Organelle isolation for proteomics: mitochondria from peripheral blood mononuclear cells

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ABSTRACT

Mitochondria play key roles in many cell functions including energy production, fatty acid metabolism, pyrimidine biosynthesis, calcium homeostasis, and aging. They also regulate crucial signaling cascades such as apoptosis and oxidative stress. The proteome is often used to investigate the functional correlations on protein levels. Based upon the human, genome there is estimated 2000 to 2500 associated mitochondrial proteins, however, just over 600-800 have been identified at the protein level. For this reason, mitochondria contain a great number of proteins that have yet to be identified and characterized. The identification of these proteins can help in discovery of biological process. This protocol focuses on step-by-step procedure of mitochondrial proteome extraction from peripheral blood mononuclear cell (PBMC) mitochondria. The isolation and preparation procedures described here require 6 hours approximately.

Keywords: Mitochondria Isolation; Proteomics; peripheral mononuclear cell (PBMC)

INTRODUCTION

Mitochondria are essential mammalian organelles surrounded by two lipid bilayers. They are central organelles controlling the life and death of the cell [1]. Based upon human genome, it is estimated that genome code 2000-2500 mitochondrial related proteins [2]. They participate in key metabolic reactions, synthesize majority of the ATP and regulate a number of signaling cascades, including apoptosis [3]. Also evidences highlight on the role of this organelle in several pathophysiological processes, including neurodegeneration [4], neuronal morphogenesis [5], plasticity and infertility[6].

Proteomic methods help us to improve our knowledge in the cell function. It could be divided into two approaches; Top-down and Bottom-up. Top-down is a method of protein identification with use of intact proteins in high resolution mass spectrometry, usually followed by tandem mass[7-8]. For Top-down proteomics, fractionation just perform on protein level[9-10]. A common method in proteomic, bottom-up, is also used for identification and characterization of proteins sequences and diagnose post-translation modification by protein digestion prior any analysis by mass spectrometry[11-12]. In this approaches, the fractionation usually could be done in two levels; protein and peptide, by a variety of gel-free and gel-based methods [13-14]. For identification of protein, intact mass peptides or fragment should be compared with protein sequence databases. Given that eukaryotic cells have organelles with different task there is a great attention to organelle proteomics. For this goal, organelles should be purified efficiently and after that we can extract its proteome biochemically [15-16]. Proteome of mitochondria differ quantitatively and qualitatively depend on the tissue and cell varieties and is regulated dynamically [17]. For example, in human cardiac mitochondria, 615 distinct types of protein have

been identified [18] and in murine 940 types of protein reported [19]. Furthermore a small percentage of the mitochondrial proteome is unique to each tissue. For example 75 proteins associated with liver, 23 proteins associated with heart, and 22 proteins associated with skeletal muscle mitochondria have been reported in rat [20]. The importance of mitochondria and its proteome cause this study with purpose of mitochondrial proteome extraction. In this study, we present a complete protocol including timing and step for the cells (PBMC). In the most protocols, mitochondria were extracted and isolated from the high mitochondrion concentration cells such as muscle, liver and cultured cells. On the other hand, a large number of these protocols associated to physiological studies and not to proteome extraction. We intend to offer a cheap, fast and efficient mitochondria preparation protocol before any fractionation in proteomics studies.

MATERIALS

reagents

- Blood for isolation peripheral blood mononuclear cell (PBMC)
- NaCl-NaHCO3 (Sigma)
- Tris base (Sigma)
- Ethylenediamineteraacetic acid (EDTA) (Sigma)
- Sodium dodecyl sulfate (SDS) (Sigma)
- Protein ladder (Fermentase page ruler unstained ladder)
- Methanol (Sigma)
- 2-propanol (Sigma)
- Acetic Acid (Sigma)
- Glycine (Sigma)
- Coomassie blue G250 (Sigma)
- Bis Acrylamide (Sigma)
- Acrylamide (Sigma)
- Tetramethylethylenediamine (TEMED) (Sigma)
- Ammonium persulfate (APS) (Sigma)
- Glycerol (Sigma)
- dH2O
- 2-β Mercaptoethanol (Sigma)
- Phosphate buffer saline (PBS) (Sigma)
- Sucrose (Sigma)
- Tricarboxylic acid (TCA) (Sigma)
- Phenylmethanesulfonylfluoride (PMSF) (Sigma)
- Neutral red (Sigma)
- Janus green B (Sigma)
- Ammonium bicarbonate (Sigma)
- Tributylphosphine (TBP) (Sigma)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma)
- CHAPS (Sigma)
- Dithiothreitol (DTT) (Sigma)
- Ficoll (Sigma)
- Protease inhibitor (Sigma)
- Phosphatase inhibitor (Sigma)
- Acetonitrile (Sigma)
- Methanol (Sigma)
- Trifluoric acid (TFA) (Sigma)

equipment

- 50 ml polypropylene Falcon tubes
- 15 ml polypropylene Falcon tubes
- 1.5 ml microfuge test tube
- Refrigerated centrifuge for 15 ml Falcon tubes and glass centrifuge tube (Kendro, D37520)
- Hamilton syringe: 10 ml and 50 ml
- Sonicator (Hielscher)
- Rubber adapter sleeve for centrifuge tube
- Refrigerated centrifuge for 1.5 ml tubes (Kendro, D37520)
- Electrophoresis tools (Paya Pajoohesh)
- Speed vacuum (Scanvac)
- HPLC (Agilent)

reagent setup

- Isolation Buffer (0.25 M Sucrose, 10 mM HEPES, pH 7.5) (21).
- Lysis Buffer (7M Urea, 2M Thiourea, 4%CHAPS) (22).
- Alkylation Reagent (1M Acrylamide)
- Mass Spectrometry Ion Trap LCQ (Thermo)
- Gel Stain Solution (Methanol, Acetic acid, Coomassie blue G250, Distilled water)
- Gel Destain Solution (Methanol, Acetic acid, Distilled water)
- Mitochondrial Stain (1% Janus green B, 10% Neutral red)
- Anti-Coagulant (1M EDTA)
- Dilute Buffer (Normal saline)

Reagent preparation		
Reagent	**formulation**	**Comments**
Isolation Buffer [23] (IB)	0.25 M Sucrose, 10 mM HEPES, pH 7.5	Dissolve 85.5 g of Sucrose and 2.38 g HEPES in 1 liter of distilled water then pH should be adjusted on 7.5 and store at -20 °C.
Lysis Buffer [24] (LB)	7M Urea, 2M Thiourea, 4%CHAPS	Dissolve 420 g Urea, 152 g Thiourea and 40 g CHAPS in 1 liter of distilled water. Store at -20 °C.
Alkylation Reagent	1M Acrylamide	Add 1/100 volume to the sample for two hours. This is for complete and particular Cysteine residue alkylation. Simultaneous reduction and alkylation could be done when the non-thiol Tributylphosphine (TBP) is substituted for DDT. Prepare it fresh, and it can be stored in room temperature and use within one month. Do not freeze.
Stain	Methanol, Acetic acid, Coomassie blue G250, Distilled water	Dissolve 450 cc methanol, 100 cc acetic acid and 2.5 g Coomassie blue G250 in 450 cc distilled water and store in room temperature.
Destain	Methanol, Acetic acid, Distilled water	Dissolve 450 cc methanol and 100 cc acetic acid in 450 cc distilled water and store in room temperature.
Mitochondrial Stain [25]	1% Janus green B	Dissolve 1 g Janus green B in 100 cc distilled water and store at room temperature. Janus green B is a specific stain for mitochondrial membrane, was used together with Neutral red which stains lysosomes and the non-mitochondrial fractions.
	10% Neutral red	Dissolve 10 g neutral red in 100 cc PBS and store at room temperature.
Anti-Coagulant	1M EDTA	Dissolve 372.2 g of EDTA in 500 ml of distilled water, adjust pH to 7.4 using Tris powder, bring the solution to 1 liter and store at 4 °C. EDTA chelates ions like $Ca^{2+}$and distort coagulation process.
Dilute Buffer	Normal saline	Because of normal saline's salt concentration, blood cells are protected from hemolysis.

PROCEDURE

(A)Lymphocytes Separation (Figure 1)

1.5ml blood contains anticoagulant EDTA is prepared.

2.Dilute blood with 5ml (8.5 g/l) of Normal saline and gently mix with blood.

3.Pour 6ml Ficoll to two 15ml Falcon tube equally (each 3ml).

4.5ml of the diluted blood is poured slowly and carefully onto a falcon containing Ficoll to avoid mixing with Ficoll. (Similarly add blood.)

5.Both Falcons centrifuge for 20 minutes at 2500 rpm, 4°C.

6.After centrifugation the layer contain lymphocyte can be seen (the thin layer called buffy coat between the Plasma and the Ficoll). PBMC should be isolated carefully from both falcon tubes and transfer to a new falcon tube.

7.The volume of Falcon tube should be achieved to 10 ml with PBS solution.

8.The Falcon centrifuges for 10min at 1500rpm, 4 °C.

9.After centrifugation, Discard PBS on PBMC pellet.

10.Resuspend the pellet in 100µl PBS remained then transfer to a new tube.

11.Wash the falcon with 100µl PBS then add to the prior tube. (Final volume should be 200 µl).

12.Centrifuge tube at 2000 rpm and 4 °C for 7 min.

13.Discard the PBS, add 200ml IB with phosphatase inhibitor, protease inhibitor and PMSF then pellet resuspend in it.

14.Mix 10µl of the cell suspension with 10µl Trypan blue (0.2w/v) by sampler, and move it to Neubauer Lam.

CRITICAL STEP Count Lymphocyte cells and announces the percent of cells according this formula: 1000 * Dilution factor * vol. of cell suspension * num. of counted cells ($m.l^{-1}$).

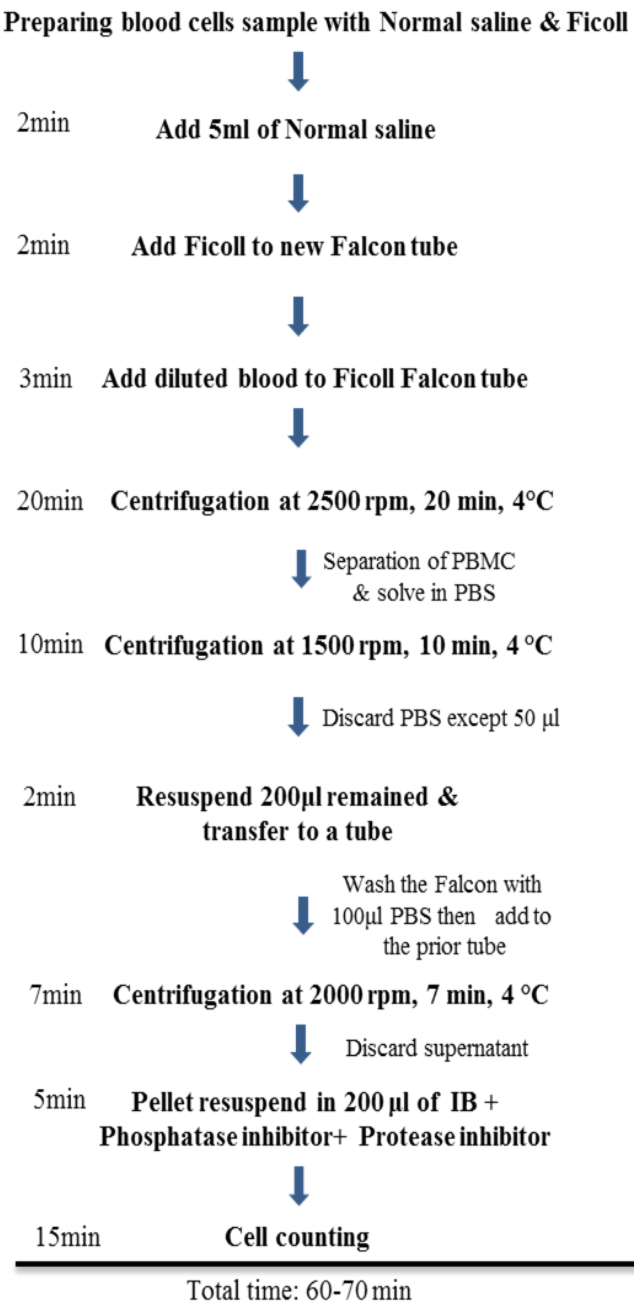

Figure 1: Lymphocytes separation protocol.

(B) Mitochondria isolation (Figure 2)

1.Cell lysis by sonication: sonicate cells in MS 72 D and 4 °C for 30 sec then vortex for 30 sec (5 times).

CRITICAL STEP Sonication usually increase the system temperature, so it should be better done in 4 °C (by mixing water and ice).

2.Cell lysis by freeze/thaw: freeze samples 5 min in -80 °C then thaw 1 min in 37 °C (10 times).

CRITICAL STEP Freezer led to create slowly ice crystals, so cell lysis can occur completely.

3.Do centrifugation for 10 min at 1000 g.

4.Transfer supernatant that contains mitochondria to 1.5ml tube.

5.Centrifuge supernatant for 25 min at 20000g.

6.Remove supernatant, the sediment is mitochondria.

7.Store the tubes contain mitochondria pellet in –80.

CRITICAL STEP Mix 20µl of supernatant step 4 with 20µl of dye solution (1% janus green B, 10% Neutral red). The mixture was incubated at room temperature for 10 min and the purity of mitochondria was determined under light microscope.

CRITICAL STEP Janus green B that is a specific stain for mitochondrial membrane, was used together with Neutral red which stains lysosomes red and can also stain Golgi apparatus [26]. Alive mitochondria appear pinky due to active electron transfer chain, whereas they seem blue in inactive manner (Figure 4).

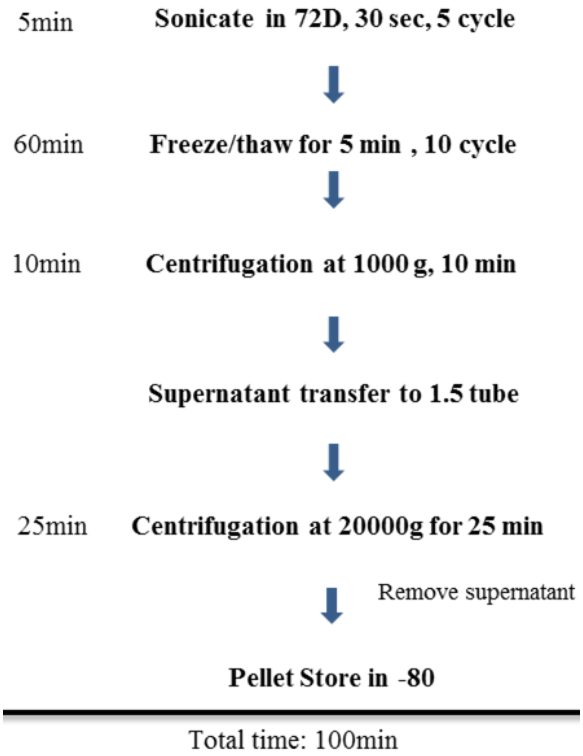

Figure 2: Mitochondria isolation protocol.

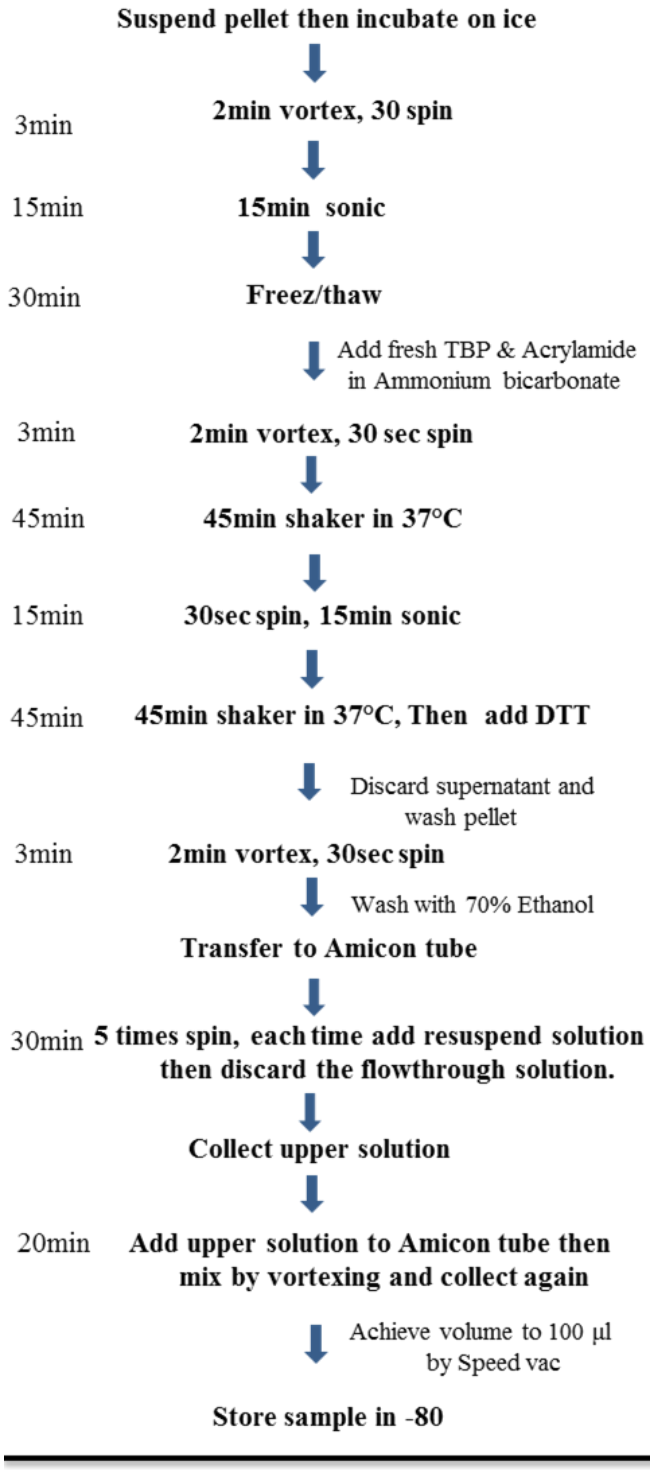

Figure 3: Protein isolation of mitochondria protocol.

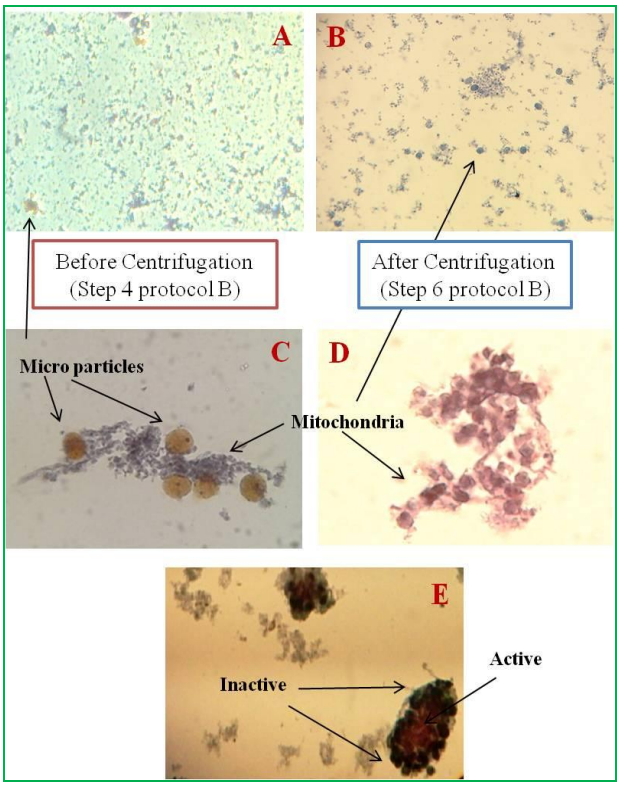

Figure 4: Isolated Mitochondria. A and B: For initial test of the purity, the specific staining of mitochondria is shown by magnification power 400×. C and D: For initial test of the purity, the specific staining of mitochondria is shown by magnification power 1000×. E: At the core of aggregated mitochondria, alive mitochondria appear pinky due to active electron transfer chain where they turned to blue (inactive manner) over time.

(C) Mitochondrial proteins isolation (Figure 3)

1.Suspend mitochondria pellet with 88.5µl of lysis buffer, 0.5µl of PMSF, 1µ of protease inhibitor and 4 µl phosphatase inhibitor then incubate on ice.

2.2 min Vortex, 30 sec Spin, then 15 min Sonic.

3.Mitochondria lyses physically by freeze/thaw: freeze samples 5 min in -80 °C then thaw 1 min in 37 °C (5 times).

4.Add 2.5 µl of fresh TBP (5mM) and 10 µl Acrylamide (1M) in 0.25 Ammonium bicarbonate to the tube.

CRITICAL STEP This step is for complete and specific alkylation and reduc**tion of Cys re**sidues.

5.2 min Vortex, 30 sec Spin, 15 min Sonic.

6.45 min Shaker in 37 °C

7.30 sec Spin, 15 min Sonic

8.45 min Shaker in 37 °C

9.Add 2.5 µl DTT (50 mM)
10.2 min Vortex, 30 sec Spin
11.Transfer supernatant to 3 kD Amicon tube.
CRITICAL STEP At this step, it is removed all contamination and small interfering such as detergents, salts and small molecules.
12.5 times spin, each time add 100-200 µl of resuspend solution (%25 Acetonitrile, 0.1% acid formic) then discard the flow through solution.
13.Collect upper solution.
14.Add 500 µl of upper solution to Amicon tube then wash it by vortexing and again collect the solution.
15.Achieve volume to 100 µl by Speed vac.
CRITICAL STEP The same volume of samples helps us to compare all samples fairly.
16.Store sample in -80.
CRITICAL STEP At this step, we measure the protein concentration for each sample that it is **8.98mg/ml**.
CRITICAL STEP The extracted samples run on SDS-PAGE to study visually **[22](Figure5**).

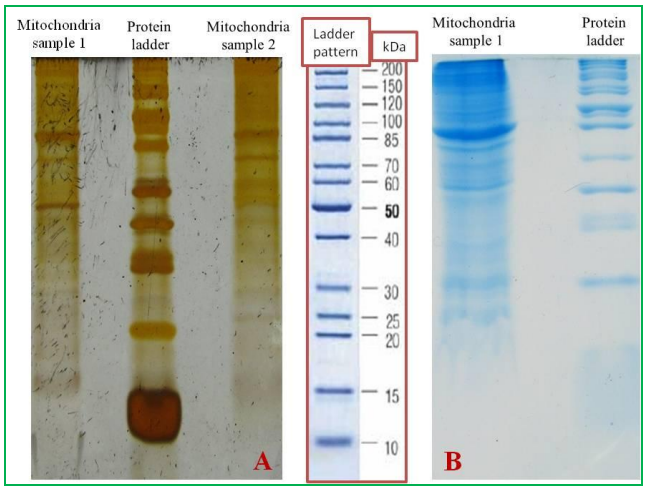

Figure 5: The molecular weight distribution of the mitochondrial samples was visualized by SDS-PAGE electrophoresis. A: Two different mitochondrial samples were loaded in SDS-PAGE (Totally 180ug proteins) and stained by silver staining method. B: One mitochondrial sample was loaded in SDS-PAGE and stained by Coomassie Brilliant Blue staining method.

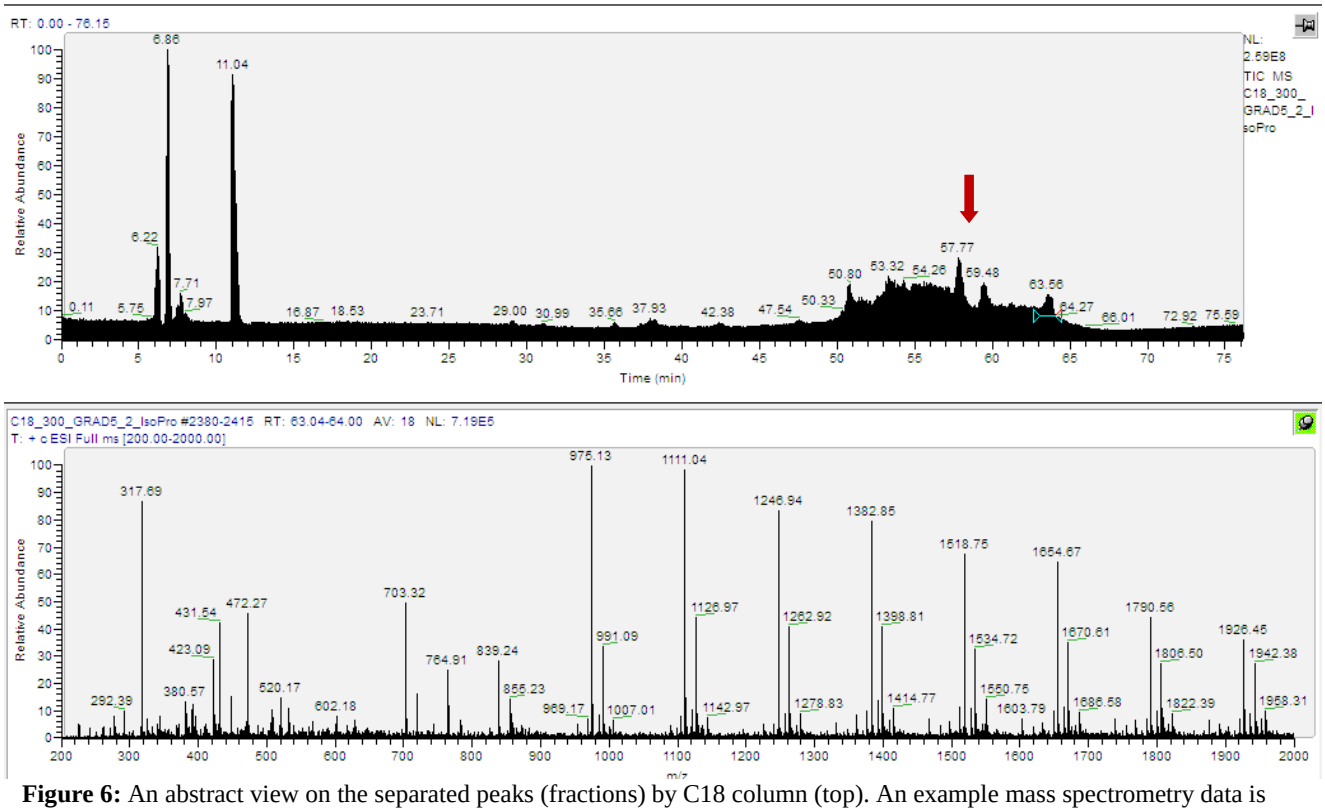

Figure 6: An abstract view on the separated peaks (fractions) by C18 column (top). An example mass spectrometry data is shown for the selected peak (bottom). The clusters (isotopic envelopes) indicate acceptable quality of protein mass data following contaminant removal.

Troubleshooting		
Step	**Problem**	**Solution**
Lymphocyte separation	Mixing PBMC with RBC	Centrifugation should be done at the lowest deceleration rate. High deceleration rate cause both disordered and incomplete separation.
	Small pellet (low number of PBMC)	a. Dilution buffer should be equal to volume of blood that it helps to get high efficiency in isolation. b. Carefully adding blood to Ficoll to avoid cell lysis followed by mixing suddenly. c. When the PBS is poured off, the PBMC pellet may be detached.
PBMC lysis	Very large pellet	In transferring the supernatant that contain mitochondria should be careful to don't pick up cell lysate pellet.
	Small pellet	a. Often mitochondria pellet are colorless or white, so it is difficult to distinguish it. Don't worry about it! b. After 30s sec vortex, it is better to spin tubes between sonicates. This work helps to mix all cells in solution that required for complete sonication.
Mitochondria isolation	Decreasing the solution in Amicon tube	It should be carefully collect the solution from up of the filter to avoid damaging it.
	Contamination	The main contamination in proteomics analysis is Keratins and plasticizers. It should be better washing by ethanol all tube before performing protocol and prohibit all source of keratins by gloves and mask.

RESULT AND DISCUSSION

In this study, we aimed to obtain a usable amount of relatively pure mitochondrial proteins for proteomics study. The quality of the obtained organelles can be checked by using conventional optical microscopy by specified staining (Figure 4). In this figure, it can be observed that intact mitochondria are active (pinky in the presence of Janus B, Figure 4E). They gradually changed to blue and inactive during microscopic imaging.

Also, the quality of the proteome extraction is obviously presented in 2D-PAGE and mass spectrometry data (Figure 5 and 6). The different fractionation methods on protein levels indicate the enough amounts and diversity of proteins following removal of contaminates.

In comparison with other proposed protocols, we present a simple, fast, efficient and low cost protocol. In the most of those protocol, we require some additional steps in differential centrifugation (Using Percoll) [20, 23-27] and in some of those, we should have special instruments [28]. Although, these suggestions help us to increase sensitivity especially in plant proteomics (in the presence of chloroplast), but we suppose that fractionation approach play important role to compensate sensitivity and specificity. This protocol is completely compatible with two proteomics approaches, top-down and bottom-up.

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