INSTRUCTIONS

2-D Sample Prep for Membrane Proteins

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Number	Description
89864	**2-D Sample Prep for Membrane Proteins,** contains sufficient components to prepare and process 25 membrane protein extracts for 2-D gel analysis

Kit Contents:

Mem-PER® Eukaryotic Membrane Protein Extraction Kit: contains sufficient reagents for extracting 25 cell pellet fractions with a packed cell weight of 10-15 mg each or 25 tissue samples weighing 20 mg each

Mem-PER® Cell Lysis Reagent, 5 ml, store at room temperature

Mem-PER® Buffer, 12.5 ml, store at 4°C

Mem-PER® Membrane Protein Solubilization Reagent, 20 ml, store at 4°C

2-D PAGEprep™ Protein Clean-up and Enrichment Kit: contains sufficient components for purifying 25 aliquots of membrane protein extract

2-D PAGEprep™ Protein Binding Resin, 0.5 ml, store at 4°C

DMSO, dimethyl sulfoxide, 50 ml, store at room temperature

Elution Buffer, 1.25 ml, store at 4°C

Spin cups, 25 cups

Collection tubes, 50 tubes

2-D Sample Buffer for Membrane Proteins: contains sufficient reagents for preparing 30 ml of 2-D Sample Buffer containing 7 M urea, 2 M thiourea, and 4% CHAPS

2-D Sample Buffer for Membrane Proteins Component A, 18 ml, store at 4°C

2-D Sample Buffer for Membrane Proteins Component B, 16.5 g, store at room temperature

Protein Desalting Spin Columns, 25 columns, each column containing ~700 µl of desalting slurry buffered in 10 mM Tris•HCl, pH 7.5 containing 0.02% sodium azide

Storage: Upon receipt store individual components as described above. Product is shipped at ambient temperature.

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Introduction

The 2-D Sample Prep for Membrane Proteins kit provides a fast, convenient and reliable method for membrane protein extraction and 2-D sample preparation. Membrane proteins are first extracted from cultured mammalian or yeast cells or from mammalian tissue using a mild detergent-based protocol. The prepared membrane extract is subsequently prepared for 2-D gel electrophoresis using a two-step process (see Additional Information Section for a schematic). The extract is first concentrated with 2-D PAGEprep™ Resin, a modified form of diatomaceous earth that selectively binds to proteins while detergents and other contaminants are removed by washing. The resulting eluate is then desalted/buffer exchanged, transferring the proteins into 2-D Sample Buffer to be electrophoresed on an immobilized pH gradient (IPG) strip. Multiple chaotropes present in the 2-D Sample Buffer improve protein solubility [1,2] and result in high-resolution 2-D gels. In addition, prepared membrane protein extract is concentrated up to six-fold, improving detection of proteins in low abundance.

Cell fractionation and sample preparation are important for optimal 2-D gel analysis; however, both procedures can be difficult when working with membrane proteins. Traditional methods for membrane protein isolation are time-consuming and require expensive ultracentrifugation equipment. Furthermore, concentration/clean-up of these hydrophobic proteins, most commonly performed using precipitation methods, involves lengthy incubation and difficult resolubilization. The 2-D Sample Prep for Membrane Proteins kit overcomes these challenges. Specifically, membrane extraction is performed with a bench-top microcentrifuge and, unlike in precipitation methods, the proteins are maintained in solution throughout the entire clean-up process. Approximately 30-100 µg of membrane proteins from cells or 50-200 µg of membrane proteins from tissues can be prepared for analysis in less than 90 minutes.

Important Product Information

- Although this sample prep kit provides reagents for the extraction of both membrane and hydrophilic proteins, it is intended for purification of the membrane fraction only. The extracted hydrophilic fraction obtained is compatible with 2-D gel electrophoresis and therefore does not require additional processing before 2-D gel analysis. Please note that this kit does NOT provide enough 2-D Sample Buffer for applications with the hydrophilic fraction.
- For optimal results, include protease inhibitors (e.g., Product No. 78415) in the Mem-PER® Reagents. Do not use protease inhibitors containing EDTA because it will reduce the binding of protein to the 2-D PAGEprep™ Resin.
- Do not exceed 10 samples in one extraction procedure because rapid resolubilization of the hydrophobic phase into the hydrophilic phase occurs at room temperature.

Additional Materials Required

- Variable-speed bench-top microcentrifuge
- Waterbath (37°C and 60°C)
- 1.5-2.0 ml microcentrifuge collection tubes (for desalting/buffer exchange)
- Reducing agent such as DTT (Product No. 20290)
- Carrier ampholytes
- Bromophenol Blue (optional)
- 405-600 µm acid-washed glass beads (for yeast only)
- Vortex with a multi-tube holder (for yeast only)
- 2 ml Dounce Tissue Grinder, such as Kontes or Wheaton Tenbroeck (soft tissues)
- Hand-held homogenizer for 0.5 to 1.5 ml samples, such as Brinkmann Polytron PT-1200CL (hard tissues)
- Tris Buffered Saline (TBS) containing protease inhibitors (tissue extraction only)

Procedure for 2-D Sample Prep for Membrane Proteins

A. Preparation of Membrane Extract from Different Sample Types

Following are four protocols: Protocol 1 is for the extraction of membrane proteins from mammalian cells; Protocol 2 is for the extraction of membrane proteins from yeast cells: Protocol 3 is for the extraction of membrane proteins from soft tissues; and Protocol 4 is for the extraction of membrane proteins from hard tissues.

Protocol 1: Mammalian Cells

1. Isolate 10 mg of mammalian cells by centrifuging harvested cell suspensions in 1.5 ml microcentrifuge tubes at 850 x *g* for 2 minutes. Carefully remove and discard the supernatant.
2. Add 150 µl of Mem-PER® Cell Lysis Reagent to the cell pellet and pipette up and down to obtain a homogeneous cell suspension. Incubate suspension 10 minutes at room temperature with occasional vortexing.

   Note: White flocculent debris appears upon addition of Mem-PER® Cell Lysis Reagent.

   Note: To check cell lysis efficiency, spot 5 µl of cell lysate onto a glass slide, add coverslip and view using a microscope. Compare with 5 µl of the same number of intact cells in 150 µl of phosphate-buffered saline (PBS) or Tris-buffered saline (TBS).
3. Place lysed cells on ice.
4. In a new tube, add 2 parts Mem-PER® Membrane Protein Solubilization Reagent to 1 part Mem-PER® Buffer, making sufficient mixture to add 450 µl to each sample (e.g., for 10 extractions, combine 3.33 ml of Mem-PER® Membrane Protein Solubilization Reagent with 1.67 ml of Mem-PER® Buffer).

   Note: Keep the prepared Mem-PER® Membrane Protein Solubilization mixture on ice at all times.
5. Add 450 µl of the solubilization mixture prepared in the previous step to each tube of lysed cells and vortex. Proceed to Section B: Phase Separation.

Protocol 2: Yeast Cells

1. Harvest yeast cells in the exponential growth phase with an OD_{600}= 0.3-1.7. Use approximately 15 mg of wet cell paste per sample.
2. Pellet cells by pulse centrifugation and carefully remove the supernatant.
3. Resuspend cells in 80 µl of Mem-PER® Cell Lysis Reagent by pipetting up and down to obtain a homogeneous cell suspension.
4. Add 150 mg of acid-washed glass beads to the cell suspension and vortex continuously for 15 minutes to lyse cells. Pellet the beads by pulse centrifugation. Transfer suspension into a new microcentrifuge tube and keep on ice.

5. In a new tube, add 2 parts Mem-PER® Membrane Protein Solubilization Reagent to 1 part Mem-PER® Buffer, making sufficient mixture to add 720 µl to each sample (e.g., for 10 extractions, combine 5.33 ml of Mem-PER® Membrane Protein Solubilization Reagent with 2.67 ml of Mem-PER® Buffer).

   Note: Keep the prepared Mem-PER® Membrane Protein Solubilization mixture on ice at all times.

6. Add 720 µl of the prepared solubilization mixture from the previous step into the tube containing the glass beads and briefly vortex to wash. Perform a pulse spin to gather beads.
7. Transfer wash into the tube containing the cell suspension (from step 4). Proceed to Section B: Phase Separation.

Protocol 3: Soft Tissues

1. Place 20 mg of soft tissue in a 1.5 ml microcentrifuge tube. Add 200 µl TBS to tissue, vortex briefly and discard wash.
2. Transfer rinsed tissue to a 2 ml tissue grinder. Add 200 µl TBS to tissue, and homogenize until an even suspension is obtained (approximately 6 to 10 strokes).
3. Transfer homogenate to a new 1.5 ml tube and centrifuge at 1,000 x *g* for 5 minutes at 4°C.
4. Discard the supernatant. Add 150 µl of Mem-PER® Cell Lysis Reagent to the pellet and pipette up and down to obtain an even suspension. Incubate suspension 10 minutes at room temperature with occasional vortexing.

   Note: White flocculent debris appears upon addition of Mem-PER® Cell Lysis Reagent.

5. Place lysed cells on ice.
6. In a new tube, add 2 parts Mem-PER® Membrane Protein Solubilization Reagent to 1 part Mem-PER® Buffer, making sufficient mixture to add 450 µl to each sample (e.g., for 10 extractions, combine 3.33 ml of Mem-PER® Membrane Protein Solubilization Reagent with 1.67 ml of Mem-PER® Buffer).

   Note: Keep the prepared Mem-PER® Membrane Protein Solubilization mixture on ice at all times.

7. Add 450 µl of the solubilization mixture prepared in the previous step to each tube of lysed cells and vortex. Proceed to Section B: Phase Separation.

Protocol 4: Hard Tissues

1. Place 20 mg of hard tissue in a 2.0 ml microcentrifuge tube. Add 500 µl TBS to tissue, vortex briefly and discard wash.
2. Add 500 µl TBS to tissue, and cut the tissue into small pieces with a clean razor blade.
3. Homogenize minced tissue with a hand-held Polytron using a low setting to prevent foaming.
4. Transfer homogenate to a new 1.5 ml tube and centrifuge at 1,000 x *g* for 5 minutes at 4°C.
5. Discard the supernatant. Add 150 µl of Mem-PER® Cell Lysis Reagent to the pellet and pipette up and down to obtain an even suspension. Incubate suspension 10 minutes at room temperature with occasional vortexing.

   Note: White flocculent debris appears upon addition of Mem-PER® Cell Lysis Reagent.

6. Place lysed cells on ice.
7. In a new tube, add 2 parts Mem-PER® Membrane Protein Solubilization Reagent to 1 part Mem-PER® Buffer, making sufficient mixture to add 450 µl to each sample (e.g., for 10 extractions, combine 3.33 ml of Mem-PER® Membrane Protein Solubilization Reagent with 1.67 ml of Mem-PER® Buffer).

   Note: Keep the prepared Mem-PER® Membrane Protein Solubilization mixture on ice at all times.

8. Add 450 µl of the solubilization mixture prepared in the previous step to each tube of lysed cells and vortex. Proceed to Section B: Phase Separation.

B. Phase Separation

1. Incubate tubes containing the cell lysate for 30 minutes on ice, vortexing every 5 minutes.
2. Centrifuge tubes at 10,000 x *g* for 3 minutes at 4°C to remove cell debris. Transfer supernatant to new tubes and incubate for 10 minutes at 37°C.

3. Centrifuge tubes at 10,000 x *g* for 2 minutes at room temperature to partition the hydrophobic fraction (the fraction containing membrane proteins) from the hydrophilic fraction.
4. Carefully remove the hydrophilic phase (top layer) from the hydrophobic protein phase (bottom layer) and save in a new tube. Perform the phase separations as quickly as possible because the interface between the layers slowly disappears at room temperature. Place the separated fractions on ice.

   Note: The majority of membrane protein will be present in the lower viscous phase.
5. Dilute 1 part Mem-PER® Buffer with 3 parts ultrapure water (e.g., for one extraction, combine 30 µl of Mem-PER® Buffer with 90 µl of ultrapure water). Then add 100 µl of this diluted Mem-PER® Buffer to the hydrophobic fraction to reduce viscosity.
6. Store the hydrophobic fraction, which contains the membrane proteins, at -70°C or proceed with Section C.

C. Purification of Prepared Membrane Extract with 2-D PAGEprep™ Resin

For a schematic of Sections C-E see the Additional Information Section.

Note: The addition order of the components is critical. DO NOT vary the procedure.

Note: Set vortex speed to two-thirds of the maximum setting to prevent sample from leaking from the spin cup.

1. Prepare 50% DMSO wash solution for use in Step 8 of this section; mix 750 µl DMSO with 750 µl of ultrapure water, which is sufficient volume to process one sample.
2. Shake and vortex the 2-D PAGEprep™ Resin to evenly disperse the resin. Pipette 20 µl of dispersed resin into spin cup inserted in a collection tube.

   Note: The slurry is viscous and will require using a cut pipette tip to transfer the support from the bottle to the tube.
3. Transfer 75-300 µl of the membrane extract (the hydrophobic fraction) from Section B to the spin cup.

   Note: Generally, 75-300 µl of extract will result in 30-100 µg of protein from cells or 50-200 µg of protein from tissues after processing with 2-D PAGEprep™ Resin. Refer to Table 1 in the Additional Information Section for approximate protein recoveries.
4. Vortex spin cup for 5 seconds to mix. Make sure that the resin is completely resuspended and the solution is homogeneous.
5. Transfer the same volume of 100% DMSO to the spin cup to make a 1:1 ratio with the membrane extract. For example, if processing 100 µl of sample, use 100 µl of 100% DMSO.
6. Vortex spin cup for 5 seconds to mix and incubate for 5 minutes at room temperature with occasional vortexing.
7. Centrifuge spin cup at 2,000 x *g* for 2 minutes. Discard flow-through and blot collection tube on a paper towel. Reinsert spin cup into the same collection tube.

   Note: The resin may appear wet following centrifugation when using large sample volumes. This appearance is caused by the detergent in the membrane extract and is a typical occurrence.
8. Add 500 µl of the 50% DMSO to the resin (prepared in Step 1) and vortex spin cup until a homogeneous suspension is obtained or up to 1 minute.

   Note: The resin may not completely resuspend.
9. Centrifuge spin cup at 2,000 x *g* for 2 minutes. Discard wash and blot collection tube on a paper towel before reinserting collection tube. Reinsert spin cup into the same collection tube. Repeat this wash two additional times for a total of three washes. If the third wash is turbid, perform a fourth wash.
10. Transfer the spin cup to a new collection tube. Add 50 µl of elution buffer to the resin, and vortex spin cup briefly to obtain a homogeneous suspension.
11. Incubate spin cup at 60°C for 5 minutes. Vortex spin cup briefly. Centrifuge spin cup at 2,000 x *g* for 2 minutes. Place the collected eluate on ice. Samples may have a yellow tint.

D. Preparation of 2-D Sample Buffer for Membrane Proteins (7 M urea, 2 M thiourea, 4% CHAPS)

Note: The following procedure prepares 1.2 ml of 2-D Sample Buffer sufficient to equilibrate one column and to adjust the volume of the sample before and after desalting. Increase volumes accordingly for multiple samples. Use 2-D Sample Buffer within 4 hours of preparation.

1. Mix the bottle containing 2-D Sample Buffer Component B (dry component) by inverting several times to obtain a homogeneous powder.
2. Weigh 0.66 g of 2-D Sample Buffer Component B into a microcentrifuge tube and add 720 µl of 2-D Sample Buffer Component A (liquid component).
3. Thoroughly mix the two components until a homogeneous suspension is obtained.
4. Keep the prepared 2-D Sample Buffer at room temperature until use.

E. Desalting Membrane Proteins

1. Invert Protein Desalting Spin Column several times to suspend slurry. Twist off bottom closure and loosen cap.

   Note: Do not snap off bottom closure. To remove closure, twist it slightly in one direction followed by the other direction.
2. Place column in a microcentrifuge collection tube and centrifuge at 1,500 x *g* for 1 minute to remove excess liquid.
3. Blot bottom of column on a paper towel to remove any excess trapped liquid. Empty the collection tube, and replace column into the tube.

   Note: Place a mark on the side of the column where the compacted resin is slanted upward. Place column in microcentrifuge with the mark facing outward in all subsequent centrifugation steps.
4. Carefully add 300 µl of 2-D Sample Buffer (prepared in Section D) to the top of the column. Do not mix. Centrifuge column at 1,500 x *g* for 1 minute to remove excess liquid. Discard flow-through and replace column into the tube. Reinsert spin cup into the same collection tube. Repeat this wash once.
5. Carefully add 300 µl of 2-D Sample Buffer to the top of the column, and centrifuge column at 1,500 x *g* for 2 minutes. Discard collection tube and transfer column to a new tube.

   Note: The amount of membrane protein required for 2-D gel analysis is dependent on the size of the IPG strip used, the gel staining method and the total number of gels that will be processed with the sample. Consult with the IPG strip manufacturer for recommendations concerning the amount of protein to apply.
6. Apply the membrane extract (~50 µl) that was eluted from the 2-D PAGEprep™ Resin (Section C) to the center of the compacted resin bed of the Desalting Column. Do not disturb the resin or allow sample to flow around the resin bed.
7. Centrifuge column at 1,500 x *g* for 2 minutes. The collected sample volume is ~60 µl and contains the desalted sample exchanged into 2-D Sample Buffer.
8. Discard desalting column after use.

F. Preparation of Desalted Membrane Sample for IPG Strip Rehydration

Note: Consult the IPG strip manufacturer for recommended total sample volume required for IPG strip rehydration and for recommended amounts of reducing agent and carrier ampholytes. This sample prep kit provides sufficient 2-D Sample Buffer for 25 samples, each applied to an 18 cm IPG strip. Fewer than 25 applications will be obtained if larger IPG strips are used.

1. Determine the final sample volume needed (see Note above). Add the prepared 2-D Sample Buffer from Section B to the desalted membrane protein sample.
2. Add reducing agent and carrier ampholytes.
3. (Optional) Add a trace amount of Bromophenol Blue to sample.
4. Rehydrate IPG strip with sample according to manufacturer's instructions.

Troubleshooting

Problem	Cause	Solution
Cloudy flow-through from the third wash of the 2-D PAGEprep™ Resin	Larger volumes of the membrane extract may require additional washes to remove all of the Mem-PER® Membrane Protein Solubilization Reagent	Perform an additional wash of the resin
Spin cup membrane clogs during 2-D PAGEprep™ Resin steps	A combination of high protein concentration and detergent	Increase the centrifugation speed to 3,000 x *g*

Additional Information

The membrane extract is prepared for 2-D gel electrophoresis using a two-step process (Figure 1). The extract is first concentrated with 2-D PAGEprep™ Resin, a modified form of diatomaceous earth that selectively binds to proteins while detergents and other contaminants are removed by washing. Generally, 75-300 µl of extract will result in 30-100 µg of protein from cells or 50-200 µg of protein from tissues after processing with 2-D PAGEprep™ Resin (see Table 1). The resulting eluate is then desalted/buffer exchanged, transferring the proteins into 2-D Sample Buffer to be electrophoresed on an immobilized pH gradient (IPG) strip.

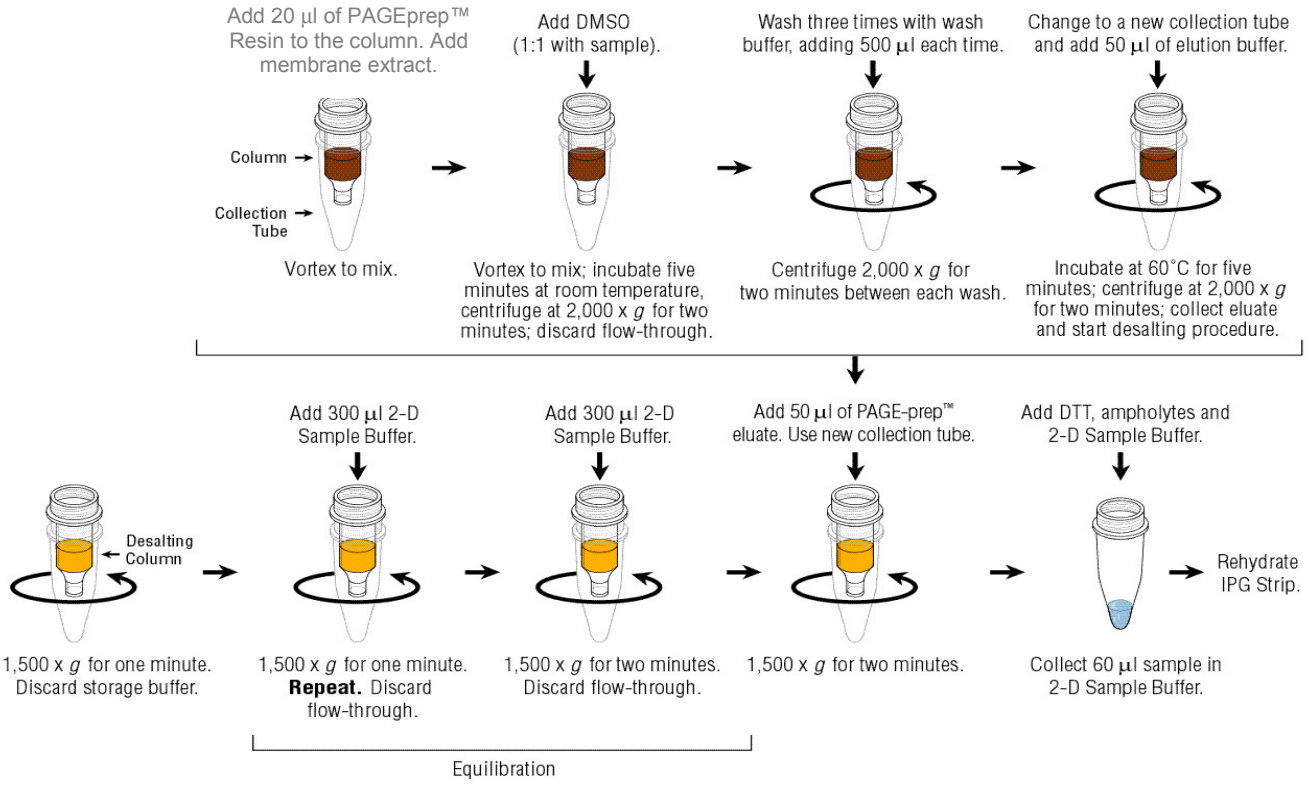

Figure 1. Protocol summary for the concentration and clean-up of membrane proteins using 2-D PAGEprep™ Resin and subsequent desalting/buffer exchange into 2-D Sample Buffer using the 2-D Sample Prep for Membrane Proteins kit.

Table 1. Approximate protein recoveries from processed membrane extract.

Mammalian C6 cells*	Approximate protein recovered**
75 µl	30 µg
150 µl	56 µg
300 µl	102 µg

*Similar results are obtained with yeast cells.
**Results will vary with different cell lines. Samples were processed with 2-D PAGEprep™ Resin and desalting/buffer exchange.

Related Pierce Products

20290	**DTT,** Cleland's Reagent, 5 g
23225	**BCA™ Protein Assay Reagent Kit,** sufficient reagents to perform 500 standard tube assays or 5,000 microplate assays
26659	**2-D Protein Molecular Weight Marker Mix,** 500 µl, sufficient reagent for up to 1,000 mini-gels stained with silver stain
26671	**ColorMeRanger™ Unstained Protein Molecular Weight Marker Mix,** 48-96 doses
24612	**GelCode® SilverSNAP® Silver Stain Kit II**, sufficient to stain 20 mini gels
78415	**Halt™ Protease Inhibitor Cocktail Kit,** EDTA-Free
89863	**2-D Sample Prep for Nuclear Proteins,** 25 applications/kit
89865	**2-D Sample Prep for Soluble Proteins,** 25 applications/kit
89866	**2-D Sample Prep for Insoluble Proteins,** 25 applications/kit

References

1. Rabilloud, T., *et al.* (1997). Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis*. **18:**307-316.
2. Lanne, B., *et al.* (2001). Thiourea enhances mapping of the proteome from murine white adipose tissue. *Proteomics*. **1:**819-828.

The most current versions of all product instructions are available at www.piercenet.com. For a faxed copy, contact customer service (in the USA call 800-874-3723) or your local distributor.