Subcellular Fractionation of Cultured Human Cell Lines

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[Abstract] Subcellular localization is crucial for the proper functioning of a protein. Deregulation of subcellular localization may lead to pathological consequences and result in diseases like cancer. Immuno-fluorescent staining and subcellular fractionation can be used to determine localization of a protein. Here we discuss a protocol to separate the nuclear, cytosolic, and membrane fractions of cultured human cell lines using a centrifuge and ultracentrifuge. The membrane fraction contains plasma membranes and ER-golgi membranes, but no mitochondria or nuclear structures. The fractions can be further analyzed using Western blotting. This protocol is based on that from Dr. Richard Patten at Abcam, and was modified and utilized in a publication by Zhiguang Huang, *et al.*

Materials and Reagents

1. Sucrose
2. HEPES
3. Potassium chloride (KCl)
4. Magnesium chloride ($MgCl_2$)
5. Ethylene diamine tetraacetic acid (EDTA)
6. Ethylene glycol tetraacetic acid (EGTA)
7. Dithiothreitol (DTT)
8. Tris (Affymetrix, catalog number: 75825)
9. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: 13565)
10. Nonidet P40 substitute (NP40)
11. Sodium deoxycholate
12. Glycerol
13. Sodium dodecyl sulfate (SDS)
14. Protease inhibitor (PI) cocktails (F. Hoffmann-La Roche, catalog number: 11836145001)
15. Methanol
16. Acetic acid
17. Brilliant Blue R (Affymetrix, catalog number: 32826)
18. Phosphate buffered saline (PBS)

19. Histone H3 antibody (Cell Signaling Technology, catalog number: 9715)
20. Alpha-tubulin antibody (GeneTex, catalog number: GTX108784)
21. Cell scraper (BD Biosciences, Falcon®, catalog number: 353086)
22. Subcellular fractionation buffer (SF buffer) (see Recipes)
23. Nuclear lysis buffer (NL buffer) (see Recipes)
24. Brilliant Blue R staining solution and destaining solution (see Recipes)

Equipment

1. 4 °C Microcentrifuge (Eppendorf, catalog number: 5415R);
2. Ultracentrifuge (Beckman Coulter, model number: Optima TLX)
3. Optional: Sonicator (Sonics, model number: VC505)
4. Whatman filter paper
5. 37 °C incubator
6. 1.5 ml Eppendorf microtubes
7. Tube roller (Maplelab-scientific, model number: MTR-1D)

Procedure

Note: Keep the sample at 4 °C on ice at all times! All buffers must be ice-cold when used. All centrifugations are done in the Eppendorf Microcentrifuge unless stated otherwise.

1. Culture cells on 100 mm culture plate until 75% confluent in a 37 °C incubator supplied with 5% CO_2. For beginners, the well-studied HEK293 and its derivatives are recommended for easy maintenance and ectopic protein expression.
   Note: This is for adherent cell. Suspension cells may need centrifugation before lysis.
2. Wash twice with ice-cold PBS and immediately add 500 µl per 100 mm plate of SF buffer and put on ice, use cell scraper to collect lysate and transfer to a 1.5 ml Eppendorf tube. If multiple samples are collected, process one specimen at a time.
3. Agitate the lysates at 4 °C for 30 min at around 30-50 rpm on the tube roller.
4. Centrifuge at 720 *x g* at 4 °C for 5 min. Carefully transfer the supernatant to a new 1.5 ml tube for future use. Keep the pellet for next step.
5. Wash the pellet with 500 µl of SF buffer and disperse the pellet with a pipette.
6. Centrifuge the pellet at 720 *x g* at 4 °C for 10 min.
7. Remove the supernatant and resuspend the pellet in NL buffer. Agitate and incubate at 4 °C for 15 min.
   Optional: Sonicate the pellet on ice (2 x 3 sec sonication, separated by 3 sec resting,

under 30% full amplitude power. On ice!). This is the nuclear fraction including nuclear membranes.

8. Centrifuge the supernatant from step 4 at 10,000 *x g* at 4 °C for 10 min.
9. Carefully transfer the supernatant to a new 1.5 ml tube. This is the cytosolic and membrane fraction.
10. Centrifuge the cytosolic and membrane fraction from step 9 in an ultracentrifuge. Ultracentrifuge at 100,000 *x g* at 4 °C for 1 h. Carefully transfer the supernatant to a new 1.5 ml tube. This is the cytosolic fraction.
11. Wash the pellet with 500 µl of SF buffer and re-suspend by pipetting.
12. Ultracentrifuge the pellet at 100,000 *x g* at 4 °C for 1 h.
13. Remove the supernatant and re-suspend the pellet in NL buffer.
    Optional: Sonicate the pellet on ice (same setting as for nuclear fraction in step 7). This is the membrane fraction.
14. Internal loading control for Western blotting could be used to make sure each fraction does not cross-contaminate others; but relative amount can also be determined between samples to ensure equal loading. For example, alpha-tubulin is used for the cytosolic fraction; histone H3 is used for the nuclear fraction; Brilliant Blue R is used for staining for the membrane fraction. Use an extra gel for the loading controls. Alpha-tubulin and histone H3 are probed after protein is transferred onto a PVDF membrane. Brilliant Blue R staining can be applied directly to the SDS-PAGE gel (Figure 1).
    Note: Brilliant Blue R is used here for monitoring relative amount of protein loading across different samples, but is unable to show cross contamination. Membrane proteins such as EGF receptor and integrins may be used to confirm cross-contamination between the membrane and other fractions. However the recycling of these membrane proteins can be an issue, which may appear to be false-positive cross-contamination.

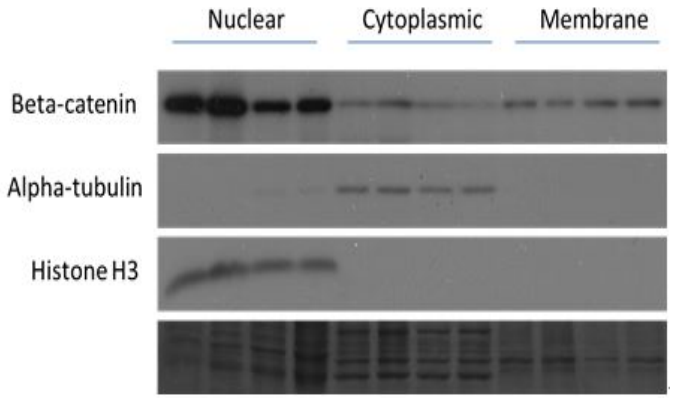

Figure 1. Western blotting of nuclear, cytoplasmic, and membrane fractions with

internal controls. Alpha-tubulin and histone H3 are used for the cytoplasmic and nuclear fractions, respectively; Brilliant Blue R staining is applied for the membrane fraction.

Recipes

1. Subcellular fractionation buffer (SF buffer)

	Stocks	50 ml 1x solution
250 mM Sucrose	-	4.28 g
20 mM HEPES (pH 7.4)	1 M	1 ml
10 mM KCl	-	0.0373 g
1.5 mM $MgCl_2$	1 M	75 µl
1 mM EDTA	0.5 M	100 µl
1 mM EGTA	0.5 M	100 µl

At time of use, add the following into 10 ml of SF buffer

	Stocks	10 ml 1x solution
1 mM DTT	1 M	10 µl
PI cocktail	40x (dissolve 1 tablet in 2 ml ddH_2O)	250 µl

2. Nuclear lysis buffer (NL buffer)

	Stocks	50 ml 1x solution
50 mM Tris HCl (pH 8)	1 M	2.5 ml
150 mM NaCl	1 M	7.5 ml
1% NP-40	20%	2.5 ml
0.5% sodium deoxycholate	10%	2.5 ml
0.1% SDS	10%	0.5 ml

At time of use, add the following into 10 ml of NL buffer

	Stocks	10 ml 1x solution
PI cocktail	40x	250 µl
10% glycerol	-	1 ml

Note: DTT can be added if further delicate experiments such as IP and ChIP are needed; current recipe without DTT is fine for Western blotting.

3. Brilliant Blue R staining solution and destaining solution

   For staining solution, dissolve 1 g of Brilliant Blue powder in 1 L of 50% Methanol/10% AceticAcid/40% H_2O (all [v/v]) solution. Stir until dissolved and (optional) filter through Whatman filter paper.

   For destaining, make a 10% AceticAcid/15% Methanol/75% H_2O solution.

Procedures: Place the gel containing the proteins of interest in a plastic container and cover with fresh staining solution. Shake it for 1 h at room temperature (or overnight). Remove the staining solution and add destaining solution. Put three sheets of fine-grade tissue paper in the container. Shake it until gel is fully destained. Constantly replace the solution and tissue paper.

Acknowledgments

This protocol is based on that from Dr. Richard Patten at Abcam (see Reference 1), and was modified and utilized in a publication by Huang *et al*. (2012).

References

1. Huang, Z., Cheng, Y., Chiu, P. M., Cheung, F. M., Nicholls, J. M., Kwong, D. L., Lee, A. W., Zabarovsky, E. R., Stanbridge, E. J., Lung, H. L. and Lung, M. L. (2012). Tumor suppressor Alpha B-crystallin (CRYAB) associates with the cadherin/catenin adherens junction and impairs NPC progression-associated properties. *Oncogene* 31(32): 3709-3720.
2. Patten, R. Procedure for separating nuclear, membrane, and cytoplasmic cell fractions using centrifugation methods.

