# ProteoExtract®
# Native Cytoskeleton Enrichment and Staining Kit

Catalog No. 17-10210

***4 x 8-well chamber slides***

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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## Introduction

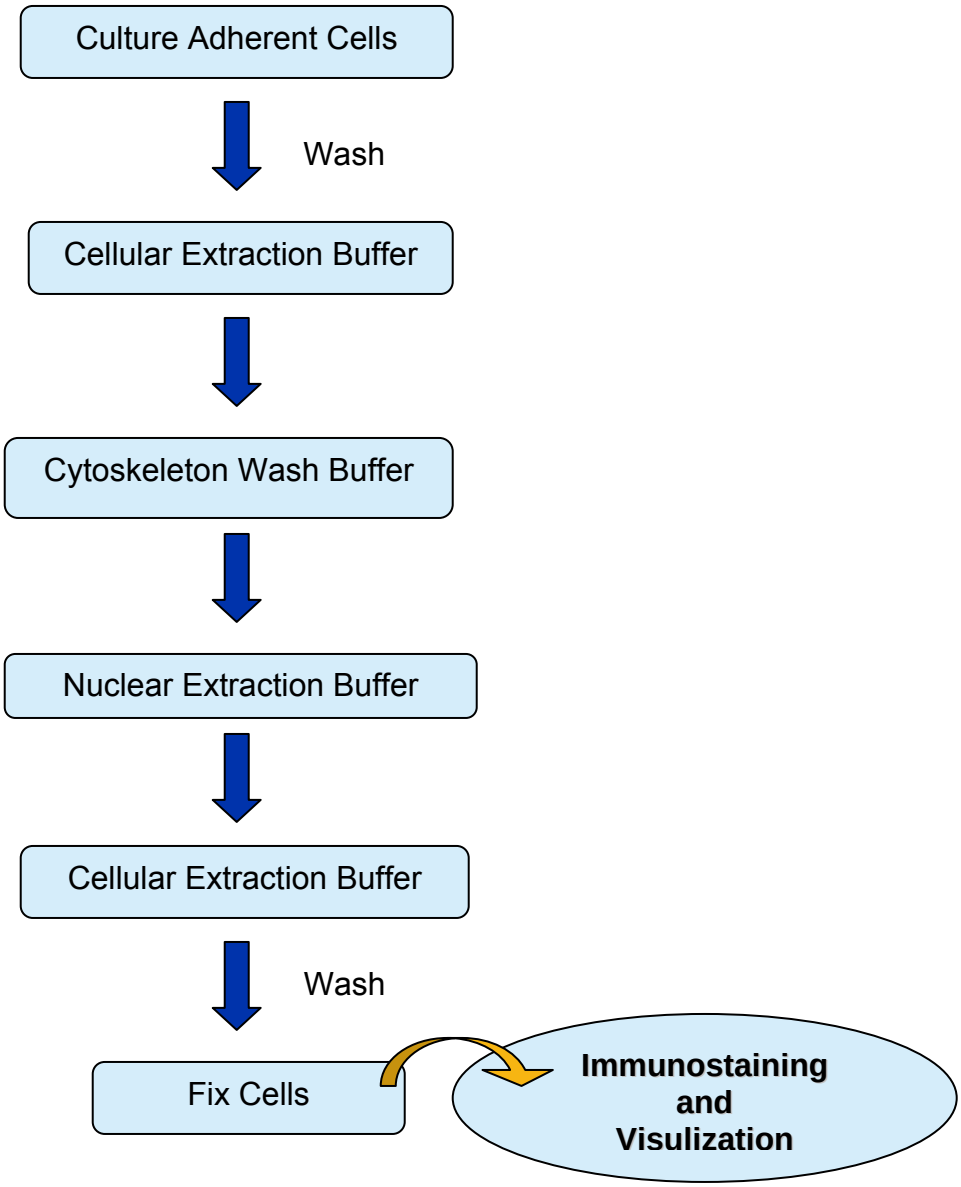
The actin cytoskeleton is a highly dynamic network composed of actin polymers and a large variety of associated proteins. The functions of the actin cytoskeleton is to mediate a variety of essential biological functions in all eukaryotic cells, including intra- and extra-cellular movement and structural 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 ProteoExtract® Native Cytoskeleton Enrichment and Staining Kit provides cytoskeleton purification detergent buffers that retain focal adhesion and actin-associated proteins while removing soluble cytoplasmic and nuclear proteins from the cell. Anti-Vinculin and anti-GAPDH antibodies are provided as markers for focal adhesions and cytosol, respectively, in immunofluorescence 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 staining whole cells.

## Protocol Flow Chart

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## Kit Components

### Compartmentalization Module (Part No. 17-10210-2)
**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). One vial 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. Donkey anti-Mouse IgG, FITC Conjugate: (Part No. CS207809). One vial containing 50 µL of 200x donkey anti-mouse FITC conjugate.

### Immunoflorescence Module (Part No. 17-10210-1)
**Store at 2-8º C**

1. 10x Blocking/Permeabilization Buffer: (Part No. CS207808). One bottle containing 12.5 mL of 10x Blocking/Permeabilization Buffer.
2. TRITC-Conjugated Phalloidin: (Part No. 90228). One vial containing 15 µg lyophilized TRITC-Conjugated Phalloidin.
3. DAPI: (Part No. 90229). One vial containing 100 µL of 200x DAPI.
4. Anti-Vinculin Mouse Monoclonal antibody: (Part No. 90227). One vial containing 100 µL of 100x anti-Vinculin mouse monoclonal IgG1 antibody.
5. Anti-Glyceraldehyde-3-Phosphate Dehydrogenase Mouse Monoclonal antibody: (Part No. CS207810). One vial containing 16 µL of 500x anti-GAPDH mouse monoclonal IgG1 antibody.

## Materials Not Supplied

1. Pipettors and tips capable of accurately measuring 1-1000 μL
2. Graduated serological pipettes
3. Distilled or deionized water
4. 4% paraformaldehyde solution for fixing cells
5. 1x Dulbecco's Phosphate Buffered Saline (D-PBS)
6. Cover slips
7. Mounting fluid (EMD Millipore Catalog No. 5013)

## Storage

Maintain the unopened kit modules (17-10210-1 & 17-10210-2) 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 50 mL of 1x Cytoskeleton Wash Buffer by adding 2.5 mL 20x Cytoskeleton Wash Buffer (Part No. CS207813) to 47.5 mL of Milli-Q or distilled water. Aliquot and store at -20º C.
3. **1x Blocking/Permeabilization Buffer:** Prepare 125 mL of 1x Blocking/Permeabilization Buffer by adding 12.5 mL of 10x Blocking/Permeabilization Buffer (Part No. CS207808) to 112.5 mL of Milli-Q or distilled water. Store at 2-8º C.

4. **TRITC-Conjugated Phalloidin:** Prepare stock solution by resuspending vial of TRITC-Conjugated Pahlloidin (Part No. 902280 into 250 µL of methanol. Store at -20º C upon reconstitution.

## Protocol

**Compartmentalization:**

1. Culture adherent cells to approximately 80-90% confluency in an 8-well chamber slide.
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 8-well chamber slide requires 4 mL 1x Cellular Extraction Buffer, 2 mL Nuclear Extraction Buffer and 12 mL Cytoskeleton Wash Buffer. To these volumes, add 4 µL, 2 µL, and 12 µL respectively of both the Protease Inhibitor Cocktail and Sodium Orthovanadate.
    b. Keep all buffers on ice until use.
3. Once cells have reached the desired confluency, gently wash cells twice using 0.5 mL cold 1x D-PBS. Aspirate after each wash.
    a. ***Note:*** *If unextracted cells are desired for some wells, the wells may be kept in D-PBS until step 11.*
4. Add 0.25 mL cold 1x Cellular Extraction Buffer to the cells and incubate for 1.5 minutes.
5. Rinse the cells by pipetting buffer up and down gently 2-3 times.
    a. ***Note:*** *This and subsequent rinse steps may be skipped if cells are not strongly adherent.*
6. Remove the buffer by aspirating.
7. Add 0.5 mL 1x Cytoskeleton Wash Buffer to the cells, and gently pipette wash buffer up and down 2-3 times. Remove the wash buffer by aspirating.
8. Add 0.25 mL Nuclear Extraction Buffer to the cells and incubate for 10 minutes. Remove the buffer by aspirating.
9. Add 0.25 mL 1x Cellular Extraction Buffer to the cells, and gently pipette wash buffer up and down 2-3 times. Remove the buffer by aspirating.
10. Wash the cells twice, each time with 0.5 mL 1x Cytoskeleton Wash Buffer. Remove the buffer by aspirating after each wash.
11. Add 0.25 mL 4% paraformaldehyde to wells. Incubate for 30 minutes at room temperature.
12. Wash the cells with 0.25 mL 1x D-PBS. Remove the buffer by aspirating.

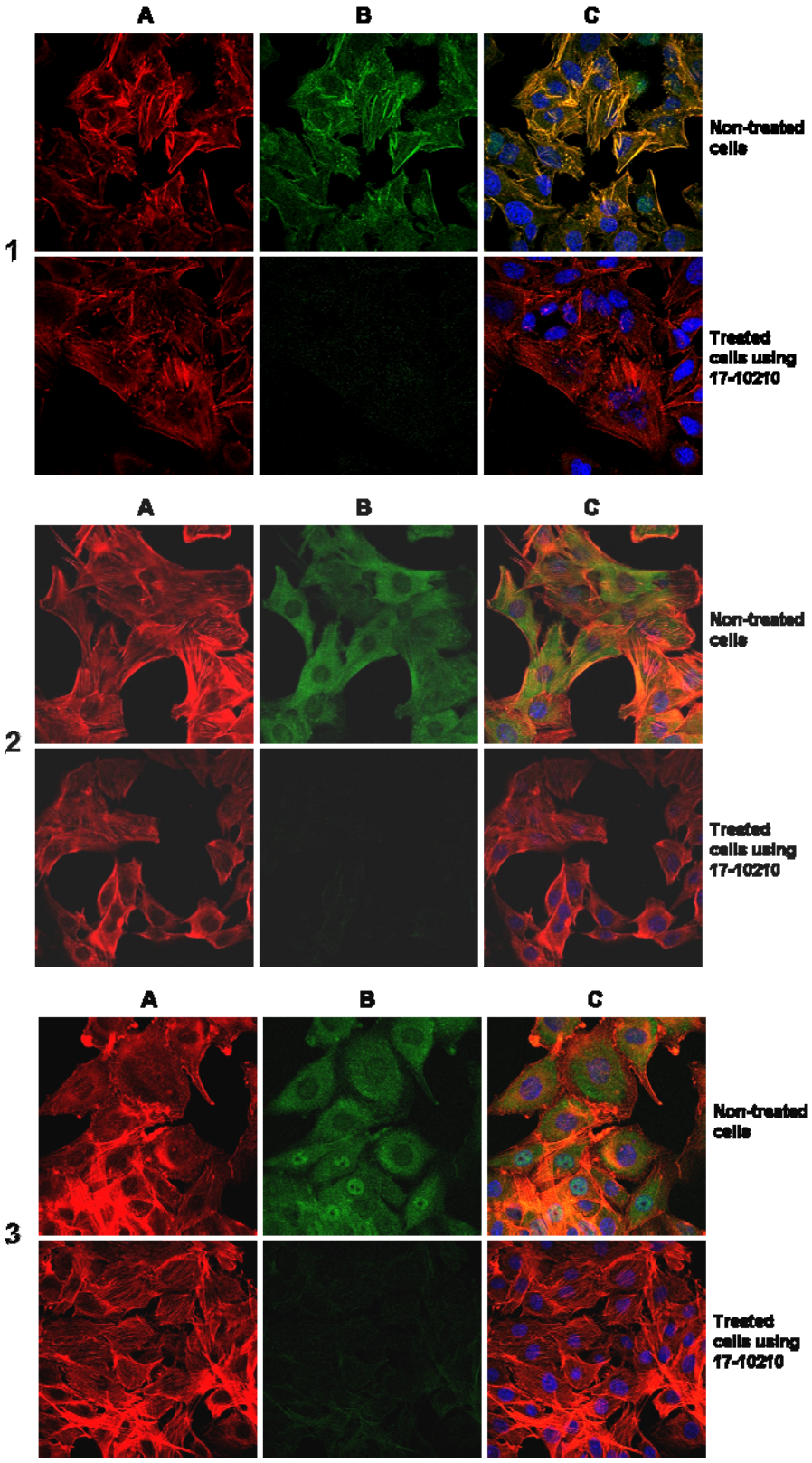
**Immunostaining and Visualization:**

1. Wash the cells twice, each time with 0.5 mL 1x Blocking/Permeabilization Buffer. Remove the buffer by aspirating after each wash.
2. Dilute primary antibodies in separate tubes of 1x Blocking/Permeabilization Buffer (0.25 mL per well). Add to appropriate wells of the chamber slide, and incubate for 1 hour at room temperature.
    a. For example: If 1 mL of each diluted antibody solution is needed, dilute anti-GAPDH antibody 1:500 into 1 mL of 1x Blocking/Permeabilization Buffer (i.e. 2 µL) and dilute anti-Vinculin antibody 1:100 into 1 mL 1x Blocking/Permeabilization Buffer (i.e. 10 µL).

    ***Note:*** *It is recommended to optimize primary antibody titer for each cell type. A typical dilution range for anti- Vinculin is 1:100-1:500.*
3. Wash the cells twice using 0.5 mL each of 1x Blocking/Permeabilization Buffer. Remove the buffer by aspirating after each wash.
4. In one tube, dilute secondary antibody 1:200, TRITC-Conjugated Phalloidin 1:100, and DAPI 1:200 in 1x Blocking/Permeabilization Buffer (0.25 mL per well).
    **IMPORTANT:** Staining solutions and cells undergoing staining in subsequent steps should be protected from light.
    b. For example: One 8-well chamber slide requires 2 mL of secondary antibody and stain solution. To 2 mL of 1x Blocking/Permeabilization Buffer, add 10 µL donkey-anti-mouse, FITC conjugate, 20 µL TRITC-Conjugated Phalloidin, and 10 µL DAPI.

    ***Note:*** *Secondary antibody and TRITC-conjugated Phalloidin dilutions may need to be optimized for each cell type. Typical dilutions range from 1:100 -1:1,000.*
5. Add 0.25 mL diluted secondary antibody and stains into each well. Incubate for 1 hour at room temperature.
6. Wash the cells twice using 0.5 mL each of 1x Blocking/Permeabilization Buffer. Remove the buffer by aspirating after each wash.
8. Wash the cells twice, each time with 0.5 mL 1x D-PBS. Remove the buffer by aspirating after each wash.
9. Mount cover slip on slide with Mounting fluid and allow to dry.
    **IMPORTANT:** Protect from light while drying. Slide can be stored at 2-8ºC if immediate visualization is not required.
10. Visualize with fluorescent microscope. Unextracted cells will have high GAPDH signal, whereas cells treated with Extraction Buffers will have little or no GAPDH signal. Some DAPI staining is expected in cells treated with Extraction Buffers, as gentle conditions for nuclear extraction are required to preserve morphology. Phalloidin will reveal filamentous actin structure, and vinculin staining of focal adhesions will be punctate.

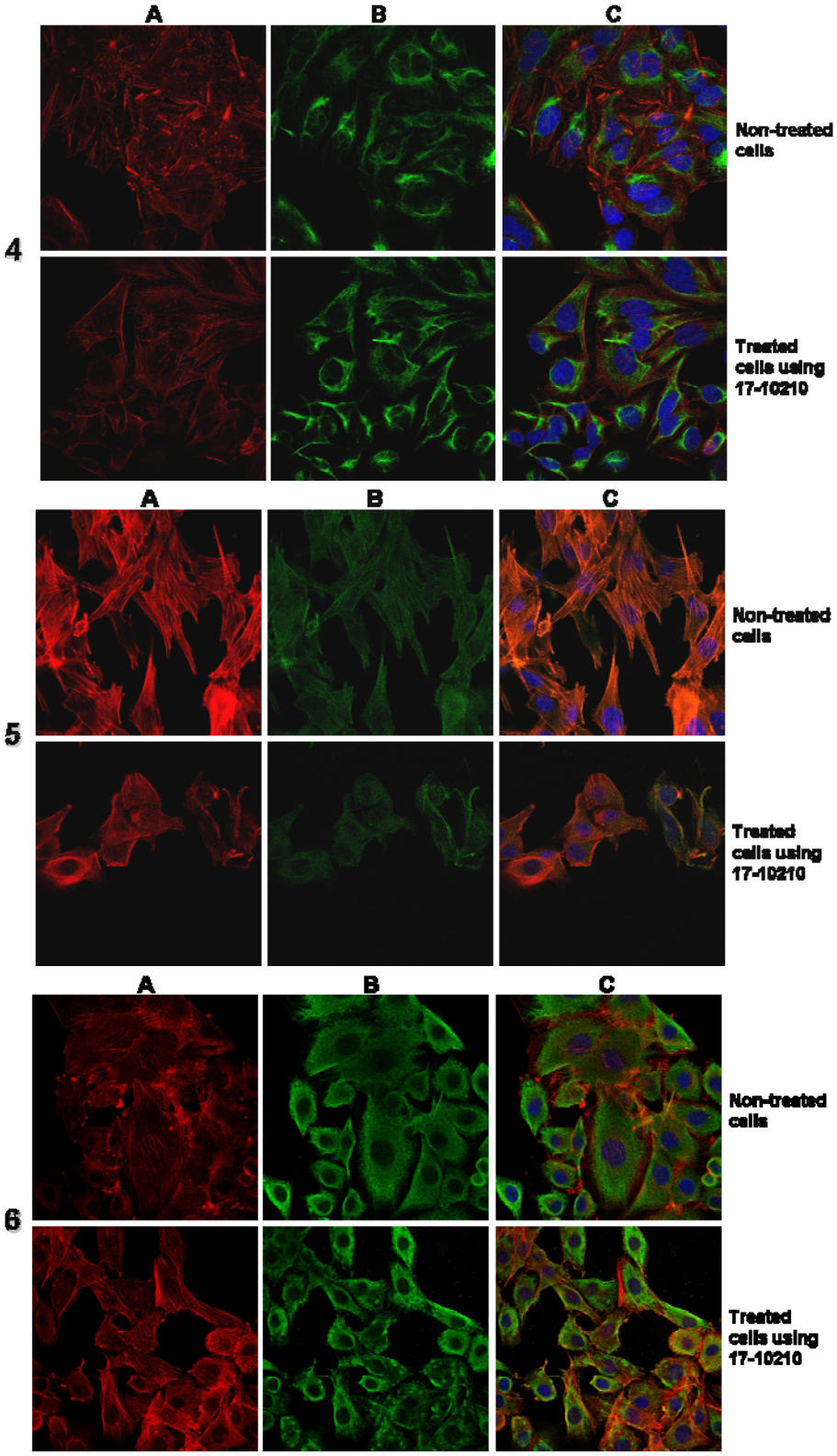
## Representative Data




**Figures 1, 2 and 3:**

Confocal fluorescence microscopy of non-treated/enriched and Cat. No. 17-10210-treated/enriched **(1)** HeLa cells, **(2)** C2C12 cells, and **(3)** L6 cells. **(A)** F-actin was detected using TRITC-conjugated Phalloidin (Part No. 90228), **(B)** cytosolic protein was detected using anti-GAPDH (Part No. CS207810) antibody as a negative control and a FITC-conjugated secondary (Part No. CS201651), **(C)** nuclear counterstaining revealed with DAPI and all images were overlaid.

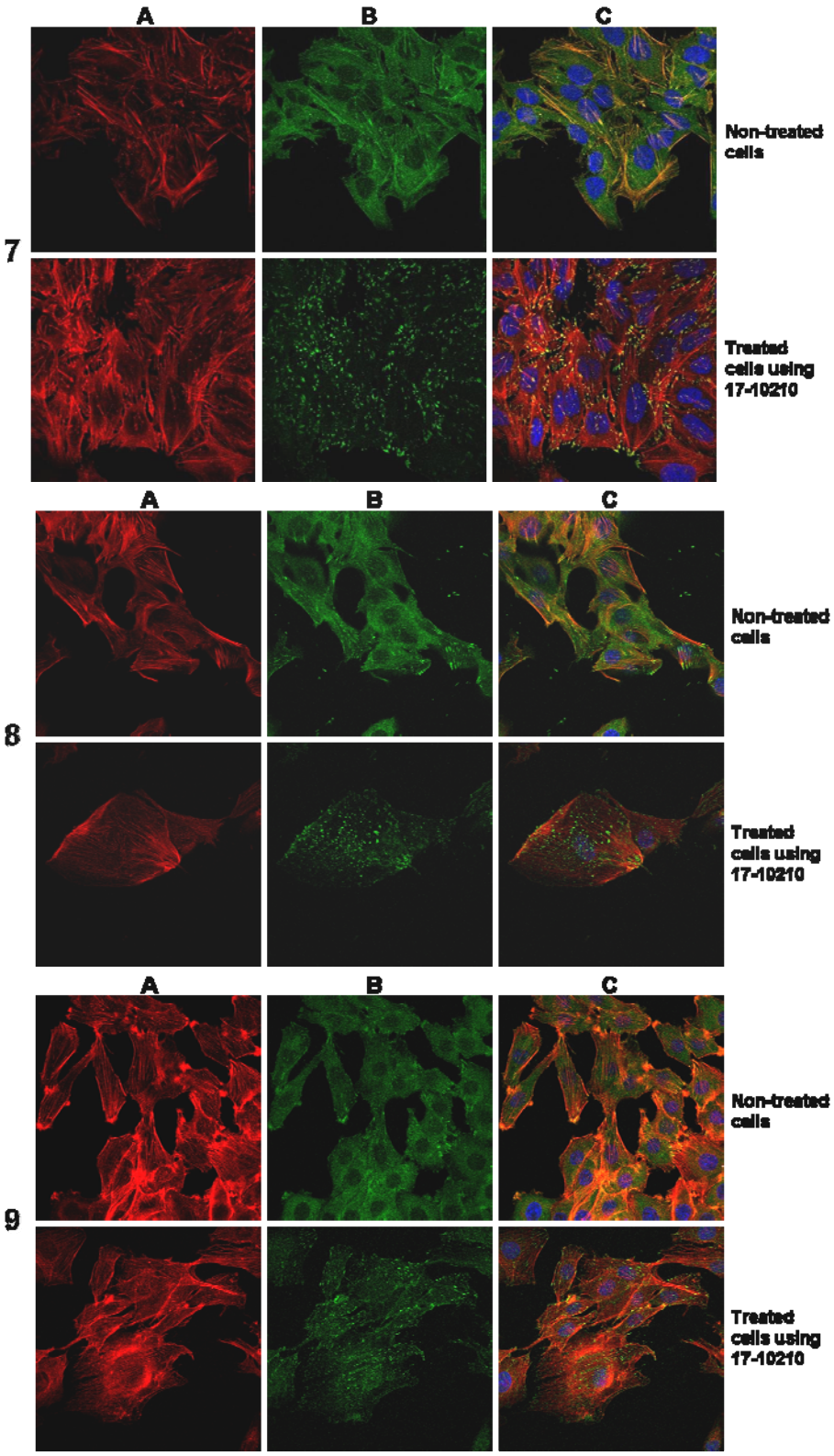
Soluble cytosolic protein fraction (GAPDH) is successfully removed after enrichment in all 3 cell types using the ProteoExtract® Cytoskeleton Enrichment and Staining Kit (Cat. No. 17-10210).




**Figures 4, 5 and 6:**

Confocal fluorescence microscopy of non-treated/enriched and Cat. No. 17-10210-treated/enriched **(4)** HeLa cells, **(5)** C2C12 cells, and **(6)** L6 cells. **(A)** F-actin was detected using TRITC-conjugated Phalloidin (Part No. 90228), **(B)** cytoskeletal protein was detected using anti-Vimentin (Cat. No. MAB3400 **(4 and 6)** and MAB1633 **(5)**) antibody and a FITC-conjugated secondary (Part No. CS201651), **(C)** nuclear counterstaining revealed with DAPI and all images were overlaid.

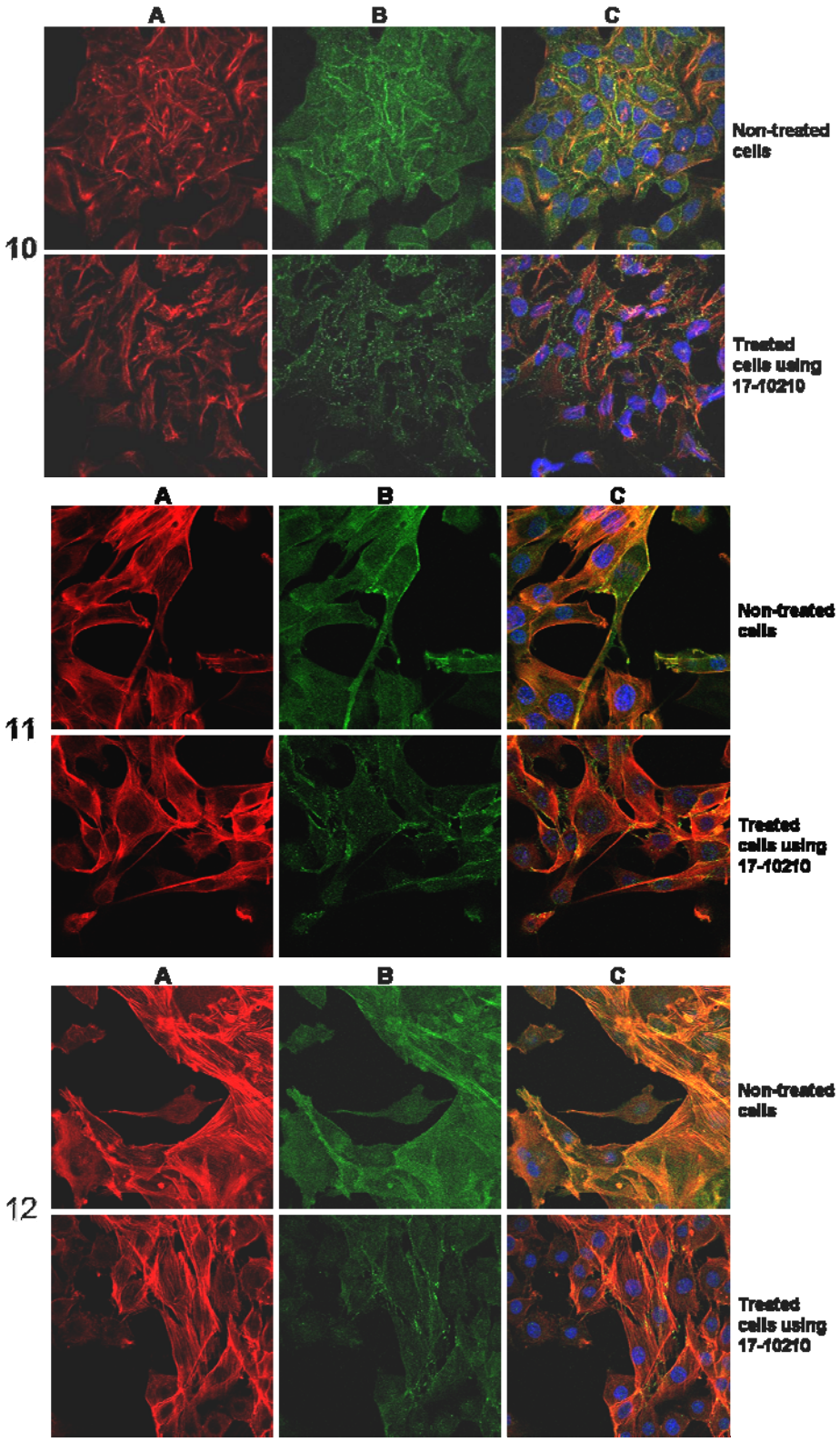
Insoluble cytoskeletal protein fraction (vimentin) is successfully retained after enrichment in all 3 cell types using the ProteoExtract® Cytoskeleton Enrichment and Staining Kit (Cat. No. 17-10210).




**Figures 7, 8 and 9:**

Confocal fluorescence microscopy of non-treated/enriched and Cat. No. 17-10210-treated/enriched **(7)** HeLa cells, **(8)** C2C12 cells, and **(9)** L6 cells. **(A)** F-actin was detected using TRITC-conjugated Phalloidin (Part No. 90228), **(B)** focal adhesion contacts were detected using anti-Vinculin (Part No. 90227) antibody and a FITC-conjugated secondary (Part No. CS201651), **(C)** nuclear counterstaining revealed with DAPI and all images were overlaid.

Background due to soluble cytosolic fraction is significantly reduced after enrichment using the ProteoExtract® Cytoskeleton Enrichment and Staining Kit (Cat. No. 17-10210), resulting in clear detection of insoluble, actin-associated proteins (vinculin).




**Figures 10, 11 and 12:**

Confocal fluorescence microscopy of non-treated/enriched and 17-10210-treated/enriched **(10)** HeLa cells, **(11)** C2C12 cells, and **(12)** L6 cells. **(A)** F-actin was detected using TRITC-conjugated Phalloidin (Part No. 90228), **(B)** cell junctions were detected using anti-beta-Catenin (Catolog No. 06-734) antibody and a FITC-conjugated secondary (Part No. CS201651), **(C)** nuclear counterstaining revealed with DAPI and all images were overlaid.

Background due to soluble cytosolic fraction is significantly reduced after enrichment using the ProteoExtract® Cytoskeleton Enrichment and Staining Kit (Cat. No. 17-10210), resulting in clear detection of insoluble, low-abundance actin-associated proteins (beta-catenin).

## References


1. Chen CS, et al. (2003). *Biochemical and Biophysical Research Communications*, 307(2):355–61.
2. Frixione E. (2000). *Cell motility and the cytoskeleton* 46 (2): 73–94.
3. Bretscher A et al. (1994). *J Cell Biol* **126**:821-825.
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8. Yamada KM, Geiger B. (1997). *Curr Opin Cell Biol* **9**:76-85.
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10. Turner CE, Burridge K. (1991). *Curr Opin Cell Biol* **3**:849-853.

## Troubleshooting Guide

| Problem | Potential Cause | Experimental Suggestions |
|---|---|---|
| No signal or weak signal in all wells | Loss of cells during compartmentalization | Some cells adhere more strongly than others. Check cells by bright-field microscopy at each step of the protocol to be certain that cells are retained. |
| | Cell type analyzed has low expression of vinculin | Use more concentrated primary antibody. Antibodies are validated with human and rodent cell lines, results with cells from other species may vary. |
| | Incorrect storage temperatures | Items are to be stored at the appropriate storage temperatures. Performance can be negatively affected if reagents are not stored and used in the appropriate time period. |
| Too much background noise signal | Primary or secondary antibody, or TRITC-phalloidin were present at higher concentration than necessary for the cell type analyzed | Test a range of the primary antibody dilutions to obtain a proper dilution with lower background noise. |
| | High GAPDH indicates incomplete extraction of cytoplasm | Increase incubation times or number of washes |
| | High signal with DAPI | Gentle extraction of nuclei better preserves cell morphology. If less interference from nucleic acids is desired, increase time of nuclear extraction and/or incubate at 37°C. |

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