# Efficient and convenient enrichment of multispanning membrane proteins for proteomic studies

## High-quality isolation of peripheral and integral membrane proteins

Membrane proteins play crucial roles in many cellular functions such as signal transduction, cell integrity, intracellular and extracellular transport, and cell-to-cell communication. Membrane proteins are classified based on their level of interaction with the phospholipid bilayer. Peripheral membrane proteins associate with the membrane surface and are usually transiently immobilized on the surface by noncovalent interactions. In contrast, integral membrane proteins, also known as transmembrane proteins, span across the phospholipid bilayer with one or more segments composed of hydrophobic residues that interact with the fatty-acyl groups of the membrane phospholipids. Transmembrane proteins are polytopic proteins that tend to aggregate and precipitate in aqueous solutions, making their isolation and extraction for proteomic studies difficult. Traditional methods for isolating these membrane proteins are tedious and time-consuming, requiring ultracentrifugation, mechanical disruption, and expensive equipment. Additional drawbacks of these methods include poor solubilization, incompatibility with downstream applications, and disruption of membrane protein complexes.

The Thermo Scientific™ Mem-PER™ Plus Membrane Protein Extraction Kit (Cat. No. 89842) enables small-scale solubilization and enrichment of integral and membrane-associated proteins from cultured cells and tissues using a simple, reagent-based procedure and a benchtop microcentrifuge. This mild detergent–based, selective extraction protocol eliminates the inconveniences of traditional membrane protein isolation procedures and allows for better reproducibility and higher throughput (Figure 1).

Here we present data to demonstrate the performance of the Mem-PER Plus kit with a sequential detergent extraction method to isolate specific multispanning membrane proteins (≥2 transmembrane domains) for downstream analysis by western blotting, immunoprecipitation, and mass spectrometry.

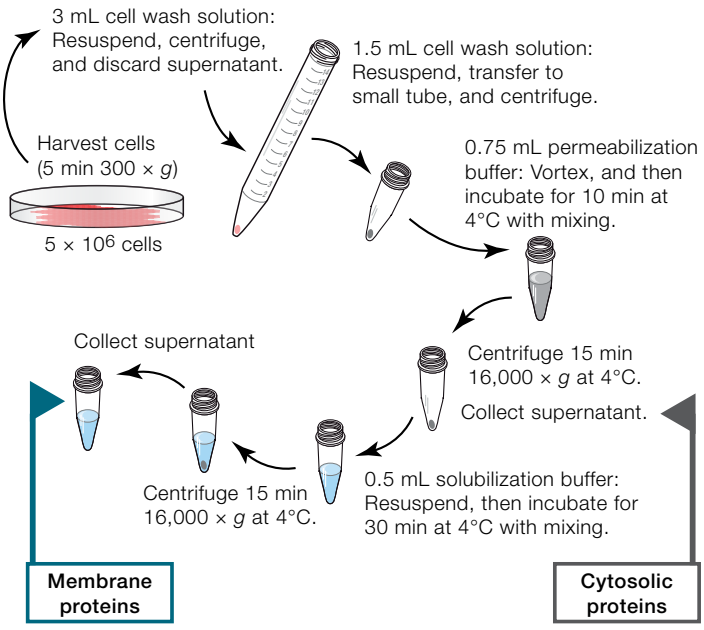


**Figure 1. Protocol summary for the Mem-PER Plus Membrane Protein Extraction Kit.**

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# Results

## Extraction of multispanning membrane proteins

We evaluated the efficiencies of membrane protein extraction from various mammalian cell lines using several commercially available kits that use different extraction methods. These include a detergent-based method using the Mem-PER Plus kit, a non–detergent-based method using EMD Millipore ProteoExtract™ Transmembrane Protein Extraction Kits (TM-PEK A and B), and a sodium carbonate–based method using the Bio-Rad ReadyPrep™ Protein Extraction Kit (Membrane II). We compared each of the resulting cytoplasmic and membrane protein fractions by probing for two integral membrane proteins containing multiple transmembrane segments by western blotting (Figure 2).

The mild detergent–based method of the Mem-PER Plus kit provided higher extraction efficiency of the multispanning membrane proteins than the other methods tested. In addition, there was higher purity of the membrane and cytosolic protein fractions—the enzymes were enriched in the membrane fraction, with less cross-contamination into the cytosolic fraction.

## Increasing the extraction efficiency of multispanning membrane proteins

To further increase the extraction efficiency of multispanning membrane proteins using the Mem-PER Plus Membrane Protein Extraction Kit, an isotonic solubilization buffer was utilized. HEK293 cells were lysed using the cell permeabilization buffer included in the Mem-PER Plus kit and subsequently solubilized with either the hypotonic solubilization buffer included in the kit or an altered isotonic solubilization buffer (150 mM NaCl). As seen in Figure 3, increasing the salt content in the solubilization buffer increased the extraction efficiency for both the $Na^+/K^+$-ATPase and ADP–ATP translocase 3 proteins. Keeping the environment balanced during solubilization allowed for better extraction of multispanning membrane proteins.

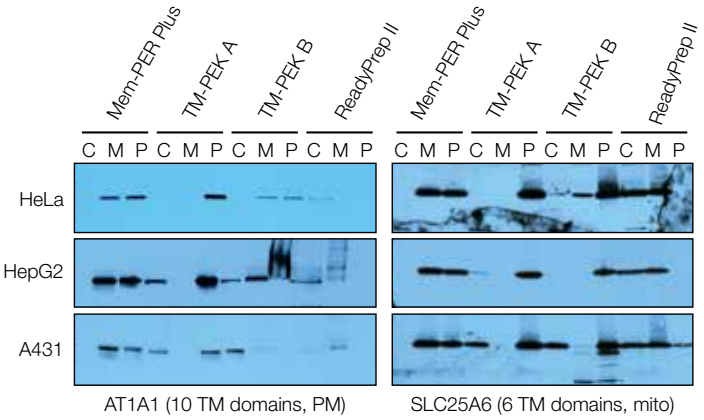


**Figure 2. Efficiencies of membrane protein extraction from various mammalian cell lines.** Membrane proteins were isolated from the mammalian cell lines HeLa, HepG2, and A431, using four commercial extraction kits. Membrane and cytosolic fractions were separated by SDS-PAGE, transferred to nitrocellulose membranes, and evaluated by chemiluminescent western blotting for the presence of the AT1A1 and SLC25A6 proteins. C = cytoplasmic fraction, M = membrane fraction, P = insoluble fraction, TM = transmembrane, PM = plasma membrane.

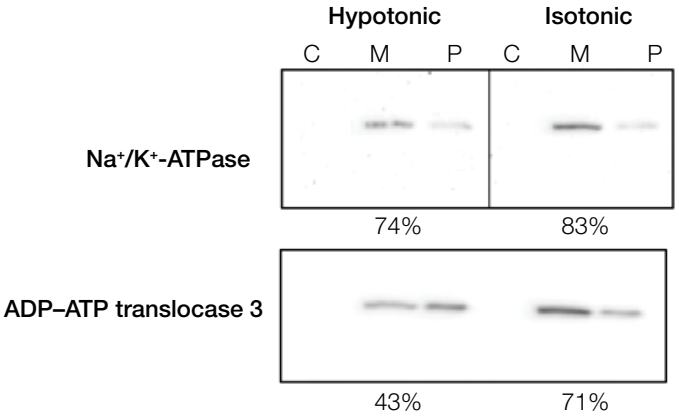


**Figure 3. Increased efficiency of extraction of multispanning membrane proteins.** Membrane proteins were isolated from 5 x $10^6$ cultured cells following the Mem-PER Plus Membrane Protein Extraction Kit protocol, using either the solubilization buffer provided or an altered isotonic solubilization buffer. Membrane fractions were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against the $Na^+/K^+$-ATPase or ADP–ATP translocase 3 proteins and HRP-tagged secondary antibodies. Blots were developed with Thermo Scientific™ SuperSignal™ West Dura Substrate. C = cytoplasmic fraction, M = membrane fraction, P = insoluble fraction.

**Table 1. Analysis of peptide digests from equal volumes of whole cell and membrane fractions from HEK292 cells, on a Velos Pro Orbitrap mass spectrometer.**

| Reagent | M-PER | Mem-PER Plus | TM-PEK A | TM-PEK B | ReadyPrep II |
|---|---|---|---|---|---|
| Total number of proteins identified | 451 | 425 | 342 | 341 | 295 |
| Integral membrane proteins identified | 30 | 90 | 14 | 34 | 46 |
| Integral membrane proteins identified, percent of total | 6.7 | 21.2 | 4.1 | 10.0 | 15.6 |

## Mass spectrometry analysis of membrane fractions

We evaluated the ability of several commercially available kits to facilitate proteome analysis of global membrane proteins by mass spectrometry. Membrane fractions from a mammalian cell line, HEK292, were isolated using the Thermo Scientific™ M-PER™ Mammalian Protein Extraction Reagent (for total soluble protein extraction), the Mem-PER Plus Membrane Protein Extraction Kit, ProteoExtract Transmembrane Protein Extraction Kits (TM-PEK A and B), and ReadyPrep Protein Extraction Kit (Membrane II) (Table 1). The membrane fractions were digested with trypsin and analyzed on a Thermo Scientific™ Velos Pro™ Orbitrap™ mass spectrometer. The total numbers of membrane proteins and integral proteins identified were compared.

As seen in Table 1, the Mem-PER Plus kit's sequential detergent extraction increased the enrichment of integral membrane proteins compared to that obtained by the non–detergent-based methods. In addition, the Mem-PER Plus sequential detergent method yielded higher sequence coverage of several integral membrane proteins containing 1–12 transmembrane domains, compared to other commercial reagents (Figure 4).

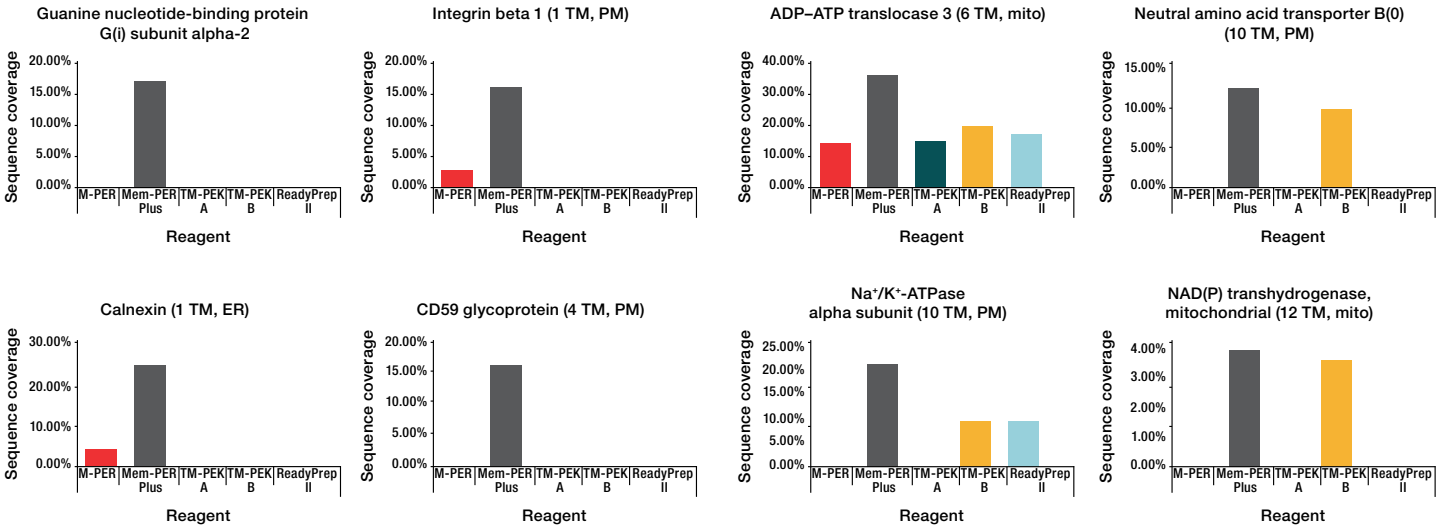


**Figure 4. Sequence coverage obtained for several membrane proteins (containing 1 to 12 transmembrane domains) extracted using commercially available kits.** TM = transmembrane, PM = plasma membrane, ER = endoplasmic reticulum.

### Immunoprecipitation of membrane complexes

One common technique used in the field of proteomics is immunoprecipitation. This application allows the identification and study of protein complexes. To evaluate native protein complexes, nondenaturing extraction buffers must be used to ensure that the protein complexes stay intact for analysis. To evaluate the compatibility of the Mem-PER Plus kit with immunoprecipitation, membrane fractions of the $Na^+/K^+$-ATPase transport complex were immunoprecipitated using an antibody against the beta-1 subunit of the complex. The immunoprecipitated proteins were digested in solution using the Thermo Scientific™ Pierce™ In-Solution Tryptic Digestion Kit and analyzed by LC-MS/MS using a Thermo Scientific™ Orbitrap Fusion™ mass spectrometer. Peptides from both the alpha (α) and beta (β) subunits were identified, indicating that the Mem-PER Plus Membrane Extraction Kit did not disrupt the $Na^+/K^+$-ATPase membrane complex (Table 2).

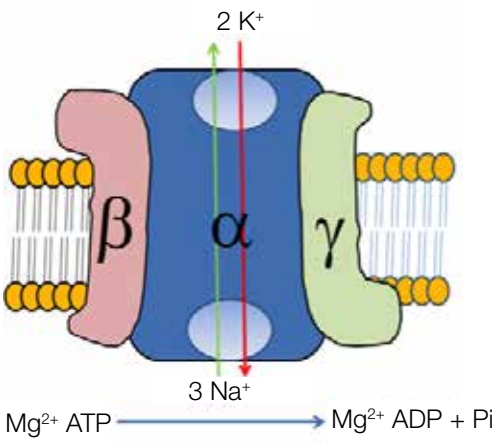


**Figure 5. The $Na^+/K^+$-ATPase transport complex consists of a catalytic subunit (α) and a regulatory subunit (β), which are both essential for function, as well as an adaptor/regulatory FXYD protein (γ subunit).** This complex pumps $Na^+$ out of the cell and $K^+$ into the cell as a function of ATP hydrolysis [1] .

**Table 2. Identification of peptides by LC-MS/MS from immunoprecipitated $Na^+/K^+$-ATPase transport complex extracted with Mem-PER Plus Kit.** Samples were processed by in-solution digestion according to the kit protocol, and analyzed on the Orbitrap Fusion mass spectrometer.

| Protein | Peptide sequence | Xcorr value |
|---|---|---|
| $Na^+/K^+$-transporting ATPase subunit beta-1 | VAPPGLTQIPQIQK | 2.67 |
| | VGNVEYFGLGNSPGFPLQYYPYYGK | 4.81 |
| | AYGENIGYSEK | 2.52 |
| | SYEAYVLNIVR | 3.17 |
| $Na^+/K^+$-transporting ATPase subunit alpha-1 | LSLDELHR | 2.20 |
| | QGAIVAVTGDGVNDSPALK | 4.03 |
| | NIAFFSTNCVEGTAR | 3.29 |
| | SPDFTNENPLETR | 4.46 |
| | TSATWLALSR | 3.18 |
| | AVFQANQENLPILK | 2.01 |

## Conclusions

The analysis of multispanning membrane proteins represents a significant technical challenge in the field of proteomics. The Mem-PER Plus Membrane Protein Extraction Kit provides a simple and efficient way to study difficult-to-isolate membrane proteins. As demonstrated here, the Mem-PER Plus Membrane Protein Extraction Kit is compatible with several downstream proteomic analyses. The extraction kit is effective in isolating integral transmembrane proteins with high efficiency while providing a nondenaturing environment in which native protein complexes can be captured by immunoprecipitation.

**References**

1. Tokhtaeva E et al. (2012) Subunit isoform selectivity in assembly of Na,K-ATPase α-β heterodimers. *J Bio Chem* 287:26115-26125.
2. Kaboord B, Smith S, Patel B et al. (2015) Enrichment of low-abundant protein targets by immunoprecipitation upstream of mass spectrometry. In "Proteomic Profiling," *Methods in Molecular Biology* 1295, 135-151. Springer Protocols, Posch, A., Ed.

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## Methods

### Membrane protein extraction

Membrane proteins were extracted from six cell lines and three tissue types using commercially available membrane protein extraction reagents: the Mem-PER Plus Membrane Protein Extraction Kit, the EMD Millipore ProteoExtract Transmembrane Protein Extraction Kits, and the Bio-Rad ReadyPrep Protein Extraction Kit (Membrane II). Membrane proteins were extracted from 5 x $10^6$ cultured cells or 50 mg of tissue according to protocols outlined in the kits. Protein in each fraction was estimated using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Cat. No. 23225), and lysates were stored at –80°C before use.

### Western blotting

Normalized samples were separated on a 4–20% Tris-glycine gel and transferred to nitrocellulose membranes using the Thermo Scientific™ Pierce™ G2 Fast Blotter. Membranes were blocked with a 1:1 solution of Thermo Scientific™ Blocker™ BSA (10X) in TBS (Cat. No. 37520) and Pierce™ 20X TBS Tween™ 20 Buffer (Cat. No. 28360), and probed with one of the following primary antibodies for 1 hour at room temperature or overnight at 4ºC: Invitrogen™ ADCY2 antibody (Cat. No. PA5-12328), SLC25A6 antibody (Cat. No. PA5-29199), Na/K-ATPase 1 antibody. Membranes were incubated with Invitrogen™ HRP-conjugated goat anti-rabbit secondary antibody for 30 minutes at room temperature (Cat. No. 32260), washed, and incubated for 5 minutes in SuperSignal West Dura Extended Duration Substrate (Cat. No. 34075). Blots were then exposed to film or imaged on the Thermo Scientific™ myECL™ Imager.

### Immunoprecipitation

HeLa cell membrane fractions generated using the Mem-PER Plus kit (125 µg) were immunoprecipitated with Thermo Scientific™ AT1B1 antibody (5 µg) (Cat. No. MA3-930) and the Thermo Scientific™ Pierce™ MS-Compatible Magnetic IP Kit, protein A/G (Cat. No. 90409) following the manufacturer's instructions.

### Mass spectrometry

HEK293 cell membrane fractions were prepared with commercially available reagents as listed in the membrane protein extraction methods above. Samples were then processed using methanol/chloroform/water extraction, and the cleaned samples were reduced, alkylated, and subjected to trypsin digestion [2]. The samples were analyzed by LC-MS/MS using the Velos Pro Orbitrap mass spectrometer. Immunoprecipitated samples were processed using the In-Solution Tryptic Digestion Kit (Cat. No. 89895) and analyzed by LC-MS/MS using an Orbitrap Fusion mass spectrometer.

### Data analysis

MS data were analyzed with Thermo Scientific™ Proteome Discoverer 1.4 software, using a custom human/mouse/rabbit protein database with the SEQUEST™ algorithm (<2 missed cleavages per peptide, false discovery rate (FDR) <0.1). Protein accessions for membrane fractions of HEK293 cells were annotated for gene ontology using QuickGO GO:0016021 integral component of membrane.

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