ProteoExtract®
Cytoskeleton Enrichment and Isolation Kit

Catalog No. 17-10195

15 reactions

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Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
www.millipore.com

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Introduction

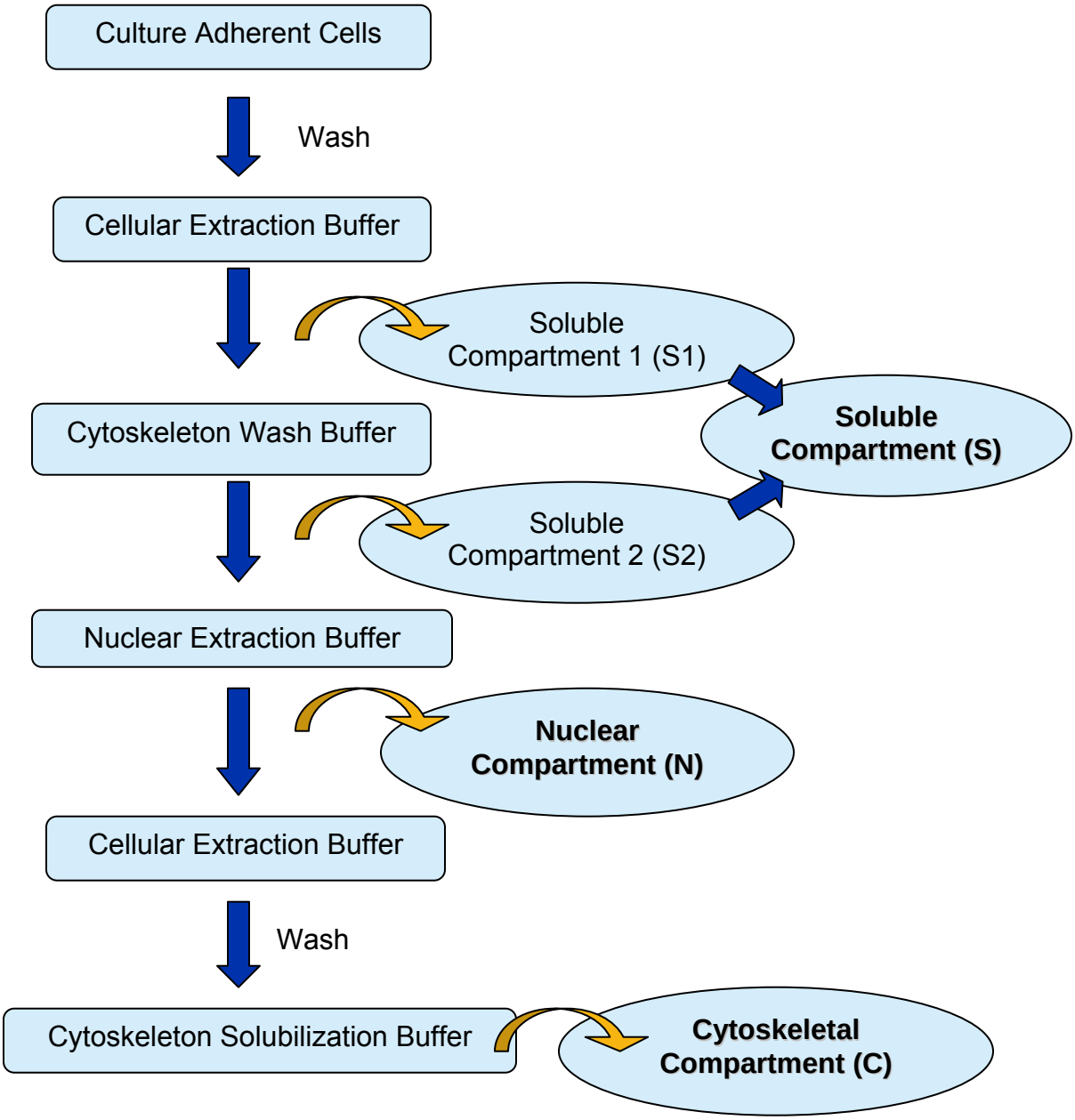
The actin cytoskeleton is a highly dynamic network composed of actin polymers and a large variety of associated proteins. The functions of the actin cytoskeleton is to mediate a variety of essential biological functions in all eukaryotic cells, including intra- and extra-cellular movement and structural Support (Chen, C.S., *et al*., 2003; Frixione E., 2000). To perform these functions, the organization of the actin cytoskeleton must be tightly regulated both temporally and spatially. Many proteins associated with the actin cytoskeleton are thus likely targets of signaling pathways controlling actin assembly. Actin cytoskeleton assembly is regulated at multiple levels, including the organization of actin monomers (G-actin) into actin polymers and the superorganization of actin polymers into a filamentous network (F-actin – the major constituent of microfilaments) (Bretscher, A., *et al.*, 1994). This superorganization of actin polymers into a filamentous network is mediated by actin side-binding or cross-linking proteins (Dubreuil, R. R., 1991; Matsudaira, P., 1991; Matsudaira, P., 1994). The actin cytoskeleton is a dynamic structure that rapidly changes shape and organization in response to stimuli and cell cycle progression. Therefore, a disruption of its normal regulation may lead to cell transformations and cancer. Transformed cells have been shown to contain less F-actin than untransformed cells and exhibit atypical coordination of F-actin levels throughout the cell cycle (Rao, J.Y., *et al.*, 1990). Orientational distribution of actin filaments within a cell is, therefore, an important determinant of cellular shape and motility.

Focal adhesion and adherens junctions are membrane-associated complexes that serve as nucleation sites for actin filaments and as cross-linkers between the cell exterior, plasma membrane and actin cytoskeleton (Yamada, K.M., Geiger, B., 1997). The function of focal adhesions is structural, linking the ECM on the outside to the actin cytoskeleton on the inside. They are also sites of signal transduction, initiating signaling pathways in response to adhesion. Focal adhesions consist of integrin-type receptors that are attached to the extracellular matrix and are intracellularly associated with protein complexes containing vinculin (universal focal adhesion marker), talin, α-actinin, paxillin, tensin, zyxin and focal adhesion kinase (FAK) (Burridge, K., *et al.,* 1990; Turner, C.E., Burridge, K., 1991).

Studying the proteins that associate with and regulate the actin cytoskeleton has been traditionally difficult because of the insolubility of the cytoskeleton in traditional detergents like Triton-X100. Work over the years has shown that many actin regulatory proteins/phospho-proteins upon activation move from the soluble cytoplasmic compartment to the insoluble actin cytoskeleton. The insolubility of these important proteins has made it difficult to study their biochemical changes, such as phosphorylation and nitrosylation, upon binding to actin. What is sorely needed is a cytoskeleton purification method that allows for the selective enrichment of cytoskeleton-associated proteins for detailed protein biochemical analyses. Such a method would provide the means to directly study this important pool of proteins in normal and diseased cytoskeletons.

The EMD Millipore ProteoExtract® Cytoskeleton Enrichment and Isolation Kit provides cytoskeleton purification detergent buffers, sufficient for extraction from ten 100 mm culture dishes, that retain focal adhesion and actin-associated proteins while removing soluble cytoplasmic and nuclear proteins from the cell. Anti-Vimentin and anti-GAPDH antibodies are provided as markers for cytoskeleton and cytosol, respectively, in western blot analysis. This kit will greatly increase the ability to detect and study the low abundance actin-associated proteins which are typically masked in conventional methods of Western blotting analysis of whole cell lysates.

Protocol Flow Chart



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Kit Components

Compartmentalization Module (Part No. 17-10195-2)
Store at -20º C

1. 10x Cellular Extraction Buffer: (Part No. CS207814). One bottle containing 1.9 mL of 10x Cellular Extraction Buffer.
2. 20x Cytoskeleton Wash Buffer: (Part No. CS207813). One bottle containing 3.5 mL of 20x Cytoskeleton Wash Buffer.
3. Nuclear Extraction Buffer: (Part No. CS207812). One bottle containing 9 mL of ready to use Nuclear Extraction Buffer.
4. Protease Inhibitor Cocktail: (Part No. 90492). Two vials each containing 100 µL of 1000x Protease Inhibitor Cocktail solution.
5. Sodium Orthovanadate: (Part No. CS207811). One vial containing 150 µL of 1000x Sodium Orthovanadate solution.
6. Cytoskeleton Solubilization Buffer: (Part No. CS207807). One bottle containing 3.8 mL of ready to use Cytoskeleton Solubilization Buffer.
7. Goat anti-Mouse IgG HRP, Secondary Antibody: (Part No. 2003482) One vial containing 30 µL of 2000X goat anti-mouse HRP conjugate.

Western Blot Detection Module (Part No. 17-10195-1)
Store at 2-8º C

1. Anti-Glyceraldehyde-3-Phosphate Dehydrogenase Mouse Monoclonal antibody: (Part No. CS207795). One vial containing 50 µL of 1000x anti-GAPDH mouse monoclonal IgG1 antibody.
2. Anti-Vimentin Mouse Monoclonal antibody: (Part No. CS207806). One vial containing 100 µL of 500x anti-Vimentin mouse monoclonal IgG1 antibody.

Materials Not Supplied

1. Pipettors and tips capable of accurately measuring 1-1000 μL
2. Graduated serological pipettes
3. Microcentrifuge tubes
4. Mechanical vortex
5. Distilled or deionized water
6. 1x Dulbecco's Phosphate Buffered Saline (D-PBS)
7. 3% Non-fat dry milk diluted in 1x TBST (containing 0.05% Tween-20).

Storage

Maintain the unopened kit modules (17-10195-1 & 17-10195-2) at the indicated temperatures. The kit may be used for up to 4 months after receipt. Avoid repeated freeze/thaw cycles, and aliquot if necessary.

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data.
- **Safety Warnings and Precautions:** This kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

Technical Notes

- All kit reagents should be rapidly thawed just prior to use, keep on ice until use.
- Do not mix or interchange reagent from various kit lots.

Reagents Preparation

1. **1x Cellular Extraction Buffer:** Prepare 19 mL of 1x Cellular Extraction Buffer by adding 1.9 mL of 10x Cellular Extraction Buffer (Part No. CS207814) to 17.1 mL of Milli-Q or distilled water. Aliquot and store at -20º C.
2. **1x Cytoskeleton Wash Buffer:** Prepare 70 mL of 1x Cytoskeleton Wash Buffer by adding 3.5 mL 20x Cytoskeleton Wash Buffer (Part No. CS207813) to 66.5 mL of Milli-Q or distilled water. Aliquot and store at -20º C.

Protocol

Compartmentalization:

1. Culture adherent cells to approximately 80-90% confluency in a 100mm cell culture dish.
2. Calculate volumes of buffers needed and add 1:1000 dilution of Protease Inhibitor Cocktail and 1:1000 dilution of Sodium Orthovanadate to all buffers.
    a. For example: One 100mm dish requires 0.5 mL 1x Cellular Extraction Buffer, 0.5 mL Nuclear Extraction Buffer, 4.5 mL Cytoskeleton Wash Buffer and 0.25 mL Cytoskeleton Solubilization Buffer. To these volumes, add 0.5 µL, 0.5 µL, 4.5 µL, and 0.25 µL respectively of both the Protease Inhibitor Cocktail and Sodium Orthovanadate.
    b. Keep all buffers on ice until use.
3. Label three 1.5 mL microcentrifuge tubes with the following: Soluble compartment (S), Nuclear compartment (N), and Cytoskeletal compartment (C).
4. Once cells have reached the desired confluency, place dish on ice and gently wash cells twice with 2 mL of cold 1x D-PBS. Aspirate completely after each wash.
5. Add 0.25 mL cold 1x Cellular Extraction Buffer to the cell. Rotate plate gently to cover cells with buffer and incubate for 1.5 minutes on ice.
6. Pipette buffer up and down gently 2-3 times. Collect buffer and place into tube labeled as Soluble compartment (S).
7. Add 0.5 mL 1x Cytoskeleton Wash Buffer to the cells and rotate plate gently to wash cells.
8. Gently collect buffer and add to tube labeled as Soluble Compartment (S).
9. Add 0.5 mL Nuclear Extraction Buffer to the cells. Rotate plate gently to cover cells with buffer and incubate for 10 minutes on ice.
10. Gently collect buffer and add to tube labeled as Nuclear Compartment (N).
11. Add 0.25 mL 1x Cellular Extraction Buffer to the cells. Rotate plate gently to cover cells with buffer, and aspirate completely.
12. Wash the cells twice with 2 mL 1x Cytoskeleton Wash Buffer. Aspirate completely after each wash.
13. Add 0.25 mL Cytoskeleton Solubilization Buffer to the cells. Rotate plate gently to cover cells with buffer.
    a. **Optional**: To increase yield of cytoskeletal proteins in Cytoskeletal Compartment (C), pre-warm Cytoskeleton Solubilization Buffer at 37ºC. Add 0.25 mL of buffer to cells and rotate gently to cover cells. Scrape cells off dish into buffer using cell scraper and collect. If the collected solution is cloudy, it will need to be sonicated before protein determination and analysis.

14. Pipette up and down. Collect buffer and add to tube labeled as Cytoskeletal compartment (C).
15. Determine protein concentrations by A_{280}.

Western Blot Detection:

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on collected compartments "S," "N," and "C." Run in duplicate.
2. Transfer proteins to a PVDF membrane. Cut PVDF membrane into two strips, each containing the three compartments "S," "N," and "C." Wash PVDF membrane twice with water.
3. Block the PVDF strips in freshly prepared TBST containing 3% non-fat dry milk (TBST-MILK), for 1 hour at room temperature with constant agitation.
4. Incubate the separate PVDF strips with

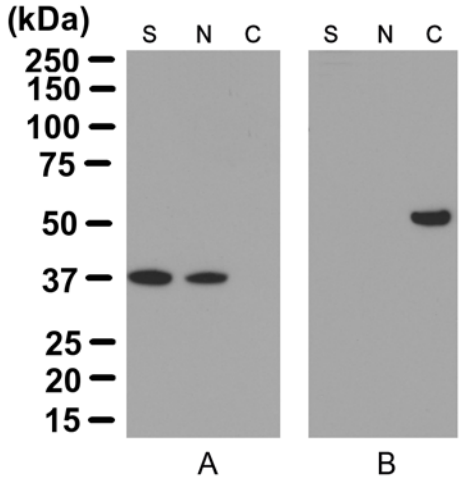
    a) 1:1,000 dilution of anti-GAPDH antibody, diluted in TBST-MILK

    b) 1:500 dilution of anti-Vimentin antibody, diluted in TBST-MILK

    for 2-3 hours at room temperature (or alternately overnight at 4º C) with agitation.

    Note: Sufficient antibody concentrates are supplied for preparing 50 mL of primary antibody incubation solution. Typically this volume would be sufficient for probing **ten** 2.75" x 3.5" blots.
5. Wash the PVDF strips once with TBST for 5 minutes followed by washing twice with distilled water for 5 minutes each.
6. Incubate both PVDF strips with goat-anti-mouse, HRP secondary antibody in TBST-MILK for 45 minutes at room temperature with agitation.
7. Wash the PVDF strips once with TBST for 5 minutes followed by washing twice with distilled water for 5 minutes each.
8. Develop the blot with the detection method of choice.

Representative Data



Representative Western Blot Detection Data

Western Blot of compartmental proteins extracted from HeLa cells using ProteoExtract Cytoskeleton Enrichment and Isolation Kit (cat. # 17-10195). Compartmental proteins were subjected to SDS-PAGE and proteins were transferred to a PVDF membrane. The membrane was probed with primary anti-GAPDH and anti-vimentin, followed by secondary antibodies. The blot was developed by enhanced chemiluminescence. Immunobloting results indicate that GAPDH (Panel A) is present in the cytoplasmic and nuclear fractions (Tristan, C., *et al.,* 2011), and the intermediate filament protein Vimentin (Panel B) is present exclusively in the cytoskeletal compartment.

Appendix

Table 1: Buffer Volumes & Dish Size

Volumes of buffers used in kit for different sized cell culture vessels.
All volumes listed in milliliters (mL).

Buffer used	Step	60mm dish	100mm dish	150mm dish
1x D-PBS	4	1	2	3
1x Cellular Extraction Buffer	5	0.15	0.25	0.4
1x Cytoskeleton Wash Buffer	7	0.3	0.5	0.75
Nuclear Extraction Buffer	9	0.3	0.5	0.75
1x Cellular Extraction Buffer	11	0.15	0.25	0.4
1x Cytoskeleton Wash Buffer	12	1	2	3
Cytoskeleton Solubilization Buffer	13	0.15	0.25	0.4

References

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8. Yamada KM, Geiger B. (1997). *Curr Opin Cell Biol* **9**:76-85.
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Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
No signal or weak signal of GAPDH or vimentin	Loss of cells during washes	Some cells adhere more strongly than others. Check cells using microscope during protocol to be certain that cells are retained.
	Primary antibody concentration too low	Titrate antibody concentration.
	Antibody not compatible with species of cell type	The antibodies have been validated with human, mouse and rat cell lines. Results with other species may vary.
Vimentin present in unexpected compartment	Cells dislodging during incubation	For loosely adherent cells, do not pipet solution directly onto cells. Centrifuge fractions to remove dislodged cells.
GAPDH present in unexpected compartment	GAPDH associating with nucleus or cytoskeleton	GAPDH has been shown to transfer to the nucleus or associate with the cytoskeleton (**ref. 11**). Check by immunofluorescence with companion staining kit (EMD Millipore ProteoExtract Native Cytoskeleton Enrichment and Staining Kit, cat. # 17-10210).
	Inadequate washing or extraction	Increase incubation times for soluble or nuclear fraction. Increase number of washes.
Protein of interest in unexpected fraction or absent, when GAPDH and vimentin are in expected fractions	Protein of interest loosely associated with cytoskeleton	This kit is designed for detecting proteins tightly associated with cytoskeleton
	Protein of interest insoluble in Cytoskeleton Solubilization Buffer	Warm Cytoskeleton Solubilization Buffer prior to addition, and incubate at increased temperature prior to collection

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