



User Protocol 444810 Rev. 29-July-04 JSW Page 1 of 13

ProteoExtract™
Native Membrane Protein Extraction Kit
Cat. No. 444810

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1. Introduction

Membrane proteins represent only one-third of the proteins encoded by the human genome, but they represent more than two-thirds of the known protein targets for drugs. Therefore, approaches to prepare and characterize membrane proteins are of significant interest for drug discovery. However, due to their hydrophobic nature, membrane proteins are more difficult to analyze than soluble proteins. In addition to the intrinsic difficulty of solubilization, membrane proteins are challenging because of their general low abundance. Thus, the challenges for sample preparation of membrane proteins are effective solubilization and selective enrichment, ideally keeping proteins in a non-denatured state.

The ProteoExtract™ Native Membrane Protein Extraction Kit (M-PEK) is designed for the isolation of native membrane proteins from mammalian cells and tissue. It has been developed for convenient high yield membrane protein extraction, providing 3-5 fold enrichment of integral membrane and membrane-associated proteins using non-denaturing conditions. The kit belongs to a range of related kits from Calbiochem all aimed at reducing sample complexity in order to improve the probability of identifying low abundance proteins (see Section 9: Related Products).

M-PEK is easy to use and allows for a fast and robust two-step extraction of membrane proteins from mammalian samples. Optimized protocols are provided for adherent tissue culture cells, suspension grown tissue culture cells, frozen cell pellets and tissue.

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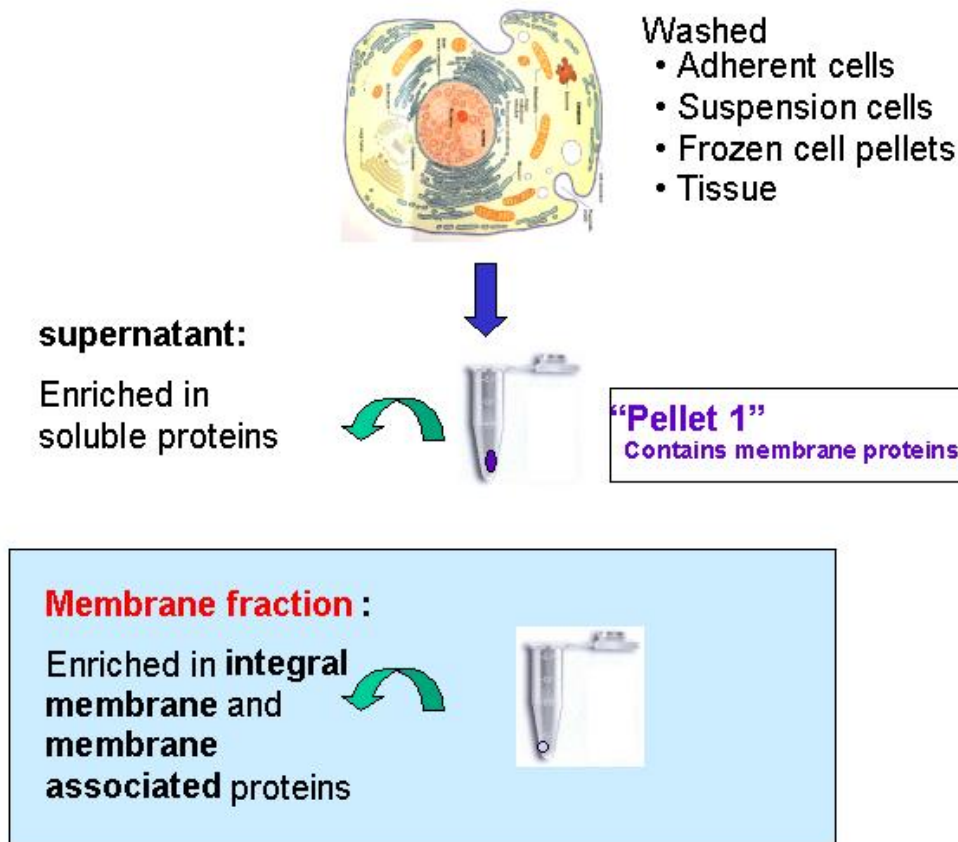


Fig. 1: Schematic Representation of the M-PEK Extraction Procedure

Robust, fast and scalable 2-step native extraction of membrane proteins that works with low speed centrifugation facilitating the processing of multiple samples simultaneously.

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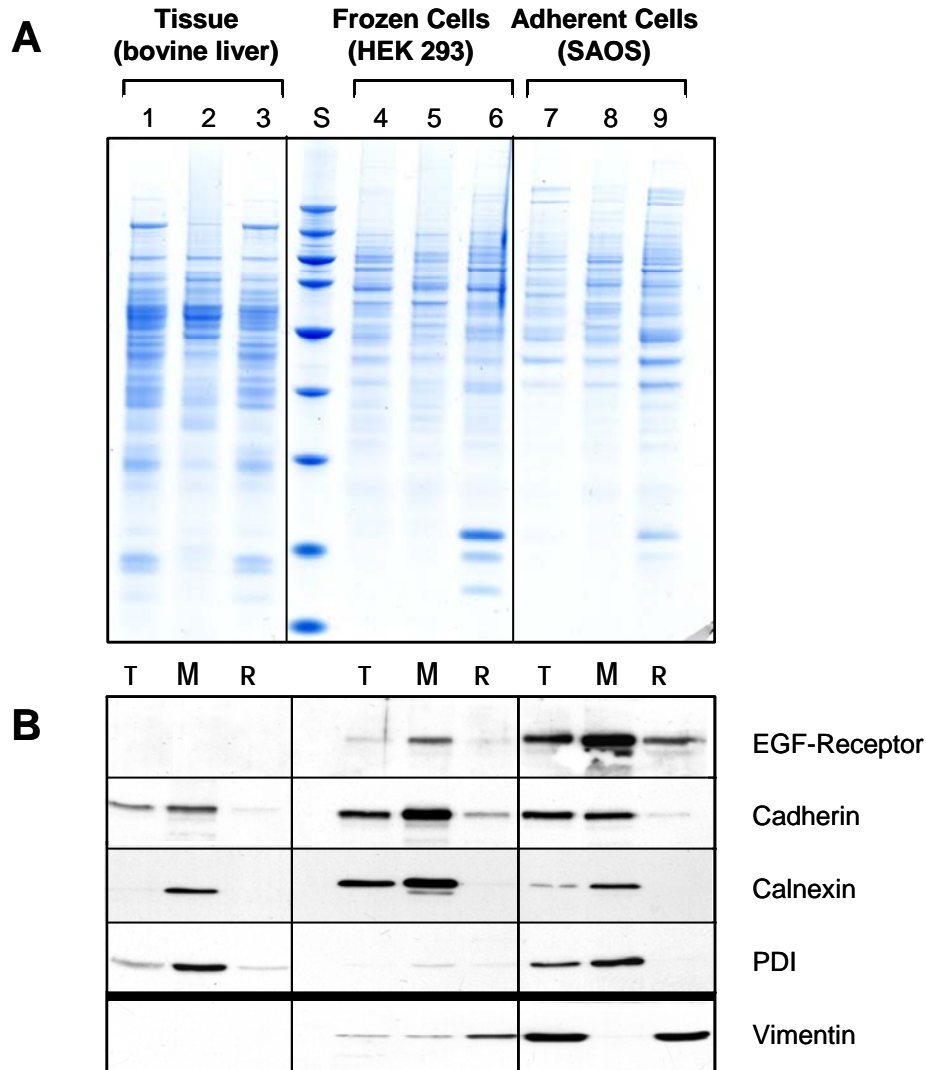


Fig. 2. Selective Enrichment of Membrane Proteins from Tissues and Cells

Frozen bovine liver tissue, cell pellet of suspension grown HEK 293 cells, and fresh adherent SAOS cells were either extracted with SDS to yield a total lysate (T) or with M-PEK to yield a membrane fraction (M) and remaining “non-membranous” proteins (R).

A: 10 µg of each extracted fraction were separated using 10% Bis-Tris SDS-PAGE and visualized by Coomassie staining. The membrane protein patterns (lanes 2, 5 and 8) are distinct from the patterns of both total and non-membranous fractions, indicating selectivity of the M-PEK extraction. The MW Standard (S) represents bands of 225, 150, 100, 75, 50, 35, 25, 15 and 10 kDa respectively.

B: Immunoblots of equivalent gels using membrane-associated and integral membrane protein markers (as indicated) demonstrates the selectivity of the M-PEK procedure. Following M-PEK extraction, all marker membrane proteins are enriched in the M fraction, whereas non-membrane proteins as e.g. vimentin are depleted in the obtained membrane fraction. Please note that the overall signal strength of the antibodies recognizing the designated marker proteins can vary, due to different expression levels in other cell types as well as due to species specificity of the antibodies utilized.

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This kit provides all required reagents, including: wash buffers and protease inhibitors. The procedure is based on a robust yet mild differential extraction procedure rather than on differential centrifugation. In contrast to the 2-phase partitioning technique, where detergents are used to separate membrane proteins based primarily on their intrinsic hydrophobicity, the kit's scalable differential extraction procedure selectively extracts integral membrane and membrane-associated proteins based on their actual association with cellular membranes. This leads to an improved reproducibility compared to 2-phase partitioning, as well as a better compatibility with downstream assays. Unlike many procedures used to isolate membrane proteins, this kit does not require sonication, extended rigorous vortexing, time-consuming ultracentrifugation, or incubation of samples at elevated temperatures. This minimizes the chances of post extraction modifications.

M-PEK extracted membrane proteins generally do not require dialysis and in most cases can be used directly for downstream applications. The reagents included are primary-amine free allowing not only a selective and effective solubilization of membrane proteins but also direct compatibility with NHS-ester labeling chemistry, protein quantitation assays (incl. BCA and modified Lowry), and common phosphatase inhibitors.

The mild, non-denaturing conditions afforded by the M-PEK procedure yield membrane proteins in their non-denatured, functional state making it particularly suitable for a variety of special proteomics applications and assays, such as enzyme activity assays including kinase assays, non-denaturing gel electrophoresis, ELISA assays and SELDI-profiling of integral and membrane associated proteins.

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2. Kit Contents

Each kit contains sufficient reagents to process 20 samples of $\sim 3\text{-}5 \times 10^6$ tissue culture cells or 25-50 mg tissue respectively.

- Wash Buffer 1 x 100 ml
- Extraction Buffer I 1 x 40 ml
- Extraction Buffer II 1 x 20 ml
- Protease Inhibitor Cocktail 1 x 450 μ l
- User Protocol

3. Storage

- Wash Buffer
Store at + 4°C.
- Extraction Buffers I & II
The Extraction Buffers I and II can be stored at + 4 °C for up to 6 month.
For prolonged storage, freeze the buffers in convenient aliquots at - 20°C. Before extraction, buffers must be thawed at room temperature (RT). After thawing, mix components by gently shaking or vortexing. Avoid repeated freezing and thawing!
- Protease Inhibitor Cocktail
The Protease Inhibitor Cocktail is supplied in DMSO and can be stored at 4°C up to 6 months. For prolonged storage, freeze the cocktail in convenient aliquots at - 20°C. During the sample preparation procedure it must be kept at RT to prevent freezing of DMSO.

4. Samples

The ProteoExtract™ Native Membrane Protein Extraction Kit is designed for the isolation of native membrane proteins from a broad range of mammalian samples, including:

- Adherent tissue culture cells
- Suspension grown tissue culture cells
- Frozen cell pellets
- Tissues

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5. Kit Components Needed for One Extraction

The amount of buffer required for each extraction is dependent upon the amount of starting cell material. Tables 1a and 1b list the sample to buffer ratio recommended for commonly used cell culture sizes (flasks & dishes) and cell pellets or tissue.

Table 1a: Buffer volumes necessary for one M-PEK extraction of adherent tissue culture cells

| Cell type | Adherent tissue culture cells | | | | | |
|-----------------------------|-------------------------------|------------------------|-------------------------|-------------------|--------------------|--------------------|
| Flask / Dish Size | T ₂₅ -flask | T ₇₅ -flask | T ₁₇₅ -flask | 35 mm | 60 mm | 100 mm |
| Wash Buffer | 2 x 2 ml | 2 x 5 ml | 2 x 10 ml | 2 x 0.8 ml | 2 x 2 ml | 2 x 6 ml |
| Extraction Buffer I | 2 ml | 6 ml | 14 ml | 0.8 ml | 2 ml | 6 ml |
| Extraction Buffer II | 1 ml | 3 ml | 7 ml | 0.4 ml | 1 ml | 3 ml |
| Protease Inhibitor Cocktail | 10 µl per fraction | 30 µl per fraction | 60 µl per fraction | 4 µl per fraction | 10 µl per fraction | 30 µl per fraction |

Table 1b: Buffer volumes necessary for one M-PEK extraction using suspension grown cells, frozen cell pellets or tissue.

| Cell type | Suspension grown cells/ frozen cell pellet | Suspension grown cells/ frozen cell pellet | Fragmented Tissue* | Fragmented Tissue* |
|-----------------------------|---|---|-----------------------|-----------------------|
| Cell Amount | 3 - 5 x 10 ⁶ cells | 1 - 2 x 10 ⁷ cells | 25 - 50 mg | 100 - 200 mg |
| Wash Buffer | 2 x 2 ml | 2 x 8 ml | 2 x 2 ml | 2 x 8 ml |
| Extraction Buffer I | 2 ml | 8 ml | 2 ml | 8 ml |
| Extraction Buffer II | 1 ml | 4 ml | 1 ml | 4 ml |
| Protease Inhibitor Cocktail | 10 µl per fraction | 40 µl per fraction | 10 µl per fraction | 40 µl per fraction |

6. Materials Required but not Provided

- Platform mixer e.g., IKA Vibramax (when extracting adherent tissue culture cells)
- Cell culture equipment, media etc. for cell growth (e. g., RPMI, DMEM).
- Micropipettes and tips, 10 µl, 200 µl and 1 ml size (e.g., Eppendorf, Gilson or equivalent)
- Cooled centrifuge and rotor for 50 or 15 ml tube size (Eppendorf, Heraeus, Nalgene, etc.)
- Cooled micro centrifuge and rotor up to 18,000 x g for 2 ml tube size (e.g. Eppendorf)
- Thermo mixer or rolling facility (e.g., Eppendorf)
- Homogenizer (e.g. potter or Dounce) or mortar.

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7. Protocols

7. 1. Extraction of Membrane Proteins from Adherent Tissue Culture Cells

The M-PEK protocol for adherent tissue culture cells is scaled for the extraction of cell monolayers from T₂₅-culture flasks (3-5 x 10⁶ cells). As a guideline to estimate the amount of starting material, roughly 0.4-2 mg membrane proteins are extracted from osteosarcoma or liver carcinoma cells, although different tissue culture cells may yield considerably different amounts of protein. Please refer to Table 1a to calculate the sample to buffer ratio needed for your actual cell amount used. For optimal cell adherence and results it is recommended to use live cells that are at approximately 80 % confluent.

1. Ensure that buffers are thawed and mixed well by vortexing. During the extraction procedure keep Wash and Extraction Buffers I and II on ice and Protease Inhibitor Cocktail at room temperature.
2. Carefully remove the growth medium without disturbing the cell monolayer.
3. Wash cells by carefully overlaying the cell monolayer with 2 ml ice cold Wash Buffer. Gently swirl the cell culture flask(s) to cover cells entirely.
4. Aspirate Wash Buffer completely without disturbing the cell monolayer.
5. Repeat washing steps 3 and 4 to remove contaminating media components. (If cells detach, transfer the cell suspension in an appropriate centrifuge tube and continue with step 7.2.4, of the protocol for suspension grown cells/cell pellets).
6. Add 10 µl Protease Inhibitor Cocktail to the wall of the cell container and immediately add 2 ml ice cold Extraction Buffer I. Carefully mix the components by swirling the flask without disturbing the monolayer. Spread the buffer until all cells are covered. Incubate for 10 min at +4 °C under gentle agitation (if cells detach, transfer the cell suspension after incubation in a appropriate centrifuge tube and continue with step 7.2.7).
7. Discard the supernatant (enriched in "soluble" proteins) or, alternatively, transfer it into a sample tube using a pipette without disturbing the cell layer. Make sure that all liquid is removed. Keep the fraction on ice, should subsequent analysis be required.
8. Add 5 µl Protease Inhibitor Cocktail to the wall of the cell container and immediately add 1 ml ice cold Extraction Buffer II. Carefully mix the components by swirling the flask without disturbing the monolayer and spread the buffer until all cells are covered. Incubate for 30 min at +4 °C under gentle agitation (if cells detach, transfer the cell suspension after incubation in a appropriate centrifuge tube and continue with step 7.2.10).
9. Transfer the supernatant (**membrane fraction** enriched in integral membrane and membrane associated" proteins) into a sample tube using a pipette without disturbing the cell debris.

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Note: For same day usage, store the extracts on ice. For long-term storage, prepare aliquots of convenient size (e. g. 100 µl) and store at or below -20°C .

7.2. Extraction of Membrane Proteins from Suspension Grown Tissue Culture Cells and frozen cell pellets

The protocol for extraction of membrane proteins from suspension grown tissue culture cells is optimized for the extraction of $3\text{-}5 \times 10^6$ cells (corresponding to roughly 25-50 mg wet weight). As a guideline to estimate the amount of starting material, roughly 0.4 mg membrane proteins are extracted from 4×10^6 human embryonic kidney cells. Different cell types may yield considerably different amounts of protein. Please refer to Table 1b to calculate the sample to buffer ratio needed, for the actual cell amount used.

For frozen cell pellets, wash cells with a suitable buffer (such as the Wash Buffer included or PBS) prior to snap-freezing (see steps 1-5). When washed cell pellets are available start with step 7.2.6.

1. Make sure that buffers are thawed and mixed well by vortexing. During the extraction procedure keep Wash and Extraction Buffers I and II on ice, and the Protease Inhibitor Cocktail at room temperature.
2. Transfer the cell culture into (an) appropriate centrifuge tube(s) and centrifuge at $100\text{-}300 \times g$ and $+4^{\circ}\text{C}$ for 10 min. Remove and discard supernatant by aspiration.
3. Wash the pellet with 2 ml ice cold Wash Buffer. Release the cell pellet by gently flicking the tube and invert the tube(s) carefully until no cell clumps are remaining.
4. Centrifuge cells at $100\text{-}300 \times g$ and $+4^{\circ}\text{C}$ for 10 min. Carefully remove the supernatant without disturbing the pellet and discard.
5. Repeat wash steps 3 and 4. After the final washing step, take care to completely remove all buffer.

Note: It is possible to snap-freeze cell pellets in liquid nitrogen at this point and to store the pellets at or below -70°C .

6. Add 10 µl Protease Inhibitor Cocktail to the wall of the tube and immediately add 2 ml ice-cold Extraction Buffer I to the cell pellet (which might be frozen). Mix and resuspend the cell pellet carefully but completely using a pipette. Incubate for 10 min at $+4^{\circ}\text{C}$ under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
7. Sediment insoluble material at $16,000 \times g$ and $+4^{\circ}\text{C}$ for 15 min.
8. Discard the supernatant (enriched in "soluble" proteins) or, alternatively, transfer it into a sample tube using a pipette without disturbing the cell layer. Make sure that all liquid is removed. Keep the fraction on ice, should subsequent analysis be required.

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9. Add 5 μ l Protease Inhibitor Cocktail to the wall of the tube and immediately add 1 ml ice-cold Extraction Buffer II to the cell pellet. Mix and resuspend the cell pellet carefully but completely using a pipette. Incubate for 30 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
10. Centrifuge insoluble material at 16,000 x g and +4 °C for 15 min.
11. Transfer the supernatant (**membrane fraction** enriched in integral membrane and membrane-associated proteins) completely into a sample tube using a pipette without disturbing the debris pellet.

Note: For same day usage, store the extracts on ice. For long-term storage, prepare aliquots of convenient size (e. g. 100 μ l) and store at or below -20° C.

7.3. Extraction of Membrane Proteins from Tissue

The protocol applies to 25 - 50 mg of fresh or snap frozen tissue. In case the amount of starting material is not limiting, it may be beneficial to use 100-400 mg in order to minimize losses that might occur during tissue homogenization. In the latter case, fewer than 20 samples total can be extracted with the provided reagents. As guideline to estimate the amount of starting material, roughly 2 mg membrane proteins are extracted from 35 mg bovine liver. Different tissue may yield considerably different amounts of protein. Please refer to Table 1b to calculate the sample to buffer ratio needed.

Following dissection of the tissue of interest, unwanted parts should quickly be removed, the tissue ideally sliced into ~2 mm³ pieces, and washed with a suitable buffer (such as the Wash Buffer included or. PBS) prior to snap-freezing (see steps 1-5). When using washed, snap-frozen tissue, start with step 7.3.6.

In case of large frozen tissue chunks, fragment the frozen tissue by transferring it into a clean disposable plastic bag, laying the bag onto a solid cold surface and covering it with a towel, and then simply hitting it with a few strokes with a solid object (such as a hammer). The frozen fragments can be transferred to a pre-weighted tube

Fresh Tissue

1. Make sure that buffers are thawed and mixed well by vortexing. During the extraction procedure keep Extraction Buffers I and II on ice and Protease Inhibitor Cocktail at room temperature.
2. Following dissection of the tissue of interest, quickly remove at +4°C unwanted materials such as connective tissue, fat, blood vessels etc. Ideally, the tissue should be sliced in ~2 mm³ pieces, and transfer into a tube containing 2 ml ice cold Wash Buffer.
3. Gently flick the tube a few times to rinse off blood cells and other loosely attached material.

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4. Centrifuge tissue pieces at 100 x g and +4 °C for 2 min. Carefully remove the supernatant without disturbing the pellet and discard.
5. Add another 2 ml ice cold Wash Buffer and repeat washing steps 3 and 4. After the final washing step, take care to completely remove all Buffer.

Note: It is possible to snap-freeze tissue in liquid nitrogen at this point and to store the pellets at or below –70°C

Frozen Tissue

6. Transfer the appropriate amount of tissue pieces (which might be frozen) into a pre-cooled homogenizer (ideally a glass potter or Dounce). Add 10 µl Protease Inhibitor Cocktail to the wall of the homogenizer and immediately add 2 ml ice cold Extraction Buffer I to the tissue pieces.
7. Carefully homogenize the tissue, using as few strokes as possible with a cold tight fitting pestle (e.g. ~10 strokes in case of bovine liver) until tissue pieces are no longer visible. The number of strokes required will depend on the structural nature of the tissue used. In case optimization should be required, efficiency can be monitored using a phase contrast microscope. Aim for small cell clumps, rather than subcellular fragmentation.
8. Transfer the homogenate as complete as possible into a pre-cooled tube and incubate for 10 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
9. Centrifuge insoluble material at 16,000 x g and +4 °C for 15 min.
10. Discard the supernatant (enriched in “soluble” proteins) or alternatively transfer it into a sample tube using a pipette without disturbing the cell layer. Make sure that all liquid is removed. Keep the fraction on ice, should subsequent analysis be required.
11. Add 5 µl Protease Inhibitor Cocktail to the wall of the tube and immediately add 1 ml ice cold Extraction Buffer II to the cell pellet. Mix and resuspend the cell pellet carefully but completely using a pipette. Incubate for 30 min at +4 °C under gentle agitation. A rotary shaker is recommended to avoid formation of cell clumps.
12. Centrifuge insoluble material at 16,000 x g and +4 °C for 15 min.
13. Transfer the supernatant (**membrane fraction** enriched in integral membrane and membrane associated” proteins) completely into a sample tube using a pipette without disturbing the debris pellet.

Note: For same day usage, store the extracts on ice. For long-term storage, prepare aliquots of convenient size (e. g. 100 µl) and store at or below –20° C.

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8. Technical Appendix

Problem

No compact pellet is formed upon centrifugation.

Detachment of the cell layer from the support occurs during washing or extraction of adherent tissue culture cells.

How do I determine the protein concentration of the membrane protein extract?

How do I prepare the M-PEK fraction for one-dimensional SDS-PAGE?

How can I concentrate the M-PEK extracted proteins?

Answer

Certain types of cells might not form compact pellets at 100 x g. In these cases increase acceleration to ~ 300 x g.

When adherent cells detach early during extracting, transfer the resulting suspension into a microcentrifuge tube and continue with the respective step of the protocol given in section 7.2 for suspension-grown cells. This does normally not affect the quality of your result.

The components in the extraction buffers are directly compatible with common protein assays, including BCA-assay (BCA Protein Assay Kit, Novagen, 71286-3), as well as with specific protein assays such as "Non-interfering Protein Assay Kit"; Calbiochem, Cat. No. 488250.

M-PEK fraction can directly be analyzed by one-dimensional SDS-PAGE: Dilute the sample with an equal volume of 2 x SDS-PAGE sample buffer (not provided: e. g. 125 mM Tris/HCl, pH 6.8; 10 % (w/v) SDS; 30 % (v/v) Glycerol; 100 mM DTT; 0.002 % (w/v) bromophenol blue) and heat to + 95 °C for 5 min.

It is possible to reduce the volume used of Extraction Buffer II, however this will decrease the yield. If the protein concentration in the extracted fraction is not sufficient for your purpose, we recommend the use of the ProteoExtract™ Protein Precipitation Kit (Cat. No. 539180) to concentrate the proteins for use in denaturing applications. Dissolve pellet in a buffer suitable for your further downstream applications. Alternatively, spin-filter concentrators (e.g. Centricon) can be used.

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9. Related Products

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| ProteoExtract™ Kits | |
| Complete Bacterial Proteome Extraction Kit | 539770 |
| Complete Yeast Proteome Extraction Kit | 539775 |
| Complete Mammalian Proteome Extraction Kit | 539779 |
| Partial Bacterial Proteome Extraction Kit | 539780 |
| Partial Yeast Proteome Extraction Kit | 539785 |
| Partial Mammalian Proteome Extraction Kit | 539789 |
| Subcellular Proteome Extraction Kit | 539790 |
| | |
| Albumin Removal Kit | 122640 |
| Albumin/IgG Removal Kit | 122642 |
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| Protein Precipitation Kit | 539180 |
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| All-in-One Trypsin Digestion Kit | 650212 |
| Phosphopeptide Capture Kit | 525250 |
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| SDS Sample Buffer | 70607-3 |
| BCA Protein Assay Kit | 71286-3 |
| Non-Interfering Protein Assay Kit | 488250 |

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