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Preparation of Mitochondria from Ovarian Cancer Tissues and Control Ovarian Tissues for Quantitative Proteomics Analysis

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Xiaoyan Cao, Ph.D.
Review Editor, JoVE

Dear Dr. Cao,

We would like to re-submit our revised manuscript (JoVE60435R1), entitled "Preparation of mitochondria from ovarian cancer tissues and control ovaries for quantitative proteomics analysis", written by Xianquan Zhan, et al., for publication in the journal JoVE.

Attached is the point-by-point response to each editorial comment.

We look forward to hearing from you.

Sincerely yours,

Xianquan Zhan, PhD, MD.
Professor of cancer proteomics and structural biology
Xiang Hospital of Central South University

TITLE:

Preparation of Mitochondria from Ovarian Cancer Tissues and Control Ovarian Tissues for Quantitative Proteomics Analysis

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KEYWORDS:

ovarian cancer, mitochondria, differential-speed centrifugation, density gradient centrifugation, Isobaric tag for relative and absolute quantification (iTRAQ) labeling, strong cation exchange (SCX), liquid chromatography (LC), tandem mass spectrometry (MS/MS), mitochondrial proteome

SUMMARY:

This article presents a protocol of differential-speed centrifugation in combination with density gradient centrifugation to separate mitochondria from human ovarian cancer tissues and control ovarian tissues for quantitative proteomics analysis, resulting in a high-quality mitochondrial sample and high-throughput and high-reproducibility quantitative proteomics analysis of a human ovarian cancer mitochondrial proteome.

ABSTRACT:

Ovarian cancer is a common gynecologic cancer with high mortality but unclear molecular mechanism. Most ovarian cancers are diagnosed in the advanced stage, which seriously hampers therapy. Mitochondrial changes are a hallmark of human ovarian cancers, and

mitochondria are the centers of energy metabolism, cell signaling, and oxidative stress. In-depth insights into the changes of the mitochondrial proteome in ovarian cancers compared to control ovarian tissue will benefit in-depth understanding of the molecular mechanisms of ovarian cancer, and the discovery of effective and reliable biomarkers and therapeutic targets. An effective mitochondrial preparation method coupled with an isobaric tag for relative and absolute quantification (iTRAQ) quantitative proteomics are presented here to analyze human ovarian cancer and control mitochondrial proteomes, including differential-speed centrifugation, density gradient centrifugation, quality assessment of mitochondrial samples, protein digestion with trypsin, iTRAQ labeling, strong cation exchange fractionation (SCX), liquid chromatography (LC), tandem mass spectrometry (MS/MS), database analysis, and quantitative analysis of mitochondrial proteins. Many proteins have been successfully identified to maximize the coverage of the human ovarian cancer mitochondrial proteome and to achieve the differentially expressed mitochondrial protein profile in human ovarian cancers.

INTRODUCTION:

Ovarian cancer is a common gynecologic cancer with high mortality but unclear molecular mechanism[1,2]. Most of ovarian cancers are diagnosed in the advanced stage, which seriously hampers therapy. Mitochondrial changes are a hallmark of human ovarian cancers, and mitochondria are the centers of energy metabolism, cell signaling, and oxidative stress[3–7]. In-depth insights into the changes of the mitochondrial proteome in ovarian cancers compared to control ovarian tissue will benefit in-depth understanding of the molecular mechanisms of ovarian cancer, and the discovery of effective and reliable biomarkers and therapeutic targets. Mitochondrial metabolism has been proposed and recognized as a target for cancer therapy, and antimitochondrial therapy might ultimately be very beneficial for preventing the recurrence and metastasis of cancer[8]. Individual metabolic profiling is also already practiced as a useful tool for cancer stratification and predictive strategies[9,10].

The long-term goal of this research is to develop and use a quantitative mitochondrial proteomics method to study ovarian cancer for clarification of mitochondrial proteome alterations between ovarian cancer and control ovarian tissues, and their molecular network alterations from a systematic multi-omics angle[11,12], which will result in the discovery of mitochondria-targeted molecular biomarkers[13] for clarification of the molecular mechanisms of ovarian cancer, prediction, and personalized treatment of ovarian cancer patients. Isobaric tags for relative and absolute quantification (iTRAQ) labeling[3,4] are an effective method to quantify the mitochondrial protein changes. Preparation of high-quality mitochondrial samples from human ovarian cancer and control ovarian tissues are the prerequisite for iTRAQ quantitative analysis of mitochondrial proteomes[3]. Mitochondrial preparation coupled with iTRAQ quantitative proteomics has been successfully used in long-term research programs about the human ovarian cancer mitochondrial proteome, including the establishment of mitochondrial proteome reference maps[3], the analysis of differentially expressed mitochondrial profiles[4,14] and post-translational modifications, including phosphorylation, which has already resulted in the discovery of important signaling pathway network changes in human ovarian cancers[5], including alterations in energy metabolism[4], lipid metabolism, and mitophagy pathway-systems[3].

Previous studies have found that differential-speed centrifugation in combination with density gradient centrifugation is an effective method to isolate and purify mitochondria from human ovarian cancer and control ovarian tissues[3–5,14]. The iTRAQ labeling coupled with strong cation exchange (SCX)-liquid chromatography (LC)-tandem mass spectrometry (MS/MS) is the key technique to detect, identify, and quantify the proteins from the prepared mitochondrial samples.

Here, detailed protocols for mitochondrial preparation coupled with iTRAQ quantitative proteomics are described. These have been successfully used in the analysis of human ovarian cancer tissue mitochondrial proteomes. The protocols include preparation of samples, differential-speed centrifugation, density gradient centrifugation, quality assessment of mitochondrial samples, protein digestion with trypsin, iTRAQ labeling, SCX fractionation, LC, MS/MS, database searching, and quantitative analysis of mitochondrial proteins. Moreover, this protocol easily translates to analyze other human tissue mitochondrial proteomes.

PROTOCOL:

Ovarian tissue samples including ovarian cancer tissues (n = 7) and normal control ovarian tissues (n = 11) were used for this protocol. The present protocol[3–5] is approved by the Xiangya Hospital Medical Ethics Committee of Central South University, China.

1. Preparation of mitochondria from human ovarian cancer tissues

1.1. Prepare 250 mL of the mitochondrial isolation buffer by mixing 210 mM mannitol, 70 mM sucrose, 100 mM potassium chloride (KCl), 50 mM Tris-HCl, 1 mM diamine tetraacetic acid (EDTA), 0.1 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 1 mM phenylmethanesulfonyl fluoride (PMSF) protease inhibitor, 2 mM sodium orthovanadate (V), and 0.2% bovine serum albumin (BSA), pH 7.4.

1.2. Place ~1.5 g of ovarian cancer tissues in a clean glass dish.

1.3. Add 2 mL of the pre-chilled mitochondrial isolation buffer to lightly wash the blood from the tissue surface 3x.

1.4. Use clean ophthalmic scissors to fully mince the tissue into about 1 mm^3 pieces, and transfer the minced tissues into a 50 mL centrifuge tube.

1.5. Add 13.5 mL of mitochondrial isolation buffer containing 0.2 mg/mL nagarse, and then use an electric homogenizer to homogenize (use scale 2, 10 s 6x, interval 10 s) the minced tissues (2 min, 4 °C).

1.6. Add another 3 mL of mitochondrial isolation buffer into the tissue homogenates and mix them well by pipetting.

1.7. Centrifuge the prepared tissue homogenate (1,300 x *g*, 10 min, 4 °C). Remove the crude nuclear fraction (i.e., the pellet) and keep the supernatant.

1.8. Recentrifuge the supernatant (10,000 x *g*, 10 min, 4 °C). Remove the microsomes (i.e., the supernatant) and keep the pellet.

1.9. Add 2 mL of mitochondrial isolation buffer and resuspend the pellet well by light pipetting.

1.10. Centrifuge the pellet suspension (7,000 x *g*, 10 min, 4 °C). Discard the supernatant and keep the crude mitochondria (i.e., the pellet).

1.11. Add 12 mL of 25% density gradient medium (i.e., Nycodenz) to resuspend the extracted crude mitochondria.

1.12. Make a discontinuous density gradient by filling a tube from bottom to top with 5 mL of 34%, 8 mL of 30%, 12 mL of 25% (containing the crude mitochondria from step 1.11), 8 mL of 23%, and 3 mL of 20% density gradient medium, and centrifuge it (52,000 x *g*, 90 min, 4 °C).

1.13. Use a long and blunt syringe to collect the purified mitochondria at the interface between the 25% and 30% density gradient medium into a clean tube.

1.14. Add mitochondrial isolation buffer into the collected mitochondria to dilute it to a three-fold volume. Centrifuge (15,000 x *g*, 20 min, 4 °C) and discard the supernatant.

1.15. Add 2 mL of mitochondrial isolation buffer to resuspend the pellet, and centrifuge (15,000 x *g*, 20 min, 4 °C). Discard the supernatant and keep the pellet.

1.16. Collect the final pellet (i.e., the purified mitochondria) and store it at -20 °C.

NOTE: Combine all purified mitochondria from the ovarian cancer tissue as the mitochondria sample for quantitative proteomics analysis.

2. Preparation of mitochondria from human control ovarian tissues

2.1. Prepare 250 mL of the mitochondrial isolation buffer as described in step 1.1.

2.2. Place ~1.5 g of the normal control ovarian tissues in a clean glass dish.

2.3. Add 2 mL of pre-chilled mitochondrial isolation buffer to lightly wash the blood from the tissue surface 3x.

2.4. Use clean ophthalmic scissors to fully mince the tissue into about 1 mm^3 pieces, and transfer the minced tissues into a 50 mL centrifuge tube.

2.5. Add 8 mL of 0.05% trypsin/20 mM EDTA in phosphate-buffered saline (PBS) to the minced control tissues, and digest (30 min, room temperature), which helps to lyse the tissues and cells and release mitochondria. Then centrifuge (200 x *g*, 5 min). Discard the supernatant, and keep the tissues and cells.

2.6. Add 13.5 mL of mitochondrial isolation buffer containing 0.2 mg/mL nagarse, and then use an electric homogenizer to homogenize (use scale 2, 10 s 6x, interval 10 s) the minced tissues (2 min, 4 °C).

2.7. Add another 3 mL of mitochondrial isolation buffer into the tissue homogenates and mix them well by pipetting.

2.8. Centrifuge the prepared tissue homogenate (1,300 x *g*, 10 min, 4°C). Remove the crude nuclear fraction (i.e., the pellet) and keep the supernatant.

2.9. Recentrifuge the supernatant (10,000 x *g*, 10 min, 4°C). Remove the microsomes (i.e., the supernatant) and keep the pellet.

2.10. Add 2 mL of mitochondrial isolation buffer to resuspend the pellet well by light pipetting.

2.11. Centrifuge the pellet suspension (7,000 x *g*, 10 min, 4 °C). Discard the supernatant and keep the crude mitochondria (i.e., the pellet).

2.12. Add 12 mL of 25% density gradient medium to resuspend the extracted crude mitochondria.

2.13. Make a discontinuous density gradient by filling a tube from bottom to top with 8 mL of 38%, 5 mL of 34%, 8 mL of 30%, 12 mL of 25% (containing the crude mitochondria from step 2.12), 8 mL of 23%, and 3 mL of 20% density gradient medium, and centrifuge it (52,000 x *g*, 90 min, 4 °C).

2.14. Use a long and blunt syringe to collect the purified mitochondria in the range from the interface between 25% and 30% to the interface between 34% and 38% into a clean tube.

2.15. Add mitochondrial isolation buffer into the collected mitochondria to dilute it to a three-fold volume. Centrifuge (15,000 x *g*, 20 min, 4 °C) and discard the supernatant.

2.16. Add 2 mL of mitochondrial isolation buffer to resuspend the pellet, and centrifuge it (15,000 x *g*, 20 min, 4 °C). Discard the supernatant and keep the pellet.

2.17. Collect the final pellet (i.e., the purified mitochondria) and store it at -20 °C.

NOTE: Combine all purified mitochondria from the control ovarian tissue as the mitochondrial

samples for quantitative proteomics analysis.

3. Verification of the quality of purified tissue mitochondrial samples

3.1. Take a tube of purified mitochondrial samples (i.e., the pellets from the ovarian cancer tissues from step 1.16 and the control ovarian tissues from step 2.17) for verification with electron microscopy (EM).

NOTE: The detailed protocol was described previously[15,16].

3.2. Prepare the mitochondrial protein extraction buffer by mixing 8 M urea, 2 M thiourea, 40 mM Tris, 1 mM EDTA, 130 mM dithiothreitol (DTT), and 4% (w:v) 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS). Adjust the pH to 8.52.

3.3. Take a tube of purified mitochondrial samples (i.e., the pellets from the ovarian cancer tissues in step 1.16 and the control ovarian tissues in step 2.17) and add mitochondrial protein extraction buffer (mitochondrial samples to protein extraction buffer, 1:5) to resuspend the pellet. Freeze the samples in liquid nitrogen 3x and store at room temperature for 2 h.

3.4. Centrifuge at 12,000 x *g*, 30 min, 4 °C, collect the supernatant (i.e., the extracted mitochondrial protein sample), and measure the protein content with a bicinchoninic acid (BCA) protein assay kit.

3.5. Prepare 50 mL of 1.5 M Tris-HCl (pH 8.8) by mixing 9.08 g of Tris and 40 mL of ddH_2O, adjusting the pH to 8.8 using HCl, and adding ddH_2O to a final volume of 50 mL. Store at 4 °C.

3.6. Prepare 50 mL of 1 M Tris-HCl (pH 6.8) by mixing 6.06 g of Tris and 40 mL of ddH_2O, adjusting the pH to 6.8 using HCl, and adding ddH_2O to a final volume of 50 mL. Store at 4 °C.

3.7. Prepare 100 mL of 30% acrylamide-bis solution by mixing 29 g of acrylamide, 1 g of bis-acrylamide, and 80 mL of ddH_2O, and adding ddH_2O to a final volume of 100 mL. Store it in a brown bottle at 4 °C.

3.8. Prepare 1,000 mL of 10x tris-glycine electrophoresis buffer by mixing 29 g of glycine, 58 g of Tris, 3.7 g of sodium dodecyl sulfate (SDS), and 800 mL of ddH_2O, and then adding ddH_2O to a final volume of 1,000 mL.

3.9. Prepare 10 mL of loading buffer by mixing 2 mL of glycerin, 0.02 g of bromophenol blue, 0.4 g of SDS, 2 mL of Tris-HCl pH 6.8, and 7 mL of ddH_2O, and then adding ddH_2O to a final volume of 10 mL.

3.10. Prepare 10 mL of 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) resolving gel by mixing 4 mL of ddH_2O, 3.3 mL of 30% acrylamide-Bis solution, 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 0.1 mL of 10% SDS, 0.1 mL of 10% ammonium persulfate, and

0.004 mL of tetramethylethylenediamine (TEMED). Pour the SDS-PAGE resolving gel solution into the plate between the glass plates to the level of the bottom of the comb. Add 2 mL of isopropyl alcohol onto the top. Leave for 30 min.

3.11. Remove the isopropyl alcohol and wash the top with ddH_2O 3x. Pour the 5% concentration gel solution containing 4.1 mL of ddH_2O, 1.0 mL of 30% acylamide-bis solution, 0.75 mL of 1 M Tris-HCl (pH 6.8), 0.06 mL of 10% SDS, 0.06 mL of 10% ammonium persulfate, and 0.006 mL of TEMED. Immediately insert the comb into the concentration gel solution, leave for 30 min, and then take out the comb.

3.12. Prepare the 1x Tris-glycine electrophoresis buffer by diluting 100 mL of 10x Tris-glycine electrophoresis buffer with 900 mL of ddH_2O and pour into the electrophoretic tank.

3.13. Mix 30 µg of the mitochondrial proteins from the ovarian cancer and control ovarian tissues from step 3.4 with loading buffer to reach a final volume of 20 µL. Boil the mixture for 5 min, then separate it with the 10% SDS-PAGE resolving gel using a constant voltage of 100 V. When the bromphenol blue reaches the bottom, stop the electrophoresis.

3.14. Prepare 1.5 L of electrophoretic transfer buffer by mixing 150 mL of 10x Tris-glycine electrophoresis buffer, 1,050 mL of ddH_2O, and 300 mL of methanol.

3.15. Soak the PVDF membrane in 100% methanol for 10 min and in electrophoretic transfer buffer for at least 5 min. Soak five sheets of filter paper in 1x elecrophoretic transfer buffer for at least 5 min.

3.16. Take out the SDS-PAGE gel containing the proteins, and soak it in electrophoretic transfer buffer for 10 min.

3.17. Make a transfer cassette by placing a wet sponge on the white plate, three sheets of wet filter paper, the PVDF membrane, the SDS-PAGE gel with the proteins, two sheets of wet filter paper, and the wet sponge from the bottom to top. Avoid any bubbles.

3.18. Place the transfer cassette into the electrophoretic tank, pour in the electrophoretic transfer buffer, and then transfer for 2 h under a constant 200 mA current.

3.19. Prepare 10x Tris-buffered saline (TBS) stock solution by mixing 12.114 g of Tris, 29.22 g of NaCl, and adding ddH_2O to a final volume of 500 mL. Prepare 2 L of 1x TBST by mixing 200 mL of 10x TBS, 2 mL of Tween-20, and 1,798 mL of ddH_2O. Prepare 100 mL of blocking solution by mixing 100 mL of TBST and 5 g of BSA.

3.20. After transfer, take out the PVDF membrane, wash lightly in TBST for 5 min, then block it in blocking solution for 1 h at room temperature.

3.21. Incubate the PVDF membrane (4 °C overnight) with different primary antibodies specific

to different subcellular organelles, including lamin B (cell nucleus; goat anti-human antibody 1: 1,000 blocking solution), flotillin-1 (cytomembrane; rabbit anti-human antibody 1:500 blocking solution), COX4I1 (mitochondrion; rabbit anti-human 1:1,000 blocking solution), GM130 (Golgi apparatus; mouse anti-human antibody 1:1,000 blocking solution), catalase (peroxisome; rabbit anti-human antibody 1:1,000 blocking solution), and cathepsin B (lysosome; rabbit anti-human antibody 1:1,000 blocking solution). Lightly wash in TBST for 5 min 3x.

3.22. Incubate the PVDF membrane for 1 h at room temperature with the corresponding secondary antibodies (rabbit anti-goat, goat anti-rabbit, or goat anti-mouse). Lightly wash in TBST for 5 min 3x. Visualize it with electrochemiluminescence (ECL).

4. iTRAQ-SCX-LC-MS/MS analysis

NOTE: The detailed procedures for section 4 refer to the iTRAQ instructions (**Table of Materials**).

4.1. Add SDT buffer (4% SDS, 100 mM Tris-HCl pH 7.6, and 100 mM DTT) to the purified mitochondrial samples (i.e., the pellets from the ovarian cancer tissues from step 1.16 and the control ovarian tissues from step 2.17). Vortex the sample until there is no visible precipitate.

NOTE: The mitochondrial sample to SDT buffer ratio should be 1:5.

4.2. Boil the SDT-treated sample for 5 min, cool down the sample on ice, and then centrifuge (2,000 x *g*, 2 min).

4.3. Collect the supernatant as the extracted mitochondrial protein samples and measure the protein content with a BCA protein assay kit.

4.4. Take SDT-extracted mitochondrial proteins (200 µg of each sample) for reduction, alkylation, digestion with trypsin, desalination, and lyophilization[3,4]. Do three replicates for each sample.

4.5. Treat the tryptic peptides (100 µg of each sample) from step 4.4 with 100 mM tetraethyl ammonium bromide solution (pH 8.5), and label the tryptic peptides with one of the 6-plex iTRAQ reagents according to its instructions[3,4]. Label each sample 3x.

4.6. Mix equally 6 labeled tryptic peptide samples (three from ovarian cancer tissues and three from control ovarian tissues), and dry with a vacuum concentrator.

4.7. Fractionate the mixed iTRAQ-labeled peptides with SCX chromatography into 60 fractions (one fraction per 1 min), then combine every two fractions as a SCX-fractionated sample (n = 30).

4.8. Subject each SCX-fractionated sample to LC-MS/MS analysis on a mass spectrometer[3,4]

coupled with a nano LC system within a 60-min LC separation gradient to obtain MS/MS data.

4.9. Search the MS/MS data to identify proteins with the search engine (**Table of Materials**).

4.10. Use the iTRAQ reporter-ion intensities to quantify each protein and determine mitochondrial differentially expressed proteins (mtDEPs) with a change of >1.5 or <-1.5 fold, and $p < 0.05$.

REPRESENTATIVE RESULTS:

There was a difference in the preparation of the mitochondria from ovarian cancer tissues and control ovarian tissues. This study found that it was much easier to prepare mitochondria from ovarian cancer tissues than from control ovarian tissues[3,4]. Some improvements had to be made to the protocol for the preparation of mitochondria from control ovarian tissues. First, prior to tissue homogenization, it was necessary to add 8 mL of 0.05% trypsin/20 mM EDTA into the PBS solution added to the minced control tissues, followed by digestion for 30 min at room temperature, and centrifugation at 200 x *g* for 5 min (see protocol step 2.5). This improved the preparation of mitochondria. Second, the discontinuous density gradient was different for the preparation of mitochondria from control ovarian tissues and ovarian cancer tissues (**Figure 1**). For ovarian cancer tissues it was prepared by adding 5 mL of 34%, 8 mL of 30%, 12 mL of 25% (containing the crude mitochondria), 8 mL of 23%, and 3 mL of 20% density gradient medium from bottom to top in a tube. The purified mitochondria were found at the interface between 25% and 30% after centrifugation (**Figure 1A**, see protocol steps 1.12 and 1.13). For control ovarian tissues it was prepared by adding 8 mL of 38%, 5 mL of 34%, 8 mL of 30%, 12 mL of 25% (containing the crude mitochondria), 8 mL of 23%, and 3 mL of 20% density gradient medium from bottom to top in a tube. In this case, the purified mitochondria were in the range from the interface between 25% and 30% to the interface between 34% and 38% after centrifugation (**Figure 1B**, see protocol steps 2.13 and 2.14).

The protocol obatined high-quality mitochondrial samples. High quality mitochondrial samples are the prerequisite for quantitative mitochondrial proteomics. This study evaluated the quality of the mitochondria that were prepared with differential-speed centrifugation and density gradient centrifugation via EM (**Figure 2**) and Western blot (WB, **Figure 3**). EM images demonstrated that in both ovarian cancers and control ovarian tissues the main organelles isolated were mitochondria, except for a small quantity of peroxisomes. The morphology of the mitochondria changed more in ovarian cancers than control ovarian tissue (**Figure 2**). WB images demonstrated that the major component in prepared mitochondrial samples from ovarian cancers and control ovaries was mitochondria, except for a small quantity of peroxisomes (**Figure 3**). The WB results were consistent with the EM results. It was reasonable for peroxisomes to be contained in prepared mitochondria, because mitochondria interact extensively with peroxisomes[3,17,18], which in turn reflect the functional completeness of the mitochondria. These results demonstrated the high quality of the prepared mitochondrial samples.

The amount of mitochondrial protein prepared with this protocol was adequate for further

analysis. It is necessary to obtain a sufficient amount of mitochondrial samples from ovarian cancer and control ovarian tissues. This study combined the mitochondrial samples prepared from seven ovarian cancer tissues, and from 11 control ovarian tissues[3]. A total of 2,409 μg of mitochondrial protein sample was obtained for ovarian cancers, and 4,440 μg of mitochondrial protein sample for control ovarian tissue (**Table 1**). Generally, for iTRAQ quantitative proteomics, each sample needs at least 600 μg proteins (200 μg proteins per each iTRAQ labeling, 3 replicates). Therefore, the prepared mitochondrial protein samples were sufficient for iTRAQ quantitative proteomics analysis.

The achievement of the maximum number of quantified proteins benefits the in-depth investigation of mitochondria in human ovarian cancer. This study detected, identified, and quantified 5,115 proteins in ovarian cancers compared to control ovarian tissue, including 2,565 (50.14%) upregulated proteins (ratio of cancers to controls >1) and 2,550 (49.86%) downregulated proteins (ratio of cancers to controls <1)[3] (**Table 2**). Further, this study determined 1,198 mtDEPs between ovarian cancers and control ovaries with >1.5 or <-1.5 fold changes ($p < 0.05$), including 523 (43.66%) upregulated proteins and 675 (56.34%) downregulated proteins[4] (**Table 2**). These data are currently the largest mitochondrial proteome profile in ovarian cancer.

FIGURE AND TABLE LEGENDS:

Figure 1: The crude mitochondria were purified with discontinuous density gradient centrifugation for ovarian cancer (**A**) **and control ovarian** (**B**) **tissues.**

Figure 2: Electron micrograph image of mitochondria isolated from ovarian cancer (**A**) **and control ovarian** (**B**) **tissues.**

Figure 3: Organelle-specific antibody-based Western blot images of mitochondria isolated from ovarian cancer (**A**) **and control ovarian** (**B**) **tissues.**

Table 1: The amount of prepared mitochondrial protein samples.

Table 2: The number of iTRAQ-identified proteins from prepared mitochondrial samples.

DISCUSSION:

Mitochondrial alterations are a hallmark of ovarian cancer. Preparation of high-quality mitochondrial samples from human ovarian cancer and control tissues for large-scale quantitative proteomics benefit the in-depth understanding of mitochondrial function in ovarian cancer pathogenesis and mitochondrial molecular network changes, and help clarify its molecular mechanism for subsequent discovery of target therapy and effective biomarkers based on mitochondria[4,5,8]. The differential-speed centrifugation in combination with density gradient centrifugation effectively isolated and purified mitochondria from human ovarian cancer and control ovarian tissues. The prepared mitochondrial samples were of very high-quality and were suitable for further quantitative proteomics analysis.

The prepared mitochondrial samples contained a small quantity of peroxisomes[3,17,18] and cytosolic proteins[19,20]. This should not be simply considered contamination, because they directly or indirectly interact or adhere with mitochondria to let mitochondria function more completely. Studies have found that mitochondria interact extensively with the actin cytoskeleton[19,20] and peroxisomes[17,18]. It is unavoidable for some cytosolic proteins and peroxisome proteins to be contained in isolated mitochondrial samples.

The key technique to detect, identify, and quantify proteins from the prepared mitochondrial samples was iTRAQ labeling-SCX-LC-MS/MS. This study identified and quantified 5,115 mitochondrial proteins[3], including 1,198 mtDEPs[4,14]. The largenumber of mitochondrial proteins found in the ovarian cancer tissues includes ones that can help to understand the role of mitochondria in ovarian cancer pathogenesis and also be a resource for the discovery of personalized target therapy based on mitochondrial metabolism[8], and even finding effective biomarkers based on mitochondrial genomics, proteomics, and metabolomics from a systematic multi-omics angle[9,11–13]. Moreover, with the introduction of proteoform and protein species concepts in the proteome, in-depth exploration of mitochondrial proteoforms or protein species might directly lead to the discovery of effective and reliable biomarkers and therapeutic targets for ovarian cancer[10,21,22].

Furthermore, the present protocols in analysis of human ovarian cancer tissue mitochondrial proteomes described here are easily translated to study other human disease mitochondrial proteomes.

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DISCLOSURES:

The authors have nothing to disclose.

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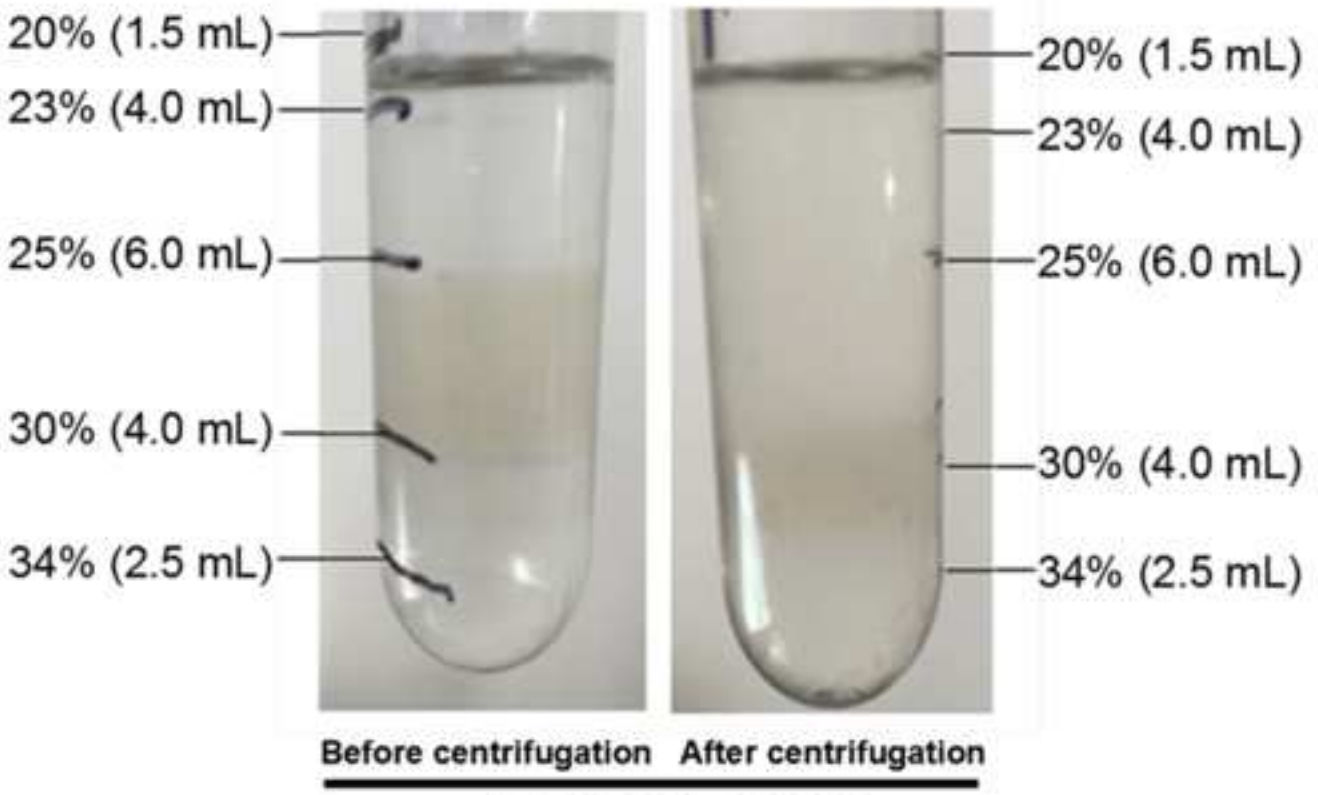
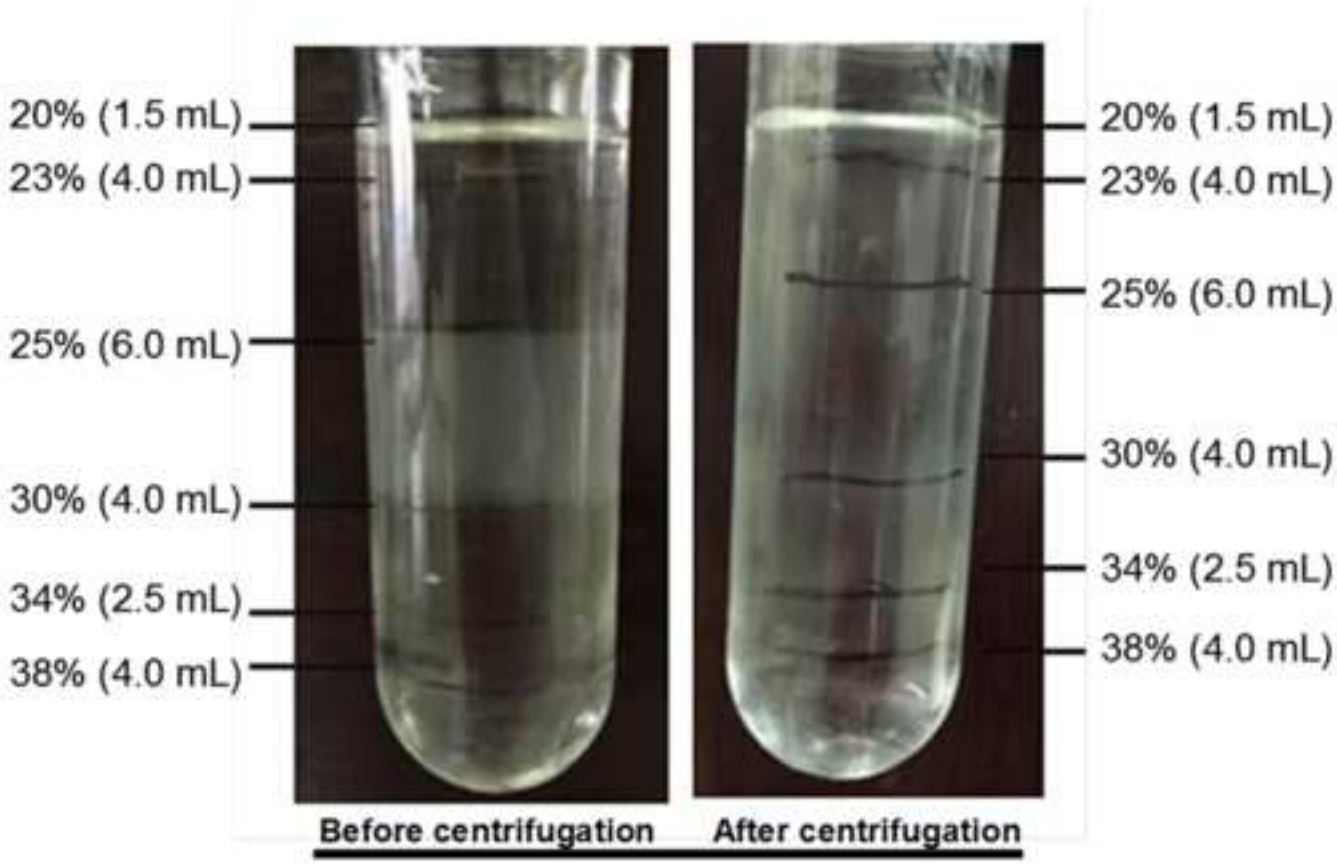
529 large-scale study of human proteoforms. *Med One*. **3**, e180008 (2018).

Figure 1

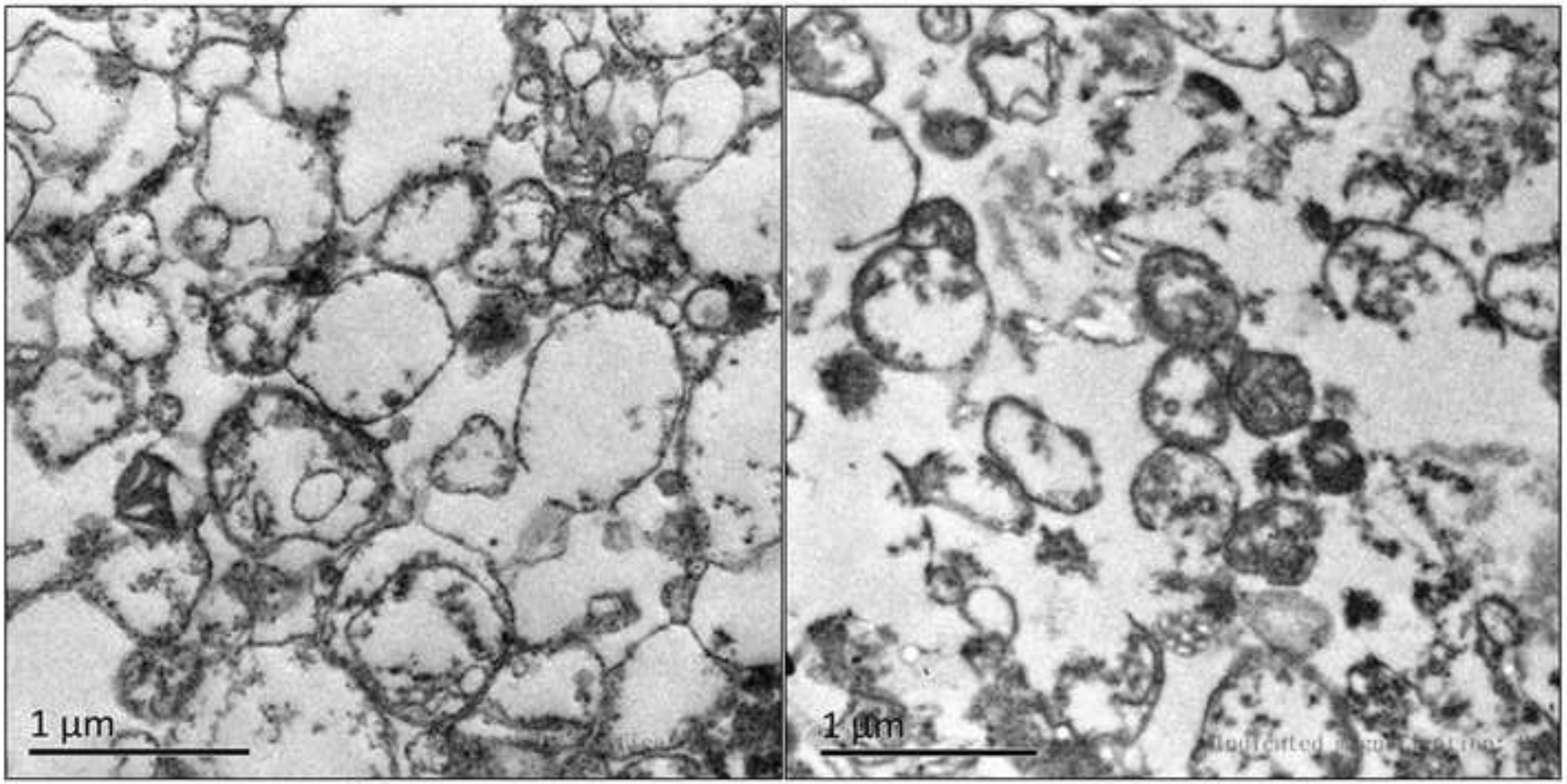


A. Ovarian cancer



B. Control ovary

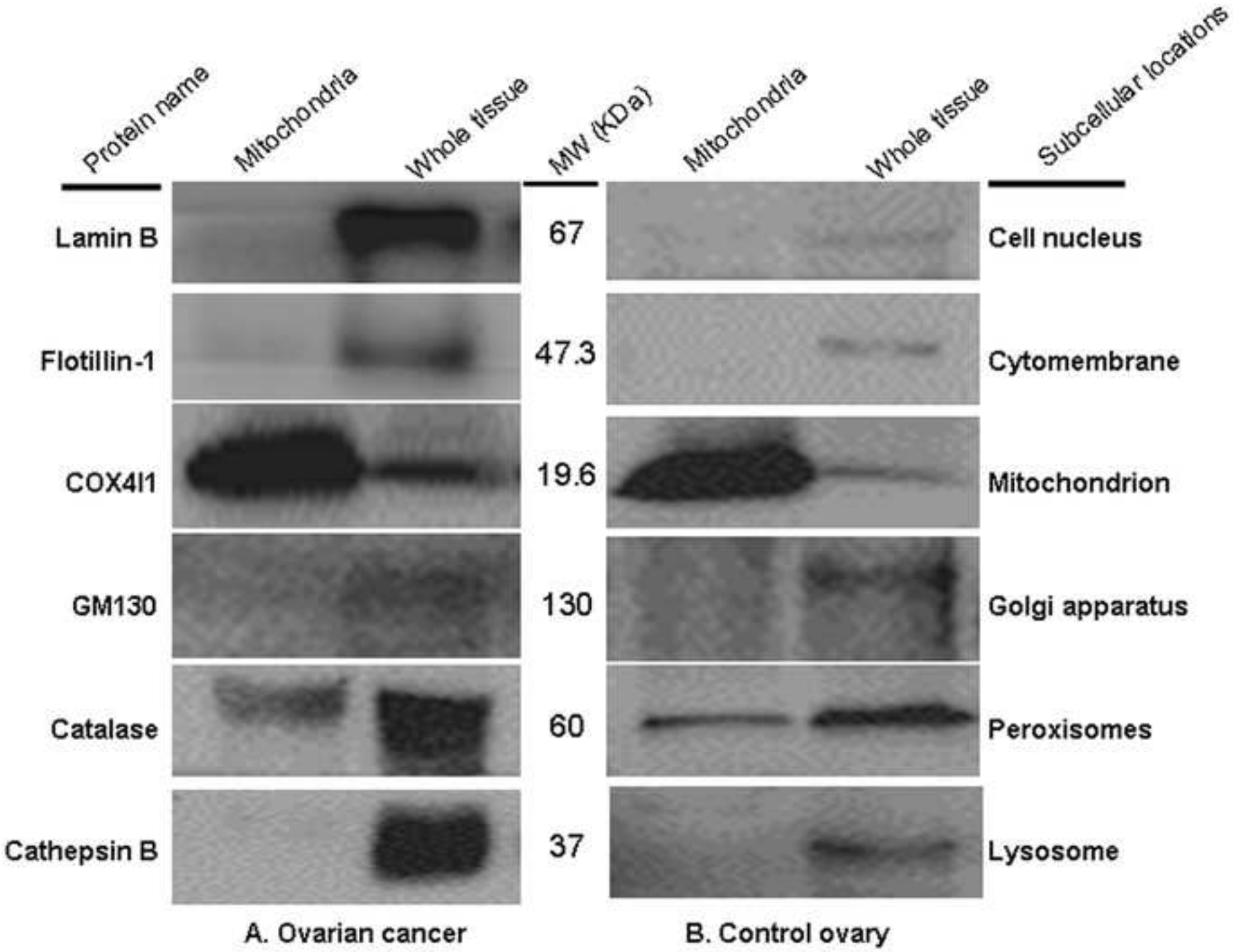
Figure 2



A. Ovarian cancer

B. Control ovary

Figure 3



Mitochondrial protein sample	Volume (µL)	Concentration (µg/µL)	Proteins (µg)
Ovarian cancer tissue	530	4.545	2,409
Control ovarian tissue	750	5.92	4,440

Table 2

Category	The number of total proteins*	The number of differentially expressed proteins#
Up-regulation	2,565 (50.14%)	523 (43.66%)
Down-regulation	2,550 (49.86%)	675 (56.34%)
Total	5,115 (100.0%)	1,198 (100.0%)

*Ratio of cancers to controls is >1 for up-regulation, <1 for down-regulation.

#Ratio of cancers to controls is >1.5 fold for up-regulation, and <-1.5 fold for

down-regulation.

Name of Material/ Equipment	Company	Catalog Number
BCA protein assay kit	Vazyme	E112
Bovine serum albumin (BSA)	Solarbio	A8020-5G
Centrifuge	XiangYi	TDZ4--WS
CHAPS	Sigma	C9426-5G
Diamine tetraacetic acid (EDTA)	Sigma	798681-100G
DTT	Sigma	10197777001
Easy nLC	Proxeon Biosystems (now Thermo Fisher Scientific)	
Ethylen glycol bis(2-aminoethyl ether)tetraacetic	Sigma	E0396-10G
Homogenizer	SilentShake	HYQ-3110
iTRAQ reagent kit	Applied Biosystems	
Low-temperature super-speed centrifuger	Eppendorf	5424R
Mannitol	Macklin	M813424-100G
MASCOT search engine	Matrix Science, London, UK; version 2.2	
Nagarse	Solarbio	P9090
N-hydroxysuccinimide (SDT)	Sigma	56480-25G
Nycodenz	Alere/Axis-Shield	1002424-1
Phenylmethanesulfonyl fluoride (PMSF) protease inhibitor	Solarbio	P0100-1ML
Potassium chloride	Macklin	P816354-25G
Proteome Discover 1.4	Matrix Science, London, UK	

PVDF membrane	Millipore	05317
Q Exactive mass spectrometer	Thermo Fisher Scientific	
SCX column	Sigma	58997
Sodium orthovanadate (V)	Macklin	S817660-25G
Sucrose	Macklin	S824459-500G
Thiourea	Sigma	62-56-6
Tris base	Sigma	10708976001
Trypsin (cell culture use)	Gibco	25200-056
Urea	Sigma	U5378-100G

Comments/Description
BCA protein assay kit is a special 3-component version of our popular BCA reagents, optimized to measure (A562nm) total protein concentration of dilute protein solutions (0.5 to 20
Heat shock fraction, Australia origin, protease free, low fatty acid, low IgG, pH 7, ≥98%
BioReagent, suitable for electrophoresis, ≥98% (HPLC) (Sigma-Aldrich) Anhydrous, free-flowing, Redi-Dri, ≥98%
1,4-Dithiothreitol
BioXtra, ≥97 .0%
Applied Biosystems iTRAQ Reagents–Chemistry Reference Guide, P/N 4351918A
Mannitol is a polyol (polyhydric alcohol) produced from hydrogenation from fructose that functions as a sweetener, humectant, and bulking agent. It has low hygroscopicity and poor oil solvency.
Purum, ≥97.0% (T)
PMSF is a protease inhibitor that reacts with serine residues to inhibit trypsin, chymotrypsin, thrombin, and papain.
Potassium chloride, KCI, also known as potassium muriate and sylvite, is a colorless crystalline solid with a salty taste that melts at 776°C (1420 OF). It is soluble in water, but insoluble in alcohol. Potassium chloride is used in fertilizers, pharmaceuticals, photography, and as a salt

It is 1 roll, 26.5 cm x 1.875 m, 0.45 µm pore size, hydrophobic PVDF transfer membrane with low background fluorescence for western blotting. It is compatible with visible and infrared fluorescent probes.

It is 5-µm particle size, length 5 cm × i.d. 4.6 mm (Supelco).

Sodium orthovanadate (Vanadate) is a general competitive inhibitor for protein phosphotyrosyl phosphatases. The inhibition by sodium orthovanadate is reversible upon the addition of EDTA or by dilution.

Vetec reagent grade, 99%

ACS reagent, ≥99.0%

TRIS base is useful in the pH range of 7.0-9.0. It has a pKa of 8.1 at 25°C.

This liquid formulation of trypsin contains EDTA and phenol red. Gibco Trypsin-EDTA is made from trypsin powder, an irradiated mixture of proteases derived from porcine pancreas. Due to its digestive strength, trypsin is widely used for cell dissociation, routine cell culture passaging, and primary tissue dissociation.

powder, BioReagent, for molecular biology, suitable for cell culture

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Author(s): Xianquan Zhan, Huanni Li, Shehua Qian, Xiaohan Zhan, Na Li.

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