## $^{35}$S-methionine metabolic labeling

1. Plate the cells in a 10 cm culture dish at previous night (80% confluent).

2. Culture O/N.

3. Wash the cells with PBS twice.

4. Add 3 ml of methionine, cysteine-free DMEM containing 10% dialyzed FCS to each dish.

5. Incubate the cells for 30 min for starvation of methionine.

6. Add 30 µl of ca. 10 µCi /µl L-[$^{35}$S]Methionine (NEN).

7. Culture the cells for 4 hr.

8. Wash the cells with cold PBS three times.

9. Scrape the cells by scraper and collect into 1.5 ml Eppendorf tube.

10. Store the $^{35}$S-methionine labeled cell pellet at -80℃ until use.

**Cell extraction and Pre-clearance**

1. Suspend ～1 x $10^7$ cells (100mm dish subconfluent) into 1ml of E1A Buffer.

2. Vortex

3. Keep on ice for 30 min.

4. c.f.g. 15,000 rpm for 10 min.

5. Keep the sup.

6. Add 50-100 µl of ProteinA Sepharose (50% suspension) into the extract (Pre-clearance step)

7. Rotate the tube for 1hour at 4℃

8. c.f.g. 15,000 rpm for 5 min.

9.Keep the sup.

**Immunoprecipitation**

1. Add IP antibody 2-4 µg to 0.5 ml of cell extract.

2. Rotate the tube for 30min. at 4℃.

3. Add 20-40 µl of ProteinA Sepharose (50% suspension) into the extract.

4. Rotate the tube for 1-4 hours at 4℃.

5. c.f.g. 12,000 rpm for 1min.

6. Remove the sup.

7. Wash the resin 4 times with E1A buffer.

8. c.f.g. 12,000 rpm for 1 min.

9. Add 20ul of 2X SDS-Sample buffer to the resin.

10. Boil for 2min.

11. c.f.g. 15,000 rpm for 1 min.

12. SDS-PAGE

13. Take an autoradiography

**E1A Buffer**

50 mM Hepes pH 7.2～7.6
250 mM NaCl
0.2 % NP-40
5 mM EDTA-2Na (pH 7.5)
10 % Glycerol
0.5 mM DTT
protease inhibitor cocktail
- 1 µg/ml pepstatin
- 1 µg/ml leupeptin
- 0.3 µM aprotinin
- 15 µM E-64

phosphatase inhibitor cocktail (in case of using phosphor-specific antibody)
- 2.5 mM Sodium pyrophosphate
- 1 mM beta-Glycerolphosphate
- 1 mM $Na_3VO_4$