

ProteoSep[®] Whole Cell Protein Preparation Protocol:

A Comprehensive Plasma (Cytosolic) + Membrane (Microsomal) Protein Preparation Protocol

The following procedure is a general protocol for extracting both plasma (cytosolic) and membrane (microsomal) proteins from cells in preparation for analysis by ProteoSep.¹⁻⁴

1. Suspend cells in the following extraction buffer and vortex vigorously.
[Minimum of 3.0mL of buffer required for $>10^7$ cells]

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 protocols.**
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 debris.
4. Resuspend debris from Step 3 in 1mL of Extraction Buffer and vortex. Centrifuge at 2800 *g* at 4°C 20 minutes. Decant supernatant and pool this supernatant with the supernatant from step 3. Discard 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 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.

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 8.**
- **For processing the membrane proteins (pellet) proceed to Step 15.**

2D ProteoSep Plasma Protein Preparation

8. 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.
9. Centrifuge the mixture at 3000 *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).
10. 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 [see table].

Buffer component	Plasma Protein Solubilization Buffer	CF Start Buffer
Urea	7.5 M	6 M
Thiourea	2.5 M	
n-OG	4%	0.1%
Glycerol	12.5%	
TCEP	6.25 mM	
Protease Inhibitor	1.25 mM	
TRIS	50 mM	
Triethanolamine		25 mM

11. 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.
12. Load the sample from step 11 onto the equilibrated PD-10 column from Step 10 and discard the eluent.
13. Elute the plasma proteins from the PD-10 column using CF start buffer and collect the first 3.5mL fraction of the eluent. Store the fraction at –20 °C until ready for use (–80 °C for long term >2 weeks storage).
14. Inject 1.0 – 3.0mL of the fraction onto the first dimension HPCF column and analyze according to the ProteoSep protocol.

Membrane Protein Preparation for CF start buffer Soluble Membrane Proteins and CF start buffer Insoluble Membrane Proteins

15. Add 2.0mL of CF start buffer (see table) and vortex to extract the carbonate-treated membrane pellet obtained in Step 7 with and centrifuge at 20,000 *g* for 1hr at 4°C. Decant the supernatant and save both the supernatant and pellet. Place the pellet and supernatant on ice prior to further processing or store at –20 °C until ready for use (–80 °C for long term > 2 weeks storage).

Buffer component	Membrane Protein Solubilization Buffer Composition	CF Start Buffer
Urea	7 M	6 M
Thiourea	2 M	
n-OG	4%	0.1%
CF START BUFFER 3-10	2%	
TCEP	10 mM	
Protease Inhibitor	2 mM	
TRIS	40 mM	
CHAPS	2% w/v	
Triethanolamine	-	25 mM

16. Treat and analyze the resulting membrane pellet from the above step according to the following:

16.1 Add 1mL of the Membrane Protein Solubilization Buffer (see table) to the membrane pellet and vortex to solubilize the membrane proteins. Store at –20 °C until ready to use (–80 °C for long term > 2 weeks storage).

16.2 Dilute the membrane extract from above 1:4 with an aqueous 0.1%TFA solution. Inject 100-200µL of the solution onto the HPRP column and analyze according to the ProteoSep protocol.

17 While centrifuging the membrane pellet, equilibrate a PD-10 column (code No. 17-0851-01, Amersham Biosciences) with approximately 25mL of CF start buffer.

18 Load the supernatant (from Step 15) onto the PD-10 column. Discard the eluent.

- 19 Elute the proteins from the PD-10 column using CF start buffer and collect the first 3.5mL fraction of the eluent. Store the fraction at -20°C until ready for use (-80°C for long term >2 weeks storage).
- 20 Inject 1.0 – 3.0mL of the fraction onto the HPCF column and analyze according to the ProteoSep protocol.
- 21 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.

References:

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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)

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

