge cells for 15 sec at max speed, 4 °C.
5. Resuspend cells in 200 µl of pre-warmed pulse medium.
6. Incubate cells for 2 min at 37 °C in a water bath (pulse).
7. Centrifuge cells for 15 sec at max speed, 4 °C.
8. Aspirate the supernatant and resuspend the cell pellet in 1.05 ml of pre-warmed chase medium.
9. Incubate labeled cells for up to 20 min at 37 °C in a water bath.
10. Take aliquots of 190 µl each at 0, 5, 10, 15, and 20 min and immediately transfer them to new 1.5 ml micro-centrifuge tubes containing 1 ml of ice-cold DPBS.
11. Centrifuge cells for 15 sec at max speed, 4 °C.
12. Aspirate the supernatant and resuspend the cell pellet in 1 ml ice-cold non-denaturing lysis buffer.
13. Incubate cell lysates for 30 min at 4 °C with slow rotation.
14. Clear cell lysates by centrifugation for 15 min at max speed, 4 °C.
15. Keep the supernatant but discard the pellet.

B. Immunoprecipitation

1. Wash 30 µl of protein A sepharose/each chase-time point twice with 0.5 ml of ice-cold DPBS by centrifugation for 1 min at 3,000 *x g*, 4 °C.
2. Resuspend resin in 0.5 ml of ice-cold DPBS supplemented with 0.001% Triton X-100 and the anti-HA antibody of choice.
3. Incubate for at least 2 h at 4 °C with slow rotation.
4. Wash resin twice with 0.5 ml of ice-cold non-denaturing lysis buffer (without protease inhibitors).
5. Add 10 µl 10% BSA.
6. Add the cleared cell lysate from each chase-time point.
7. Incubate for at least 2 h at 4 °C with slow rotation.
8. Wash resin twice with 0.5 ml of ice-cold non-denaturing lysis buffer (without protease inhibitors) but containing 0.1% instead of 0.5% Triton X-100.
9. Wash resin once with 0.5 ml of ice-cold DPBS.
10. Resuspend resin in 20 µl of 4x LDS sample buffer.
11. Boil samples for 5 min.

C. SDS-PAGE and Fluorography

1. Load 15 µl of every sample onto protein mini-gels.
2. Run for ~3 h at constant 50 mA/gel.
3. Fix gels with 10 ml fixation solution for at least 30 min at room temperature with slow rocking.
4. Dry gels in a gel drier for 1.5 h at 80 °C (in the case of 1 mm gels).
5. Expose films to the radioactive gels overnight at room temperature.
6. I usually develop my films using a Kodak X-OMAT 2000A processor.
7. Please refer to Figure 1 on our prior publication (Magadan *et al.*, 2013) for representative results and conclusions.

Recipes

1. DMEM media with 7.5% fetal bovine serum
   Supplement DMEM media with 7.5% fetal bovine serum by adding ~40 ml commercial stock to 500 ml liquid media
2. Pulse medium
   DMEM media without methionine and cysteine supplemented with 0.20 mM L-cysteine and 0.2 mCi/ml [^{35}S]-methionine
3. Chase medium
   DMEM media supplemented with 7.5% fetal bovine serum and 67 mM L-methionine
4. Non-denaturing lysis buffer
   0.5% Triton X-100
   50 mM Tris-HCl (pH 7.5)
   300 mM NaCl
   5 mM EDTA
   Complete mini, EDTA-free protease inhibitor cocktail
5. Fixation solution
   50% methanol
   10% acetic acid
6. L-cysteine
   Dissolve 12.116 mg commercial powder in 500 ml DMEM media without methionine and cysteine to obtain a new media supplemented with 0.20 mM L-cysteine
7. [^{35}S]-methionine
   Add 19.6 µl of the 5 mCi/0.49 ml stock solution to 1 ml DMEM media without methionine to get a 0.2 mCi/ml working solution
8. L-methionine

Dissolve 10 mg commercial powder in 1 ml DMEM media + 7.5% fetal bovine serum to obtain a chase media supplemented with 67 mM L-methionine

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References

1. Magadan, J. G., Khurana, S., Das, S. R., Frank, G. M., Stevens, J., Golding, H., Bennink, J. R. and Yewdell, J. W. (2013). Influenza A virus hemagglutinin trimerization completes monomer folding and antigenicity. *J Virol* 87(17): 9742-9753.

