Metabolic labeling & Immunoprecipitation

(by Jun Takagi§6/15/2000)

Purpose and backgrounds

About nuclide...

35-S

Decay: (b-ray, 0.167 MeV)

Half life: 87.5 days

Thick acrylic shield can block most of the emission. Because of the low energy, it is impossible to detect spillage/contamination by usual survey meter. Wipe test + LSC is necessary.

Chemical properties...

Use either ^{35}S-methionine or cysteine or both. Recommend using Expre^{35}S^{35}S Protein labeling mix (NEN). Before opening the cap of the vial, thaw the solution completely and open the cap in the hood (at hot lab) so that any vaporized materials can be trapped in the hood.

Principle of radiolabeling

Cells incorporate ^{35}S-methionine or cysteine during the protein synthesis. Thus it is essential to use Met,Cys-free medium and dialyzed FCS during the labeling. Short period of incubation with ^{35}S-methionine or cysteine will result in radiolabeling (pulse), and additional incubation with excess concentration of unlabeled Met+Cys (chase) is needed for complex glycoproteins like integrins to get expressed as a maturated form. Typically, overnight chase is recommended for metabolic labeling of fully processed form of integrins, but optimum time may vary depending on each protein.

Materials

•Complete medium: DMEM supplmented w/ 10% FCS, NEAA, Na-pyruvate, and Gln

•Expre^{35}S^{35}S Protein labeling mix (NEN,#NEG-072), 7.9mCi/ml

• Transfection reagents: $CaCl_2$, x2HBS, DNA

•Labeling Medium:

RPMI1640(Met,Cys-free; Sigma R-7513) supplemented w/ 10% dialyzed FCS, & Gln

• Chase Medium:

Labeling medium (above) + 500μg/ml Cysteine-HCl, 100μg/ml methionine, sterile filtered

• Tris-buffered saline (TBS): 20mM Tris, 150mM NaCl, pH 7.4

• X1000 Protease inhibitor soln: 500mM PMSF (Sigma P-7626) in EtOH, 10mg/ml leupeptin (Sigma L-2023) in water, 10mg/ml pepstatin A (P-4265) in dimethylformamide

• IP buffer: 1% Triton x-100, 0.05% NP-40 in TBS

• x2 IP buffer: 2% Triton x-100, 0.1% NP-40 in TBS

• ProteinG agarose (GIBCO #15920-010)

• Gel fixation solution: 7% acetic acid, 25% MeOH in water

• Gel soaking solution: 1% glycerol, 5% PEG 8000 in water

Procedure

Transfection of 293 cells (refer to the protocol #4, "Transient Transfection into 293T Cells")

Prepare transfected cells in 6-well plate. Use 2μg DNA/subunit/well. After 7-9h incubation with DNA:calcium phosphate, rinse once and replace with fresh medium (3ml/well).

Metabolic labeling

1. Transfected cells -> 24h after medium change, rinse cells twice with Labeling Medium.
2. Add 1.5ml/well Labeling Medium
3. Add 20-30μl (~250μCi) of [^{35}S] methionine/cysteine /well
4. Culture 1h at 37°C **(pulse)**
5. Add 1.5ml/well Chase Medium
6. Culture o/n **(chase)**
7. Harvest metabolically labeled cells (and supernatant)

Immunoprecipitation

i) preparation of cell lysate ***(for membrane or cytosolic proteins)***

1. After removing the medium, detach cells by suspending in TBS, transfer to microfuge tube
2. Spin down & resuspend in 0.5 ml TBS
3. Add 1μl each of x1000 protease inhibitor soln
4. Solubilize cells by adding 0.5 ml x2 IP buffer
5. on ice for 20min
6. Centrifuge at maximum speed for 15min
7. Save supernatant **(^{35}S-labeled cell extract)**

ii) preparation of culture supernatant ***(for secreted proteins)***

1. Harvest medium (into 15ml tube)
2. Spin down to remove cell debries
3. Adjust pH by adding 50μl 1M Tris pH 8.0
4. Save supernatant **(^{35}S-labeled culture sup)**

iii) IP

1. Prepare 1.7ml Eppendorf tubes (make sure that cap closes tightly)
2. Add 100-400μl of ^{35}S-labeled culture sup or cell lysate
3. Add mAb (1μg for purified IgG, 1μl for ascites, or 50μl for hybridoma supernatant)
4. Add 20μl of ProteinG-agarose (50% slurrry)>>>use wide-hole tips for transferring gel suspension
5. Adjust total vol to 500μl with TBS (for secreted proteins) or IP buffer (for cellular proteins)
6. Incubate at 4°C for 1-3h

(Use rocking-type mixer rather than horizontal shaker. The suspension must be mixed well during the incubation. USE SPECIAL CAUTION TO AVOID RADIOACTIVE SPILLAGE. Always place protective sheets.)

7. Spin down @ 6000rpm for 3min (use centrifuge allowed for radioactive stuff)
8. Remove supernatant (Leave ~30μl of solution above the pellet. Don't try to remove ALL of the solution because you will lose your agarose by doing so.)
9. Resuspend in 500μl of TBS (or IP buffer) and spin down

(repeat this washing step 3 times)

10. Remove final wash solution roughly with P1000 pipet, then completely with narrow-mouth, gel-loading tip attached to P200 pipet so that you won't take out ProteinG beads.
11. To the slightly moist pellet of beads,add 30μl SDS-PAGE sample buffer (either reducing or nonreducing, depending on the purpose)
12. Wait for 5min, then boil for 1min
13. Spin down, and load 5-10μl onto SDS-PAGE gel
14. After electrophoresis, fix gel in fixation soln. for 30min
15. Remove solution and put gels into enhancer solution (EN^{3}HANCE; NEN) for 1h
16. Transfer EN^{3}HANCE solution to waste bottle, soak gels into soaking solution (30min)
17. Dry gels
18. Autoradiography (use intensifying screen)