

Fast and Gentle Enrichment Assays for Isolating and Staining Native Protein-Cytoskeleton Associations

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肌動蛋白細胞骨架 (Actin cytoskeleton) 在所有真核細胞中具有多種非常重要的功能，包括細胞內及細胞外的運動和結構性支持。Actin cytoskeleton 的組成受到非常多層級的訊息調控，以確保其功能能正常運作。其中包含將 actin monomers (G-actin) 整合成 actin polymers，藉由 actin side-binding or cross-linking proteins 再將 actin polymers 整合到 filamentous network (microfilaments 主要是由 F-actin 構成)。

Actin cytoskeleton 會快速改變形狀，以回應外在的刺激及細胞周期的進程。細胞內 actin filaments 的取向分佈，是決定細胞形態和活力的重要因素。因此，如果正常的調控機制被干擾，可能導致細胞轉化及癌症的形成。研究發現轉化細胞含有較少的 F-actin，而 F-actin 的表現量也與細胞周期呈現非典型協同作用。

Focal adhesions & adherens junctions 是一種膜蛋白複合體，它是 actin filaments 核心位置，也是細胞外牆、細胞膜和 Actin cytoskeleton 的交聯劑 (cross-linker)。Focal adhesions 會將外基質 (ECM) 連結到細胞內的肌動蛋白細胞骨架。Focal adhesions 也是一個信號轉導站，主導細胞粘著的訊號傳遞。Focal adhesions 組合可接觸到細胞外基質的 integrin-type receptors 來接受外在信號，然後 integrin 再連結到細胞內的蛋白複合體。包含 vinculin (universal focal adhesion marker), talin, α -actinin, paxillin, tensin, zyxin and focal adhesion kinase。

由於 Actin cytoskeleton 不溶於一般萃取蛋白的溶劑 (例如 Triton-X100)，使得 Actin cytoskeleton 相關蛋白或調控機制的研究相當困難進行。很多 Actin 調控蛋白及磷酸化蛋白在細胞質內是可溶的，一旦活化而連結到不可溶的 Actin cytoskeleton，會導致無法偵測到很多生化改變 (例如 phosphorylation and nitrosylation)。

Merck Millipore 提供一個可以純化細胞骨架的方法，此方法可選擇性地強化細胞骨架相關蛋白，以利進行後續詳細的蛋白質生化分析。這種方法不會破壞原本的細胞骨架或原本蛋白質與骨架的關係，對於要直接研究正常和病變細胞細胞骨架的差異，是一個非常重要的工具。

Materials and Methods

Reagents

The ProteoExtract® Cytoskeleton Enrichment and Isolation Kit (#17-10195) provides the necessary extraction buffers to quickly, gently and selectively remove soluble cytoplasmic and nuclear proteins from the cell, while retaining focal adhesion and actin-associated proteins. Vimentin and GAPDH antibodies are also provided as markers for the cytoskeleton and cytosol, respectively, for Western blot analysis. The enriched cytoskeleton proteins can also be subjected directly to mass spectrometry analysis, or further enzyme digested prior to analysis. The ProteoExtract® Native Cytoskeleton Enrichment and Staining Kit also provides the necessary reagents to gently enrich and retain the cytoskeleton and associated proteins in their native conformations. Additionally, the staining kit provides labeled phalloidin and labeled antibodies against vinculin and GAPDH as markers for visualization and colocalization of the actin cytoskeleton, focal adhesions and cytosol, respectively, for immunofluorescence analysis.

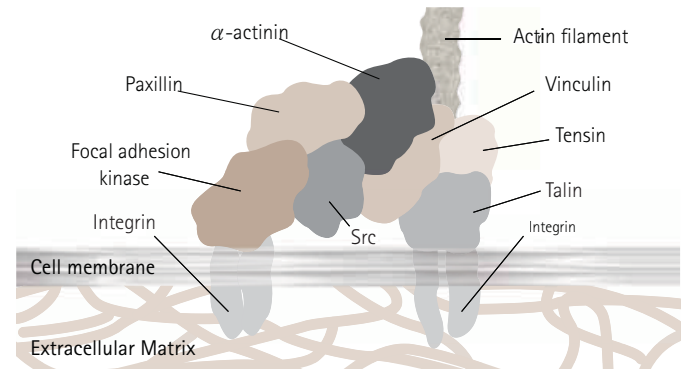


Figure 1. Composition of Focal Adhesion.

Cell culture and cytoskeleton enrichment

Human HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) complete medium in 100 mm culture dish until 80–90% confluence. The cells were gently washed twice with 2 mL of cold 1X Dulbecco's Phosphate Buffered Saline (DPBS). Cold 1X Cellular Extraction Buffer (0.25 mL per plate) was added to the cells and incubated for 1.5 minutes on ice. Buffer was collected and labeled as Soluble Compartment (S). 0.5 mL 1X Cytoskeleton Wash Buffer was added to the cells and then pooled with the Soluble Compartment. Nuclear Extraction Buffer (0.25 mL per plate) was added to the cells, and incubated on ice for 10 minutes. This fraction was collected and labeled as Nuclear Compartment (N). Cells were washed twice with 2 mL of 1X Cytoskeleton Wash Buffer.

Enriched cytoskeleton isolation

To isolate the enriched Cytoskeleton compartment, 0.25 mL of Cytoskeleton Solubilization Buffer was added to the cells following the Cytoskeleton Wash Buffer step, and pipetted up and down for maximum solubilization. This fraction was collected and labeled as Cytoskeleton Compartment (C). Protein concentrations of all collected compartments were determined by A280 and stored at -80°C until further application as described below.

Enriched native cytoskeleton fixation

To visualize the enriched native cytoskeleton, human HeLa cells were grown in DMEM complete medium in 8-well glass chamber slides until 80–90% confluence. Extraction of soluble and nuclear compartments was performed as above, except that these fractions were discarded by aspirating at each step. The adherent insoluble cytoskeleton remaining attached to the chamber slide was fixed by addition of 0.25 mL 4% paraformaldehyde to each well following the last Cytoskeleton Wash Buffer step to fix the cells. After a 30-minute incubation at room temperature, the cells were washed with 0.25 mL of 1X DPBS per well.

Immunostaining and visualization

After fixing the native cytoskeleton, the cells were washed twice with 0.5 mL 1X Blocking/Permeabilization Buffer, and incubated with 0.25 mL/well diluted primary antibody in 1X Blocking/Permeabilization Buffer for 1 hour at room temperature. Washes and incubation with a dye-conjugated secondary antibody, TRITC-conjugated phalloidin (1:100) and DAPI (1:200), each provided in the staining kit, were performed to stain the actin cytoskeleton and remaining cell nucleus. Cover slips were mounted on slides with mounting fluid and visualized with a fluorescence microscope.

Western blot analysis

Following the enrichment of the cytoskeleton, collected cell compartments (S, N and C) were subjected to SDS-PAGE gel and transferred to a PVDF membrane. GAPDH and vimentin antibodies (provided in the kit) were used to detect cytosolic and intermediate filament proteins, respectively, using standard Western blotting procedures.

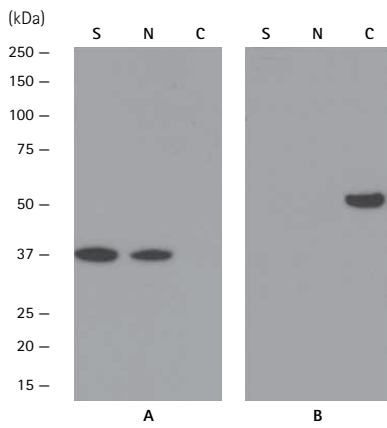


Figure 3. Western blot of compartmental proteins extracted from HeLa cells using ProteoExtract® Cytoskeleton Enrichment and Isolation Kit (Catalog No. 17-10195). Results indicate that GAPDH (Panel A) is present in the soluble cytoplasmic (lane S) and nuclear (lane N) fractions [11], and the intermediate filament protein Vimentin (Panel B) is present exclusively in the cytoskeletal compartment (lane C).

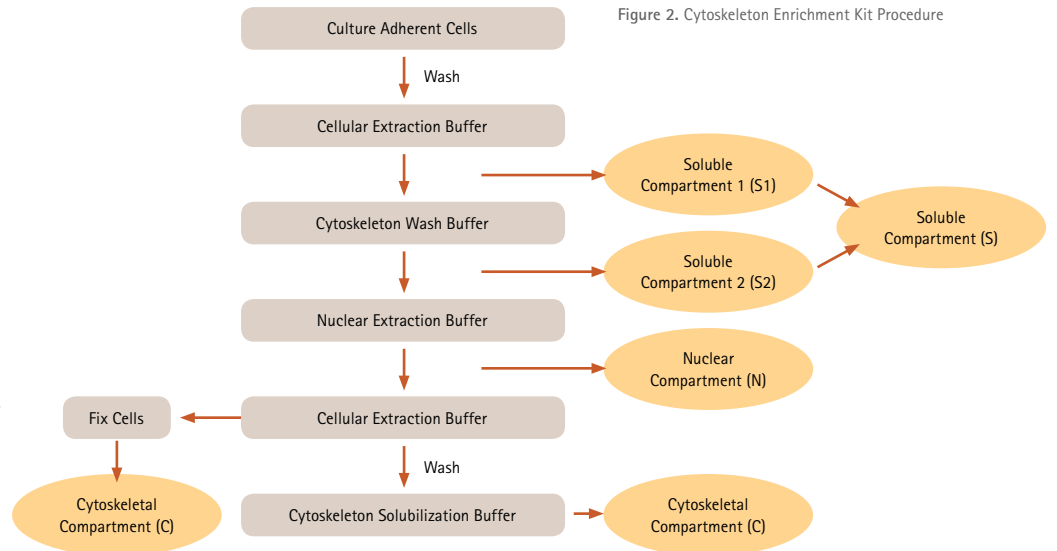


Figure 2. Cytoskeleton Enrichment Kit Procedure

Results

We utilized these fast and convenient methods to enrich and either isolate or stain the native cytoskeleton-associated proteins, minimizing interference by soluble cytoplasmic and nuclear proteins (Figure 2). For biochemical analysis, fractions were collected in a stepwise process, whereas for imaging, the native cytoskeleton was chemically fixed on the culture surface. The entire extraction process could be completed in approximately 20 minutes. Western blot analysis indicated that GAPDH was present in the cytoplasmic and nuclear fractions, whereas the intermediate filament protein vimentin was present exclusively in the cytoskeletal compartment (Figure 3). The ability to detect and study the low abundance cytoskeleton-associated proteins was greatly increased with this cytoskeleton enrichment and isolation method.

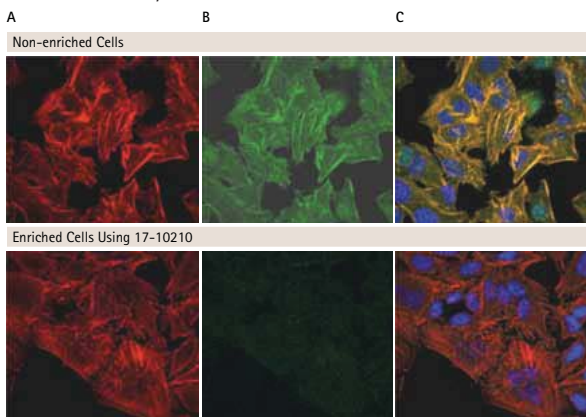


Figure 4. Confocal fluorescence microscopy of non-treated/enriched and treated/enriched HeLa cells. (A) F-actin was detected using TRITC-conjugated Phalloidin, (B) cytosolic protein was detected using GAPDH antibody as a cytosolic control and a FITC-conjugated secondary, (C) nuclear counterstaining was revealed with DAPI and all images were overlaid. Soluble cytosolic protein fraction (GAPDH) was successfully removed after enrichment using the ProteoExtract® Cytoskeleton Enrichment and Staining Kit.

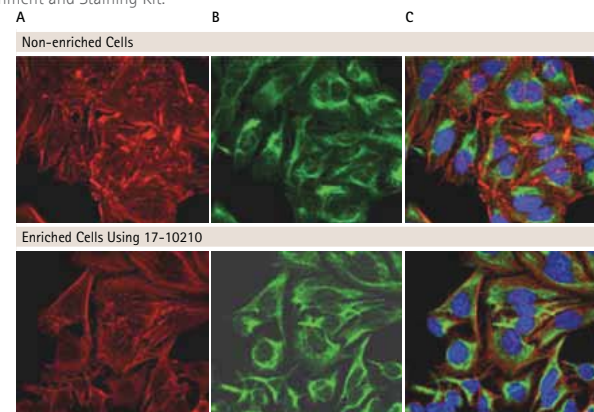


Figure 5. Confocal fluorescence microscopy of non-treated/enriched and treated/enriched HeLa cells. (A) F-actin was detected using TRITC-conjugated Phalloidin, (B) cytoskeletal protein was detected using Vimentin antibody and a FITC-conjugated secondary, (C) nuclear counterstaining was revealed with DAPI and all images were overlaid. Insoluble cytoskeletal protein fraction (vimentin) was successfully retained after enrichment using the ProteoExtract® Cytoskeleton Enrichment and Staining Kit.

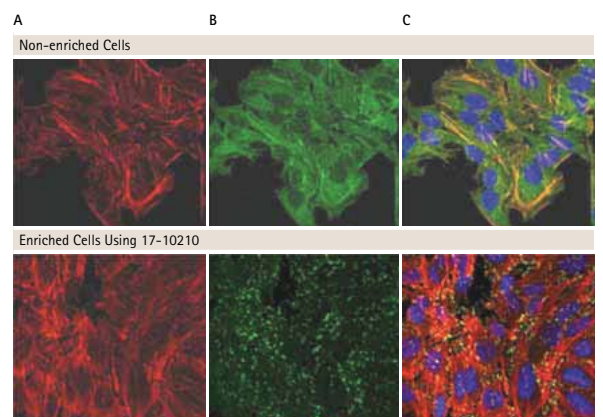


Figure 6. Confocal fluorescence microscopy of non-treated/enriched and treated/enriched HeLa cells. (A) F-actin was detected using TRITC-conjugated Phalloidin, (B) focal adhesion contacts were detected using Vinculin antibody and a FITC-conjugated secondary, (C) nuclear counterstaining was revealed with DAPI and all images were overlaid. Background due to soluble cytosolic fraction was 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).

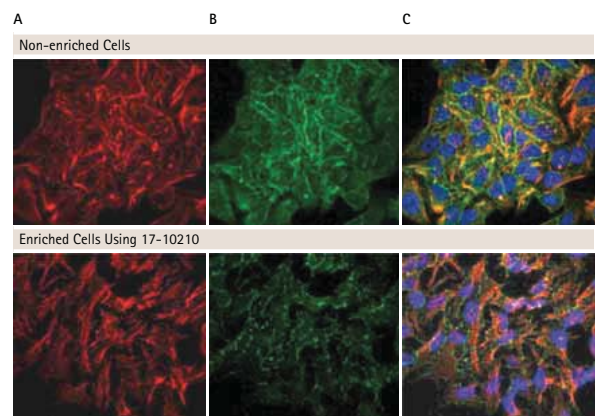


Figure 7. Confocal fluorescence microscopy of non-treated/enriched and treated/enriched HeLa cells. (A) F-actin was detected using TRITC-conjugated phalloidin, (B) cell junctions were detected using -Catenin antibody and a FITC-conjugated secondary, (C) nuclear counterstaining was revealed with DAPI and all images were overlaid. Background due to soluble cytosolic fraction was significantly reduced after enrichment using the ProteoExtract® Cytoskeleton Enrichment and Staining Kit, resulting in clear detection of insoluble, low-abundance actin-associated proteins (-catenin).

Conclusions

EMD Millipore's ProteoExtract® Cytoskeleton Enrichment and Isolation Kit offers a convenient, efficient tool to purify cytoskeleton proteins with a gentle detergent buffer treatment for subsequent biochemical analysis. The ProteoExtract® Native Cytoskeleton Enrichment and Staining Kit preserves focal adhesion and cytoskeletal structure for imaging, and greatly reduces background emanating from soluble cytoplasmic proteins in the cell. Both kits will greatly enhance the ability to detect and analyze the low abundance actin-associated proteins which are typically masked in conventional methods.

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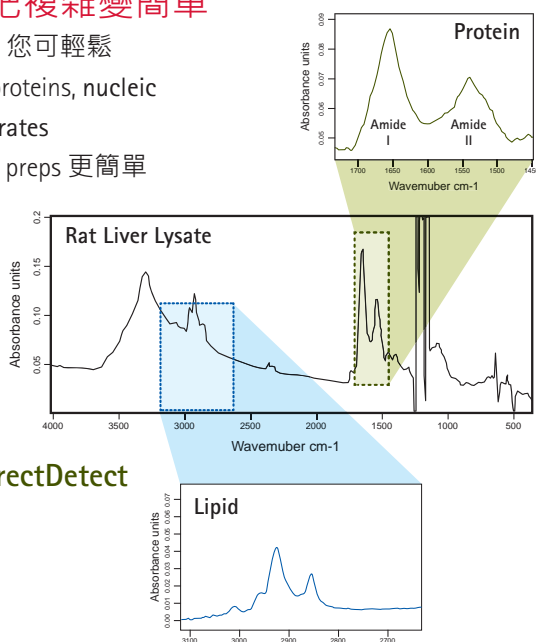


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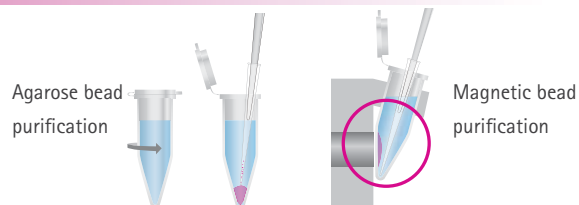
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