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Sample Preparation for Proteomic Analysis of Isolated Mitochondria and Whole-Cell Extracts

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Christos Themistokleous[1], Miratul M K Muqit[1]

[1]Medical Research Council Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK

Christos Themistokleous
University of Dundee

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We use this protocol and it's working

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Abstract

Mass spectrometry-based proteomics relies on precise sample preparation to ensure reliable analysis of proteomic changes, including post-translational modifications (PTMs). This protocol outlines a streamlined workflow for processing isolated mitochondria (or other organelles) and whole-cell extracts from cultured cells or mouse tissues. Key steps include robust lysis with 2% SDS, high-energy sonication, and protein capture on S-trap columns to remove interfering substances. Optimized trypsin/Lys-C digestion and sequential peptide elution enable high-quality mass spectrometry data acquisition. This method is adaptable for diverse proteomic and PTM studies.

Materials

Equipment

- Bioruptor, Diagenode
- DynaMag™ Magnet, Thermofisher scientific, #12320D or #12321D

Equipment

Magnet — NAME

DynaMag™-2 Magnet — TYPE

Thermo Fisher — BRAND

12321D — SKU

https://www.thermofisher.com/order/catalog/product/12321D — LINK

- Block heater
- Microcentrifuge
- ThermoMixer, Eppendorf
- SpeedVac vacuum concentrator, Thermo Scientific
- Ultrasonic bath sonicator

Materials

- Sodium Dodecyl Sulfate (SDS), C12 **Thermo Fisher Catalog #**28312
- HEPES, #15630106
- Triethylammonium bicarbonate buffer (TEAB), #T7408
- Trifluoroacetic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T6508
- Formic acid, #10780320
- Acetonitrile (ACN), VWR #10055454
- Bond-Breaker™ TCEP Solution, Neutral pH **Thermo Fisher Scientific Catalog #**77720
- Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**144-48-9

- Methanol **Thermofisher Catalog #**A456-1
- LC-grade Water **Fisher Scientific Catalog #**10777404
- Pierce™ Trypsin/Lys-C Protease Mix, MS-Grade **Thermo Fisher Catalog #**A41007
- cOmplete™ EDTA-free Protease Inhibitor Cocktail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11873580001
- PhosSTOP **Merck Catalog #**4906845001
- Benzonase nuclease **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E1014-5KU
- Micro BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #**23235
- SafeSeal reaction tube 1.5 ml PP PCR Performance Tested Low protein-binding **Sarstedt Catalog #**72.706.600
- 2ml tubes
- TipOne bevelled 1000µl, 200µl and 20µl pipette tips (Starlab).
- S-Trap™ Micro Columns

Protein Solubilisation Quantification

1h 20m 30s

1

Note

This step continues from the cell and tissue Mito-IP protocols, where you ended with Mito-IP, Control-IP, and whole-cell input samples. It covers sample preparation for proteomic analysis of isolated mitochondria and whole-cell extracts.

Mito-IP Immunopurification of mitochondria from MitoTag cells:
DOI: dx.doi.org/10.17504/protocols.io.n2bvj9q8blk5/v1

Tissue Mito-IP Immunopurification of mitochondria from MitoTag mice:
DOI: dx.doi.org/10.17504/protocols.io.5qpvo98rdv4o/v1

2 Resuspend the whole-cell input samples and the dry bead slurry of Mito-IP or Control-IP in 100 µL of HEPES lysis buffer.

- Lysis buffer: [M] 20 millimolar (mM) HEPES pH 8 in water, 2% v/v SDS, Protease inhibitors, Phosphatase inhibitors.

3 Incubate On ice for 00:10:00 .

10m

4 Place the IP samples on a block heater for a few seconds to reduce SDS precipitation. Immobilise the beads by placing the tubes on a magnetic separator (e.g., Dyna-Mag) for 00:00:30 and move the supernatant into a new 1.5 ml tube.

30s

5 Sonicate IP and input samples using a bioruptor to lyse and dissolve proteins.

- Bioraptor settings: At high energy, 15 cycles, 30 sec on 30 sec off, 4 °C .

Note

If the samples are gelatinous, Benzonase should be included in the Lysis buffer to break down the DNA. This is helpful if the samples are from cultured cells.

6 Clarify the input samples by centrifugation at 17000 x g, 00:10:00 and move the supernatant into a new tube. 10m

7 **Quantify using a micro-BCA assay kit.**

7.1 Create albumin protein standards (1000, 750, 500, 250, 125, 62.5, 31.25, 15.6, 0 ng/µl) by diluting them in lysis buffer.

7.2 In a 384-well plate, pipette 5 µL of the sample and standards into wells in duplicates. Use input dilution 1:10 (for tissues) or 1:5 (for cells). Do not dilute the IP samples.

7.3 Mix the BCA Reagent A and B at a ratio of 50:1.

7.4 Add 40 µL of the BCA reagent mix to each of the wells.

7.5 Incubate at 37 °C for 01:00:00 . 1h

7.6 Record the 562 nm absorbance of the plate.

7.7 Calculate the concentration of the samples using a standard curve.

8 Move 5 µg of IP and input samples in a new tube to begin the S-trap protocol.

Reduce and Alkylate 1h

9 **Reduction:** 30m

Add TCEP to the samples to a final concentration of [M] 5 millimolar (mM) and place on a thermomixer at 1100 rpm, 60°C, 00:30:00 .

10 Cool samples to Room temperature and turn the thermomixer down to 25 °C.

11 **Alkylation:** 30m

Add IAA to the samples to a final concentration of [M] 40 millimolar (mM) and place on a thermomixer at 1100 rpm, 25°C, 00:30:00 and shielded from light.

12 **Optional:** Spin IP samples down, max speed for 5 min. Move supernatant into a new tube (this is to ensure total removal of any debris from the magnetic beads, as they can clog the column of the Mass Spectrometer).

13 Add SDS to a final concentration of 5% (v/v).

14 **Acidification:**

Add TFA to a final concentration of 1% (v/v).

Trap Proteins 2m

15 Add 6x the current volume of S-Trap Wash Buffer and mix well.

Wash buffer: [M] 100 millimolar (mM) TEAB (final) in 90% methanol. Dilute the above TEAB stock with MeOH: e.g. to 1 mL [M] 1 Mass Percent TEAB, add MeOH until the final volume is 10 mL.

16 Place S-trap columns on 2 ml tubes to receive waste flow-through.

17 **Apply the sample to the column.**

17.1 Load 150 µL sample onto the S-trap column and spin for 1000 x g, 00:01:00. 1m

17.2 Repeat until all the sample has passed through the column.

18 **Clean protein:**

18.1 150 µL wash with washing buffer.

18.2 Spin for 1000 x g, 00:01:00 . 1m

18.3 Repeat for a total of 5 washes.

> Note
>
> Discard the flowthrough before reaching the column.

19 Transfer the column to a new 1.5 ml tube.

Incubate and Digest Protein 9h

20

> Note
>
> It is recommended to have 1:10 ratio of trypsin e.g. for 10 µg of protein you would supplement with 1 µg of Trypsin + Lys C. For S-Trap micro columns, it is recommended to have at least 1 µg of trypsin irrespective of sample amount i.e. for anything 10 µg less starting material.

21 **Digestion buffer:**

Dissolve Trypsin/Lys-C Mix in [M] 50 millimolar (mM) TEABC solution, to 25 µL concentration.

22 **On-column digestion:**

22.1 Add 60 µL (1.5 µg) Trypsin/Lys-C Mix on the column.

22.2 Quick spin for the buffer to wet and pass through the column.

22.3 Recollect it from the tube and add it back on the column. Release it as close as possible to the filter to prevent bubbles without touching the matrix. Remove any air bubbles atop the trap by flicking the column. Then, cap the column loosely.

23 Incubate on thermomixer with a cap at 47 °C for 01:00:00 and then at 22 °C Overnight with no agitation. 9h

Peptide Elution 4m

24 Spin down the columns for 1000 x g, 00:01:00 . 1m

25 **Elution 1:** Add 60 µL TEAB [M] 50 millimolar (mM) and spin 1000 x g, 00:01:00 . 1m

26 **Elution 2:** Add 60 µL 0.15% Formic acid & spin 1000 x g, 00:01:00 . 1m

27 Move the column to a new tube and keep the flow-through.

28 **Elution 3:** Add 60 µL elution buffer (80% ACN, 0.15% formic acid) & spin 1000 x g, 00:01:00 . 1m

29 Repeat the step 28 (elution 3).

30 Pool together both flowthroughs with the eluted peptides and place the tubes on dry ice.

31 Vacuum-dry the samples and store them at -80 °C or continue for injection preparation.

Prepare Samples for Injection

46m

32 Resuspend the samples in 60 µL LC buffer containing 3% (v/v) ACN and 0.1% (v/v) FA in LC-MS grade H_2O.

33 Incubate the samples on a thermomixer for 00:30:00 at Room temperature . 30m

34 Sonicate using the water bath sonicator for 00:15:00 . 15m

35 Spin down for 00:01:00 , max speed. 1m

36 **Optional:** Estimate peptide concentration of each sample using a NanoDrop instrument by measuring the solution absorbance A280 at 224 nm wavelength. *

37 Move samples to appropriate vials and inject into the Mass Spectrometer or store them in a freezer until injection.