

## 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 [<sup>35</sup>S] methionine during mRNA translation. Then, the neosynthesized 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 -[<sup>35</sup>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<sub>2</sub>
18. EGTA
19. Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>
20. NaF

21. Aprotinin
22. Leupeptin
23. PMSF
24.  $\text{Na}_3\text{VO}_4$
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  $1 \times 10^7$  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  $\mu\text{Ci}/\text{sample}$  [ $^{35}\text{S}$ ]-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  $\mu$ l 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 overnight at 4 °C by rotation with 3  $\mu$ g antibody against protein of interest (here 30  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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 [<sup>35</sup>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 chemoluminescent 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<sub>2</sub>
  - 1 mM EGTA
  - 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>

- +add extemporary
  - 10 mM NaF
  - 1 mM DTT
  - 10 µg/L aprotinin
  - 10 µg/L leupeptin
  - 1 mM PMSF
  - 1 mM Na<sub>3</sub>VO<sub>4</sub>
- 2. HNTG buffer
  - 50 mM HEPES (pH 7.0)
  - 10% glycerol
  - 0.3% Triton X-100
  - 150mM NaCl
  - 1 mM NaVO<sub>4</sub>
- 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<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O qsp 1 L
- 7. 1x transfer buffer
  - Glycine 14 g
  - Tris base 3 g
  - 20% Ethanol 200 ml
  - H<sub>2</sub>O qsp 1 L

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