Protein Translation Study – Label Protein with S35 Methionine in Cells

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[Abstract] To follow protein synthesis, cells should be incubated with radioactive amino acid such as [35S] methionine during mRNA translation. Then, the neosynthetized protein will be identified by an autoradiography after immunoprecipitation with a specific antibody and separation on a polyacrylamide denaturing gel.

Materials and Reagents

1. Methionine-free medium DMEM (Sigma-Aldrich, catalog number: D0422)
2. Fetal calf serum (Hyclone, catalog number: SV30160.03)
3. Fetal bovine serum (FBS)
4. Penicillin/streptomycin/glutamine
5. Phosphate buffered saline (PBS) (Life Technologies, Invitrogen™, catalog number: 10010-056)
6. Protein assay kit (DC Protein Assay Kit I-500) (Bio-Rad, catalog number: 0111EDU)
7. Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, catalog number: sc-2003)
8. EasyTaq -[^{35}S]-Methionine, 5 mCi (185 MBq), stabilized aqueous solution (Perkinelmer, catalog number: NEG709A005MC)
9. Hybond ECL Nitrocellulose Membrane (Amersham, catalog number: RPN68D)
10. Kodak Biomax XAR film (Sigma-Aldrich, catalog number: F5763)
11. Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, catalog number: WBKLS0500)
12. Anti-MDM2 (Santa Cruz, California, USA)
13. HEPES
14. NaCl
15. Glycerol
16. Triton X-100
17. $MgCl_2$
18. EGTA
19. $Na_4P_2O_7$
20. NaF

21. Aprotinin
22. Leupeptin
23. PMSF
24. Na_3VO_4
25. 2-mercaptoethanol
26. Acrylamide
27. Bromophenol blue
28. Ammonium persulfate (APS)
29. Lysis buffer (see Recipes)
30. HNTG buffer (see Recipes)
31. Protein A or G agarose beads (see Recipes)
32. 2x Laemmli buffer (see Recipes)
33. SDS-polyacrylamide gel (see Recipes)
34. 10x electrophoresis buffer (see Recipes)
35. 1x transfer buffer (see Recipes)

Equipment

1. Centrifuges
2. Vortexer
3. Tissue culture hood and incubator
4. Radioactive material and room
5. Western-Blot apparatus
6. Developer
7. Hamilton syringe
8. Spectrophotometer
9. Rocker
10. T25 flask

Procedure

A. Metabolic labeling

1. Wash 1 x 10^7 cells/sample in 30 ml methionine-free medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin/glutamine for 3 times.
2. Harvest cells for 60 min in 10 ml methionine-free medium in T25 flask.
3. Resuspend cells in 10 ml medium containing 250 uCi/sample [^{35}S]-methionine for 30 min.
4. Wash cells twice in 10 ml PBS and centrifuge them at 1,200 rpm for 10 min.

5. Lyse the cells with 500 µl of lysis buffer for 30 min by vortexing 15 sec every 5 min at 4 °C.
6. Centrifuge at 10,000 *x g* for 30 sec to pellet the DNA at 4 °C.
7. Determine the protein levels in the supernatant by DC Protein assay kit I.
8. Take same amount of protein extracts (about 1 mg) for immunoprecipitation after protein quantification.

B. Immunoprecipitation
1. Incubate equal amount of lysates onvernight at 4 °C by rotation with 3 µg antibody against protein of interest (here 30 µl of anti-MDM2 antibody) in eppendorf tubes.
2. Spin down the lysates (in order to collect all the drops in the cap after rotation).
3. Add 30 µl of Protein A/G PLUS-Agarose slurry volume by pipetting with tips (edge already cut off) (see Recipes 3) and incubate 2 h on a rocker at 4 °C.
4. Wash immunoprecipitates (beads) with 500 µl HNTG buffer for 4 times by centrifuging beads at 10, 000 *x g* for 30 sec at 4 °C.
5. At the end aspirate HNTG buffer with Hamilton syringe.
6. Add 30 µl of Laemmli buffer 2x on beads.
7. Boil the sample for 5 min.

C. Resolving protein of interest on SDS-polyacrylamide gel electrophoresis and autoradiography
1. Resolve the protein on SDS-polyacrylamide gel electrophoresis under denaturing conditions.
2. Transfer it onto nitrocellulose membrane and newly synthesized [^{35}S]methionine-protein will be visualized after exposure to X-AR films.
3. Verify the immunoprecipitation loading by incubating overnight with appropriate primary and secondary antibodies (see Western-blot protocol).
4. Detect the protein with a chemoluminecent HRP substrate detection kit.

Recipes

1. Lysis buffer
   50 mM HEPES (pH 7.0)
   150 mM NaCl
   10% glycerol
   1% Triton X-100
   1.5 mM $MgCl_2$
   1 mM EGTA
   10 mM $Na_4P_2O_7$

+add extemporary

10 mM NaF

1 mM DTT

10 µg/L aprotinin

10 µg/L leupeptin

1 mM PMSF

1 mM Na_3VO_4

2. HNTG buffer

   50 mM HEPES (pH 7.0)

   10% glycerol

   0.3% Triton X-100

   150mM NaCl

   1 mM $NaVO_4$

3. Protein A or G agarose beads

   Wash the beads twice with PBS

   Restore to 50% slurry with PBS

   (It is recommended to cut the edge of the tip to pipet)

4. 2x Laemmli buffer

   4% SDS

   20% glycerol

   10% 2-mercaptoethanol

   0.004% bromophenol blue

   0.125 M Tris-HCl

5. SDS-polyacrylamide gel

   10% PAGE

   H_2O 4 ml

   30% Acrylamide 3.3 ml

   1.5 M Tris (pH 8.8) 2.5 ml

   10% SDS 0.1 ml

   10% ammonium persulfate (APS) 0.1 ml

   10% TEMED 0.012 ml

   STACKING

   H_2O 5.6 ml

   Acrylamide (30%) 1.7 ml

   0.5M Tris (pH 8.8) 2.5 ml

   10% SDS 0.1 ml

10% Ammonium persulfate (APS) 0.125 ml

10% TEMED 0. 015 ml

6. 10x electrophoresis buffer

   Glycine 144 g

   Tris base 30 g

   20% SDS 50 ml

   H_2O qsp 1 L

7. 1x transfer buffer

   Glycine 14 g

   Tris base 3 g

   20% Ethanol 200 ml

   H_2O qsp 1 L

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References

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