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 ~108 cells in 3.0 cc of the **extraction buffer** below and vortex.
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 40C for 20 minutes, decant supernatant into a separate vial and place in an ice bath at 40C. Save the debris.
4. Re-suspend debris from Step 3 in 1.0cc of Extraction Buffer and vortex. Centrifuge at 2800 g at 40C for 20 minutes. Decant supernatant and pool this supernatant with the supernatant from step 3. Discard the debris.
5. Add 5cc 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 40C for 1 hr.
6. Ultracentrifuge solution at >100,000 g for 1 hr at 40C and decant supernatant into a separate vial and place in an ice bath 40C. Save the pellet.
7. Re-suspend membrane pellet in 1cc of 50mM TRIS, pH 7.3 and ultracentrifuge at >100,000 g for 20min at 40C.

Extraction Buffer composition:

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

Decant supernatant and pool this supernatant with the supernatant from step 6.

8. Store the resulting membrane protein pellet at −20 oC until ready for use (−80 oC for long term >2 weeks storage).

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

9. 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, 9cc of ethanol would be required for a 3cc sample volume). Vortex and allow the mixture to stand on ice at 4oC for 30min.

10. Centrifuge the mixture at 2800 g for 30 minutes at 4oC and discard supernatant. Store the pellet at −200C until ready for use (−80 oC for long term >2 weeks storage).

11. While centrifuging the ethanol precipitated proteins, equilibrate a PD-10 desalting column (code No. 17-0851-01, Amersham Biosciences) with 25cc of CF start buffer.

12. 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.

13. Load the sample from step 12 onto the equilibrated PD-10 column from Step 11 and discard the eluent.

Buffer component	Plasma Protein Solubilization Buffer Final Composition	Buffer Component Amount per 50 cc solution in Distilled Water
Urea	7.5 M	22.52 g
Thiourea	2.5 M	9.52 g
n-octlyglucoside	4%	2.0 g
Glycerol	12.5%	6.25 cc
Protease Inhibitor	1.25 mM	2.0 cc
TRIS	50 mM	0.242 g

14. Elute the plasma proteins from the PD-10 column using CF start buffer and collect the first 3.5 cc fraction of the eluent and dilute to 5.0 cc with CF start Buffer.

15. Inject the 5.0 cc 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.

16. Extracting CF start buffer soluble membrane proteins
    a. Add 2.5 cc of CF start buffer to the carbonate-treated membrane pellet obtained in Step 8.
    b. Vortex vigorously and centrifuge at 20,000 g for 1hr at 4°C.
    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 –200C until ready for use (–80 oC for long term >2 weeks storage).

17. While centrifuging the membrane pellet (Step 16 b), equilibrate a PD-10 column (code No. 17-0851-01, Amersham Biosciences) with approximately 25 cc of CF start buffer.

18. Load the supernatant (from Step 16 c) 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 3.5 cc fraction of the eluent and dilute to 5.0 cc with start buffer.

20. Inject 5.0 cc of the CF soluble membrane protein fraction onto the CF column and analyze according to the ProteoSep protocol.

21. Extracting CF start buffer Insoluble Membrane Proteins: Treat the resulting start buffer insoluble membrane protein pellet from the Step 16 as follows:
    a. Add 2.0 cc of the Membrane Protein Solubilization Buffer (see table on the right) 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)
    b. Take 500 uL of the membrane extract from 21.1

Buffer component	Plasma Protein Solubilization Buffer Final Composition	Buffer Component Amount per 50 cc solution in Distilled Water
Urea	7.0 M	21.03 g
Thiourea	2.0 M	7.61 g
n-octlyglucoside	4% w/v	2.0 g
SB 3-10	2% w/v	1.0 g
Protease Inhibitor	2 mM	3.0 cc
TRIS	40 mM	0.193 g
CHAPS	2% w/v	1.0 g

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.

c. Using the single injection command (Blue arrow on PF2D menu bar) for well H1, inject 100 - 250µL 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.

d. 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

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 cc of a non-detergent lysis buffer consisting of:

- 20mM HEPES,
- pH 7.85,
- 30 mM NaCl,
- 10% glycerol,
- 2 mM EDTA,
- 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.

References:

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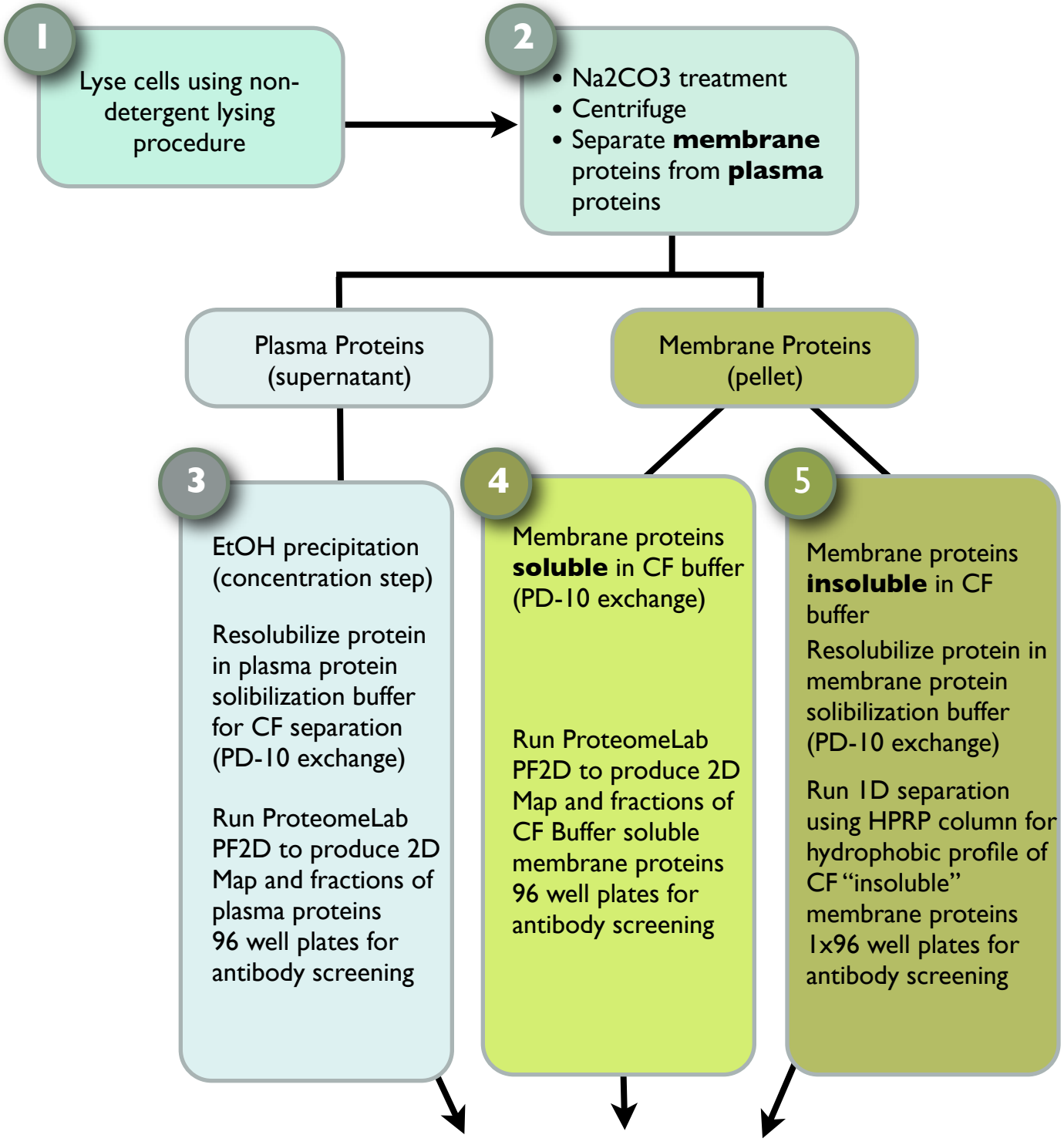
Flow chart for comprehensive protein profiling of both membrane and plasma proteins



17x96 well plates for antibody screening and protein identification

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