

ProteoSep

Tech Note #3 – A Comprehensive Plasma (Cytosolic) + Membrane (Microsomal) Protein Preparation Protocol

The following procedure is a general protocol for separate and comprehensive extraction of both plasma (cytosolic) and membrane (microsomal) proteins from cells in preparation for analysis using ProteoSep and a PF2D¹⁻⁴. See the Flow Chart at the end to view the overall procedure.

I. Separation of Plasma Proteins from Membrane Proteins.

1. Suspend $\sim 10^8$ cells in 3.0 mL of the extraction buffer below and vortex.

Extraction Buffer composition:

50mM TRIS, pH 7.3
250mM Sucrose
2mM EDTA
2mM Protease inhibitor

2. Lyse cells using conventional freeze thaw (acetone/dry ice), mechanical (French press, dounce, etc.) and/or ultrasonic methods. **Do not use detergent lysing protocols⁵.**
3. Centrifuge at 2800 *g* at 4°C for 20 minutes, decant supernatant into a separate vial and place in an ice bath at 4°C. **Save the debris.**
4. Resuspend debris from Step 3 in 1.0mL of Extraction Buffer and vortex. Centrifuge at 2800 *g* at 4°C for 20 minutes. Decant supernatant and pool this supernatant with the supernatant from step 3. Discard the debris.
5. Add 5mL of ice-cold aqueous 0.1 M Na₂CO₃ solution (pH >11) to the pooled supernatants obtained in Step 4 and agitate gently on ice at 4°C for 1 hr.
6. Ultracentrifuge solution at >100,000 *g* for 1 hr at 4°C and decant supernatant into a separate vial and place in an ice bath 4°C. **Save the pellet.**
7. Resuspend membrane pellet in 1mL of 50mM TRIS, pH 7.3 and ultracentrifuge at >100,000 *g* for 20min at 4°C. Decant supernatant and pool this supernatant with the supernatant from step 6.
8. Store the resulting membrane protein pellet at -20 °C until ready for use (-80 °C for long term >2 weeks storage).

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The pooled supernatants contain the soluble plasma (cytosolic) proteins and the pellet contains the membrane (microsomal) proteins.

- For processing the plasma proteins (supernatant) proceed to Step 9.
- For processing the membrane proteins (pellet) proceed to Step 16.

II. Plasma Protein Solubilization

- Precipitate the plasma proteins from the pre-chilled supernatants in step 7 by adding cold, absolute ethanol in a 1:3 sample to ethanol ratio (for example, 9mL of ethanol would be required for a 3mL sample volume). Vortex and allow the mixture to stand on ice at 4°C for 30min.
- Centrifuge the mixture at 2800 g for 30 minutes at 4°C and discard supernatant. Store the pellet at –20°C until ready for use (–80 °C for long term >2 weeks storage).
- While centrifuging the ethanol precipitated proteins, equilibrate a PD-10 desalting column (code No. 17-0851-01, Amersham Biosciences) with 25mL of CF start buffer.

Buffer component	Plasma Protein Solubilization Buffer Final Composition	Buffer Component Amount per 50 mL solution in Distilled Water
Urea	5.0 M	15.01g
Thiourea	2.5 M	9.52 g
n-octylglucoside	4%	2.0 g
Glycerol	12.5%	6.25 mL
Protease Inhibitor*	1.25 mM	2.0 mL
TRIS	50 mM	0.242 g

*[Sigma P2714 vial contents dissolved in 10 mL DI water then diluted to 100 mL total volume with DI water.]

- Suspend the ethanol precipitated protein pellet obtained from Step 9 in 2.5ml of Plasma Protein Solubilization Buffer (see table) and vortex until pellet is dissolved.
- Load the sample from step 12 onto the equilibrated PD-10 column from Step 11 and discard the eluent.
- Elute the plasma proteins from the PD-10 column using CF start buffer and collect the first 3.5 mL fraction of the eluent and dilute to 5.0 mL with CF start Buffer.

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- Inject the 5.0 mL diluted eluent from Step 14 onto the first dimension HPCF column and analyze according to the ProteoSep protocol.

Note: It is not recommended that samples be stored for long periods of time in Start buffer. Once the PD-10 exchange is finished it is recommended that you proceed directly to the CF fractionation in the ProteoSep analysis.

III. Membrane Protein Solubilization:

Separating CF start buffer soluble membrane proteins from start buffer insoluble membrane proteins.

- Extracting CF start buffer soluble membrane proteins
 - Add 2.5 mL of CF start buffer to the carbonate-treated membrane pellet obtained in Step 8.
 - Vortex vigorously and centrifuge at 20,000 *g* for 1 hr at 4°C.
 - Decant the supernatant and save both the supernatant and pellet. Place the pellet and supernatant on ice prior to further processing. The Pellet can be stored at –20°C until ready for use (–80 °C for long term >2 weeks storage).
 - While centrifuging the membrane pellet (Step 16 b), equilibrate a PD-10 column (code No. 17-0851-01, Amersham Biosciences) with approximately 25 mL of CF start buffer.
 - Load the supernatant (from Step 16 c) onto the PD-10 column. Discard the eluent.
 - Elute the proteins from the PD-10 column using CF start buffer and collect the first 3.5 mL fraction of the eluent and dilute to 5.0 mL with start buffer.
- Note: It is not recommended that samples be stored for long periods of time in Start buffer. Once the PD-10 exchange is finished it is recommended that you proceed directly to the CF fractionation in the ProteoSep analysis.
- Inject 5.0 mL of the CF soluble membrane protein fraction onto the CF column and analyze according to the ProteoSep protocol.

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Buffer component	Membrane Protein Solubilization Buffer Final Composition	Buffer Component Amount per 50 mL solution in Distilled Water
Urea	5 M	15.01g
Thiourea	2 M	7.61 g
n-octylglucoside	4% w/v	2.0 g
SB 3-10	2% w/v	1.0 g
Protease Inhibitor	2 mM	3.0 mL
TRIS	40 mM	0.193 g
CHAPS	2% w/v	1.0 g

21. Extracting CF start buffer Insoluble Membrane Proteins

Treat the resulting start buffer insoluble membrane protein pellet from the Step 16 as follows:

- 21.1 Add 2.0 mL of the Membrane Protein Solubilization Buffer (see table above) to the membrane pellet and vortex to solubilize the remaining membrane proteins. Store at -20°C until ready to use (-80°C for long term > 2 weeks storage)
- 21.2 Take 500 μL of the membrane extract from 21.1 and dilute it 1:4 with an aqueous 0.1%TFA solution (Mobile Phase A of the 2nd Dimension HPRP analysis) and pipette it into well H1 of a 96 well CF collection Plate.
- 21.3 Using the single injection command (Blue arrow on PF2D menu bar) for well H1, inject 100 - 250 μL of the solution onto the HPRP column to obtain a hydrophobicity profile of the start buffer insoluble proteins that are not separated by CF in the ProteoSep protocol.
- 21.4 Alternatively you can add the well H1 to the 2nd dimension analysis of the ProteoSep run from Step 20 by changing one of the non-analyzed wells (e.g. D5) in the sequence table to "H1" then saving the sequence and adding the corresponding sequence run number to the analysis (double green arrow on PF2D menu bar – range radio button and enter sequence number corresponding to well H1 input).

Combine the analyses for the CF start buffer soluble and CF start buffer insoluble proteins into the ProteoVue[®] software to generate a comprehensive (microsomal) membrane protein map.

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References:

1. Fujiki, Y., Hubbard, A.L., Fowler, S. & Lazarow, P.B. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93**, 97-102. (1982)
2. Molloy, M. P., Herbert, B. R., Slade, M. B., Rabilloud, T. *et al.*, *Eur. J. Biochem.*, **267**, 2871-2881 (2000)
3. Molloy, Mark P., *Analytical Biochemistry*, **280**, 1-10 (2000)
4. O'Neil, K.A., Miller, F.R., Barder, T.J., and Lubman, D.M., *Proteomics* **3**, 1256 – 1269 (2003)
5. For difficult to Lyse samples like embryo's, certain tissue samples or bacteria spores you may need a slightly stronger non-detergent lysing buffer with higher salt content. In this case:
 - a. homogenize the sample briefly with a pipette
 - b. Pipette in 2.5 mL of a non-detergent lysis buffer consisting of:
 - 20mM HEPES, pH 7.85,
 - 30 mM NaCl,
 - 10% glycerol,
 - 2 mM EDTA,
 - 1 mM protease inhibitor
 - c. Sonicate on ice with continuous pulses for 3-4x10 seconds using a Cell Disruptor B15 and 3 mm tip.
 - d. Continue to Step 3 of the protocol.

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Flow Chart for Comprehensive Protein Profiling of Both Membrane and Plasma Proteins

