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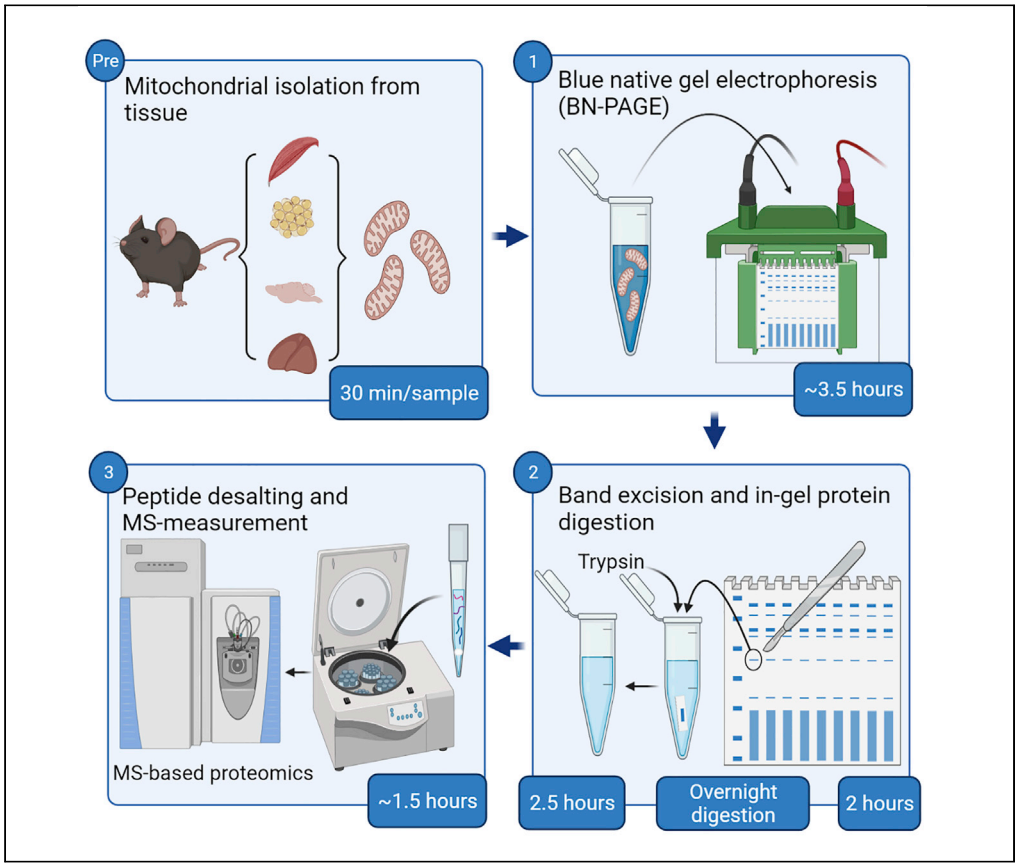
Protocol to characterize mitochondrial supercomplexes from mouse tissues by combining BN-PAGE and MS-based proteomics



The assembly of mitochondrial respiratory complexes into supercomplexes has significant implications for mitochondrial function. This protocol details mitochondrial isolation from mouse tissues and the use of blue native gel electrophoresis (BN-PAGE) to separate pre-identified mitochondrial supercomplexes into different gel bands. We then describe the excision of the individual bands, followed by in-gel protein digestion and peptide desalting for mass spectrometry (MS)-based proteomics. This protocol provides a time-efficient measurement of the abundance and distribution of proteins within known supercomplexes.

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Highlights

Protocol to study mitochondrial supercomplexes composition from mouse tissue

MS-based proteomics of visible mitochondrial supercomplexes separated on BN-PAGE

Measurement of the abundance and distribution of proteins within known supercomplexes

This protocol can be applied to protein complexes visible by Coomassie blue staining

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Protocol to characterize mitochondrial supercomplexes from mouse tissues by combining BN-PAGE and MS-based proteomics

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SUMMARY

The assembly of mitochondrial respiratory complexes into supercomplexes has significant implications for mitochondrial function. This protocol details mitochondrial isolation from mouse tissues and the use of blue native gel electrophoresis (BN-PAGE) to separate pre-identified mitochondrial supercomplexes into different gel bands. We then describe the excision of the individual bands, followed by in-gel protein digestion and peptide desalting for mass spectrometry (MS)-based proteomics. This protocol provides a time-efficient measurement of the abundance and distribution of proteins within known supercomplexes.

For complete details on the use and execution of this profile, please refer to Gonzalez-Franquesa et al. (2021).

BEFORE YOU BEGIN

This protocol contains a preliminary step to isolate mitochondria from muscle tissue (Figure 1). However, mitochondria can also be isolated from other tissues. If mitochondria are already isolated and/or frozen, you can proceed directly to "step-by-step method details".

All animal experiments were approved by the Danish Animal Experimental Inspectorate and complied with the European Convention for the Protection of Vertebrate Animals Used for Scientific Purposes. Animals used in these experiments were 10- to 14-week old C57BL/6JBomTac female mice from Taconic.

Mitochondrial isolation from muscle tissue

Timing: 30 min/sample

This step is adapted from the protocol described in (Tonkonogi and Sahlin, 1997)

1. Prepare the buffers and tubes needed for the procedure (see materials and equipment).

Note: Homogenizer, pestle, and scissors have to be washed first in distilled water (ddH_2O), then in 0.1 M HCl to remove ions, and finally by 20-30 washes in ddH_2O. Fill the homogenizer tube with isolation buffer and keep the scissors in a 1.5 mL tube also filled with isolation buffer. Both tubes should be maintained cold on ice.

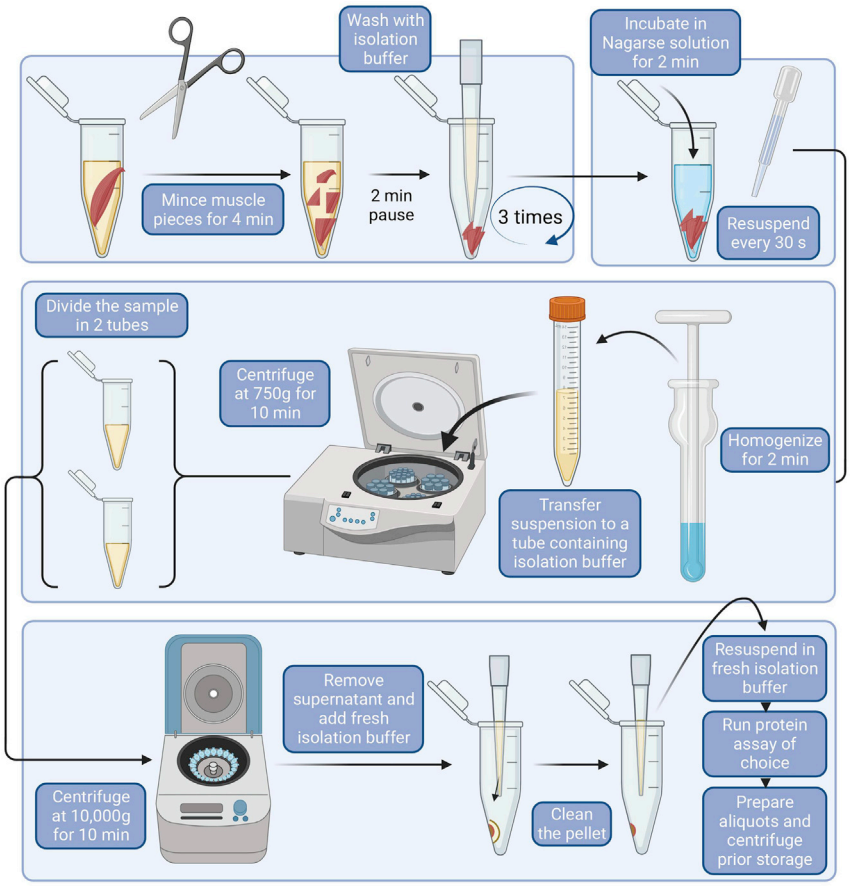

Figure 1. Mitochondrial isolation schematic workflow
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2. Place the muscle in a 2 mL tube containing 1 mL of isolation buffer.

   Note: The expected mitochondrial yield per mg of muscle tissue is approximately 500 μg of mitochondrial protein from 100 mg of muscle tissue. Therefore, from 100 mg of muscle tissue, 6 aliquots of 75 μg of mitochondrial protein can be isolated. For mouse skeletal muscle this equates to approximately half a *quadriceps*, one *triceps brachii* or *tibialis anterior* muscle per tube, or two *soleus* or *extensor digitorum longus* muscles per tube.

   ⚠ CRITICAL: From this step on, all actions should be performed on ice. All time periods should be carefully followed.

   Note: If the procedure is combined with mitochondrial functional assays, it is recommended that all tubes are left open to avoid changes in pressure inside the tube that could affect the viability of mitochondria.

3. Use scissors to mince the muscle inside the tube for 4 min. The resulting muscle pieces should be approximately 1–2 mm in length.
4. Allow the muscle pieces to settle in the bottom of the tube for 2 min. Carefully remove the supernatant using a pipette and flush the muscle pieces with 1 mL of isolation buffer. Repeat this step two more times for a total of three washes.

5. Remove the supernatant and add 1 mL of Nagarse solution. Incubate for 2 min and carefully re-suspend every 30 s using a Pasteur pipette.

   Note: The muscle pieces tend to precipitate to the bottom of the tube, therefore it is necessary to resuspend the pieces every 30 seconds to ensure an optimal digestion

6. Transfer the suspension to the Potter-Elvehjem glass vessel using the Pasteur pipette.

   Note: Make sure that all the muscle pieces are transferred into the glass vessel. If needed, add 500 μL of Nagarse solution to resuspend the remaining fragments.

7. Connect the Potter-Elvehjem plain plunger to the VOS 16 Overhead stirrer and set to speed level 3 (approximately 360 rpm). Insert the plain plunger into the glass vessel and homogenize for 15 s followed by 5 s breaks for a total of 2 min. During homogenization continuously move the glass vessel up and down.

   Note: The plain plunger needs to fit tightly into the homogenization vial, while any overhead stirrer that can connect to the plain plunger and rotate at similar speeds can be used.

8. Transfer the suspension into a 15 mL tube containing 3 mL of isolation buffer. Centrifuge the tube (750g, 10 min, 4°C).
9. Transfer the supernatant into two 2 mL tubes and spin down (10,000g, 10 min, 4°C).

   Note: Mitochondria should be visible as a brown pellet in the bottom of the tube surrounded by clear cell debris. The solution is divided into two tubes to facilitate pellet clean up.

10. Remove the supernatant and add 650 μL of isolation buffer. Clean the aureole of cell debris from the pellet by gently pipetting buffer onto the surroundings of the pellet using a 10–100 μL pipette set at maximum of 60 μL of volume. It is important that the mitochondrial pellet is not resuspended during the clean-up. Discard the supernatant containing the cell debris.

    ⚠ CRITICAL: If when cleaning the pellet it is accidentally resuspended into solution, repeat the centrifugation in step 9 and resume pellet clean up (step 10).

11. Add 650 μL of fresh isolation buffer and dissolve the mitochondrial pellet by gentle mixing with a p100 or p200 pipette. Collect both solutions into one 1.5 mL tube.
12. Determine the protein concentration using protein assay of your choice and prepare aliquots containing 75 μg of mitochondrial protein.

    Note: Please note that isolation buffer contains 0.2% of BSA. Correct sample protein concentration by subtracting the value from a blank measurement of only the isolation buffer.

13. Spin down the aliquoted tubes (7,000g, 4 min, 4°C). Ensure that the mitochondrial pellet is visible and remove the supernatant.

    ⏸ **Pause point:** At this stage, mitochondrial pellets can be stored at −80°C and returned to at a later date for electrophoresis and/or additional experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Murine Skeletal muscles e.g. triceps brachii (C57BL/6JBomTac mus musculus, 0-14 weeks old, female)	This study	N/A
Isolated mitochondria from murine triceps brachii (C57BL/6JBomTac mus musculus, 0–14 weeks old, female)	This study	N/A
Chemicals, peptides, and recombinant proteins		
Protease from Bacillus licheniformis Type VIII, lyophilized powder, 7–15 units/mg solid (Nagarse protease)	Sigma-Aldrich	Cat#P5380-25MG
Acetonitrile (ACN)	Merck	Cat#1.00030.2500
Formic Acid	Thermo Fisher Scientific	Cat#28905
Methanol	Sigma-Aldrich	Cat#34860-2.5L-R
Trifluoroacetic Acid (TFA)	Merck	Cat#8082600501
Acetic Acid	Sigma-Aldrich	Cat#33209-5L
Ethanol 96%	VWR	Cat#20823.362
Trypsin from porcine pancreas Proteomics Grade, BioReagent, Dimethylated	Sigma-Aldrich	Cat#T6567
Ammonium Bicarbonate (ABC)	Sigma-Aldrich	Cat#09830-500G
Sucrose	Millipore	Cat#84100
Potassium Chloride (KCl)	Sigma-Aldrich	Cat#P9541-500G
Tris Hydrochloride (Tris-HCl)	Roche	Cat#10812846001
Potassium Dihydrogen Phosphate (KH_2PO_4)	Supelco	Cat#1048730250
Ethylene Glycol bis (2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)	Millipore	Cat#4100-50GM
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat#A7030-100G
Critical commercial assays		
NativePAGE Sample Prep Kit, containing 5% Digitonin, NativePAGE 4X Sample Buffer, and NativePAGE 5% G-250 Sample Additive	Invitrogen	Cat#BN2008
NativePAGE Running Buffer (20X)	Invitrogen	Cat#BN2001
NativeMark Unstained Protein Standard	Invitrogen	Cat#LC0725
Coomassie Brilliant Blue G-250	SERVA	Cat#17524
NativePAGE 3–12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 15-well	Invitrogen	Cat#BN1003BOX
Other		
XCell SureLock Mini-Cell	Invitrogen	Cat#EI0001
PowerPac Basic Power Supply	BIO-RAD	Cat#1645050
Prot/Elec Pipet Tips, 1–200 µL	BIO-RAD	Cat#2239915
Heraeus Multifuge X3R with 15 mL tube rotor and plate rotor	Thermo Fisher Scientific	Cat#75004515
Centrifuge 5427 R - Microcentrifuge	Eppendorf	Cat#5409000210
Glass Homogenizer and Overhead Stirrer	N/A	N/A
SPE C18 Disks (C18 filters)	Empore	Cat#66883-U
VOS 16 Overhead stirrer	VWR	N/A
Tissue grinder Potter-Elvehjem type 5 mL, glass vessel	VWR	Cat#432-0201
Tissue grinder Potter-Elvehjem type 5 mL, plain plunger	VWR	Cat#432-0207
Centrifuge concentrator plus with plate and tube rotor	Eppendorf	Cat#5305000304
Nanodrop OneC Spectrophotometer	Thermo Fisher Scientific	Cat#ND-ONEC-W
Eppendorf ThermoMixer C	Eppendorf	Cat#5382000015
Eppendorf SmartBlock plates	Eppendorf	Cat#5363000039
Eppendorf SmartBlock 2.0 mL	Eppendorf	Cat#5362000035
Prot/Elec Pipet Tips, 1–200 µL	BIO-RAD	Cat#2239916
Chemidoc XRS	BIO-RAD	Cat#1708265

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
HPLC system, e.g., EASY-nLC nano-flow UHPLC	Thermo Fisher Scientific	Cat#LC140
ReproSil-Pur C18-aq 1.9 µm beads	Dr. Maisch	Cat#r119.aq
Mass spectrometer, e.g., LTQ Q-Exactive HFX Orbitrap	Thermo Fisher Scientific	Cat#0726042
Software and algorithms		
ImageLab Software	BIO-RAD	Cat#1709690 www.bio-rad.com/product/image-lab-software?ID=KRE6P5E8Z

MATERIALS AND EQUIPMENT

Mitochondrial isolation

Isolation buffer		
Reagent	Final concentration	Amount
MQ-H_2O	n/a	500 mL
Sucrose	100 mM	17.115 g
KCL	100 mM	3.728 g
Tris-HCl	50 mM	3.728 g
KH_2PO_4	1 mM	0.068 g
EGTA	0.1 mM	0.019 g
BSA	0.2%	1 g
Total	n/a	500 mL
Prepare fresh.		

Nagarse solution		
Reagent	Final concentration	Amount
Bacillus licheniformis Type VIII (7–15 units/mg)	0.2 mg/mL	2 mg
Isolation buffer (above)	n/a	10 mL
Total	n/a	10 mL
Prepare fresh.		

BN-PAGE gel electrophoresis

Native PAGE anode buffer		
Reagent	Final concentration	Amount
MQ-H_2O	n/a	950 mL
NativePAGE™ Running Buffer (20X)	1X	50 mL
Total	n/a	1,000 mL

Dark blue cathode buffer		
Reagent	Final concentration	Amount
Native PAGE anode buffer 1X	n/a	220 mL
Coomassie Brilliant Blue G/250	1X	0.044 g
Total	n/a	220 mL

Light blue cathode buffer		
Reagent	Final concentration	Amount
Native PAGE anode buffer 1X	n/a	200 mL
Dark blue cathode buffer	n/a	20 mL
Total	n/a	220 mL

Note: All buffers should be at 4°C before use. It is preferable to prepare the BN-PAGE electrophoresis buffers fresh on the experimental day, although they can be prepared the day before and stored at 4°C. Both dark and light blue cathode buffers should be discarded after use.

Proteomics sample preparation

Note: Unless indicated, all buffers in this section can be stored at room temperature (RT; 15/25°C) and are stable for several months.

50mM Ammonium Bicarbonate (ABC)		
Reagent	Final concentration	Amount
MQ-H_2O	n/a	1L
ABC	50mM	3.953 g
Total	n/a	1 L

50 mM Acetic Acid		
Reagent	Final concentration	Amount
MQ-H_2O	n/a	997.14 mL
Acetic Acid	50mM	2.86 mL
Total	n/a	1 L

Trypsin solution 0.5 μg/μL		
Reagent	Final concentration	Amount
50 mM Acetic Acid (above)	50 mM	2 mL
Trypsin	0.5 μg/μL	1 mg
Total	n/a	2 mL

Prepare aliquots of 25 and/or 100 μL that can be stored at −20°C for several months. Once an aliquot has been thawed, it can now be stored at 4°C for a maximum of two weeks.

ABC/Ethanol 1:1		
Reagent	Final concentration	Amount
50mM ABC (above)	n/a	10 mL
Ethanol 96%	n/a	10 mL
Total	n/a	20 mL

Buffer 1		
Reagent	Final concentration	Amount
MQ-H_2O	67%	67 mL
ACN	30%	30 mL
TFA	3%	3 mL
Total	n/a	100 mL

Buffer 2		
Reagent	Final concentration	Amount
MQ-H_2O	19.5%	19.5 mL
ACN	80%	80 mL
Acetic Acid	0.5%	0.5 mL
Total	n/a	100 mL

Buffer 3		
Reagent	Final concentration	Amount
MQ-H_2O	99.5%	99.5 mL
Acetic Acid	0.5%	0.5 mL
Total	n/a	100 mL

Digestion buffer		
Reagent	Final concentration	Amount
50 mM ABC (above)	n/a	1.5 mL
Trypsin solution 0.5 μg/μL (above)	0.005 μg/μL	15 μL
Total	n/a	1.515 mL
Prepare fresh. Store at 4°C for a maximum of two weeks.		

Elution buffer		
Reagent	Final concentration	Amount
MQ-H_2O	39.5%	39.5 mL
ACN	60%	60 mL
Acetic Acid	0.5%	0.5 mL
Total	n/a	100 mL
Prepare fresh. Discard after use.		

A* buffer		
Reagent	Final concentration	Amount
MQ-H_2O	94.9%	94.9 mL
ACN	5%	5 mL
TFA	0.1%	0.1 mL
Total	n/a	100 mL

STEP-BY-STEP METHOD DETAILS

Blue native-polyacrylamide gel electrophoresis (BN-PAGE)

Timing: between 2.5–3 h (day 1)

This step allows for the separation of mitochondrial supercomplexes based on their molecular weight (Acín-Pérez et al., 2008). Using a molecular weight standard and Coomassie staining, the bands containing mitochondrial supercomplexes can be visualized and the protein composition of the different supercomplexes can be inferred (Figure 2).

This step is adapted from the protocol described in Jha et al. (2016)

1. Prepare the samples using 75 μg of mitochondrial protein as starting material.

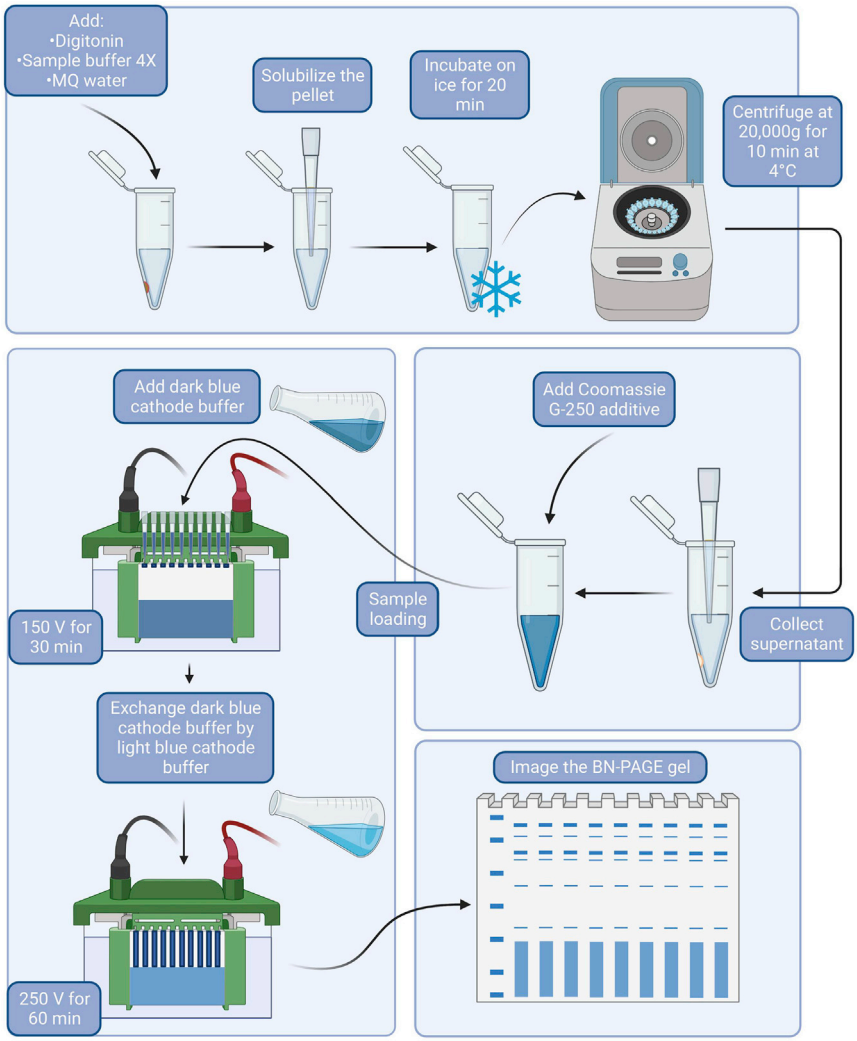


Figure 2. BN-PAGE schematic workflow
Created with BioRender.com

a. Add 6 μL of digitonin stock to the mitochondrial pellet (for a final concentration of 1.5% digitonin).
b. Add 5 μL of NativePAGE™ 4x Sample Buffer.
c. Add 9 μL of MQ-H_2O to make a final volume of 20 μL.

⚠ CRITICAL: The digitonin concentration should be adjusted depending on the protein concentration, such that the digitonin/protein ratio is kept between 4-8 g/g to allow for efficient solubilization of membrane proteins. In this protocol, we use 6 μL of a 5% digitonin stock, which corresponds to a digitonin/protein ratio of 4 g/g. For tissues with high mitochondrial content (e.g. heart), 8 g/g digitonin has been reported to give a better band separation and resolution than 4 g/g digitonin (Jha et al., 2016). For other applications, it is recommended to run a titration prior to experimentation.

2. Solubilize the pellet by gentle mixing with a 20p pipette.

Note: Avoid the formation of bubbles while mixing.

3. Incubate the solubilized mitochondria on ice for 20 min.
4. Centrifuge (20,000g, 10 min, 4°C)
   a. Collect 15 μL of the supernatant and transfer it into new tubes.
5. Add 1.5 μL of NativePAGE™ 5% G-250 Sample Additive sample additive to the collected supernatant.

   Note: The Coomassie G-250 sample additive should be such that the final G-250 concentration is $^1/_4$ the digitonin concentration. In this case, if 6 μL of 5% digitonin were used, the volume of NativePAGE™ 5% G-250 Sample Additive should be 1.5 μL.

6. Set up the electrophoresis system (XCell SureLock Mini-Cell) and place the NativePage 3–12% gradient gel inside the cell (Figure 3).

   Note: Be sure to remove the white tape on the bottom of the gel and the comb from the wells.

7. Wash the wells of the gel by gently pipetting 1 mL of dark blue cathode buffer into the wells.
8. Add 20 mL of dark blue cathode buffer in the inner chamber of the electrophoresis system and check for leakage.
9. Load 15 μL of the sample into the gel wells using gel-loading tips (Figure 3).

Figure 3. Electrophoresis system setup and sample loading
For a technical demonstration of the protocol, 75 μg of isolated mitochondria from three animals were loaded in different wells from a BN-PAGE gel. The fourth well was loaded with 10 μL of NativePAGE™ 5% G-250 Sample Additive as a loading control.

Note: To our knowledge, only unstained protein standards are available for high molecular weights such as the NativeMark™ Unstained Protein Standard. In order to visualize this protein standard, a process of staining the gel in a different Coomassie than the one used in this protocol is required (e.g. G-250 Coomassie is used for the gel whereas R-250 is used for this standard). This staining process, in which the gel is incubated in acetic acid and other chemicals. Thus, if a molecular weight standard is required, we recommend running two gels, one for staining and one for in-gel digestion.

Note: Avoid the formation of bubbles while loading the sample into the wells.

10. Fill the inner chamber with 180 mL of dark blue cathode buffer and the outside chamber with 600 mL of anode buffer.

Note: While filling the electrophoresis system chambers, be sure to use cold buffers and not to disturb the samples in the wells.

11. Place the electrophoresis system inside an ice-containing bucket and, if possible, place it inside a cold chamber at 4°C (please see troubleshooting 1 for more details).
12. Plug the electrophoresis system to a PowerPac™ Basic Power Supply and turn it on. Run the gel at 150 V for 30 min (Figure 4).
13. After 30 min, carefully remove the dark blue buffer and fill the inner chamber with 200 mL of light blue cathode buffer.
14. Run the gel at 250 V for 60 min. If better separation of the bands is desired, then the gel can be run for a maximum of 150 min at 250 V.

Note: Running times longer than 150 min will not substantially improve the separation and resolution of the bands.

15. Take the gel out of the cassette. Hold the gel by the bottom part, which is thicker and reduces the chance of breakage.
16. Rinse the gel using abundant MQ-H_2O before imaging (Figure 5).
17. Image using a camera or the Coomassie protocol in a Chemidoc XRS.

Note: If imaging using a Chemidoc XRS, open the ImageLab software and create a new protocol, then under application menu, select the protein gels tab and then Coomassie Blue.

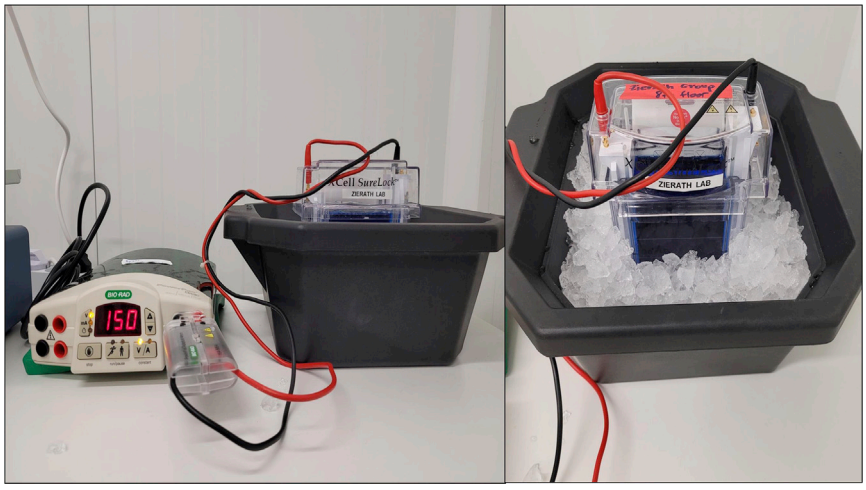


Figure 4. Running the BN-PAGE

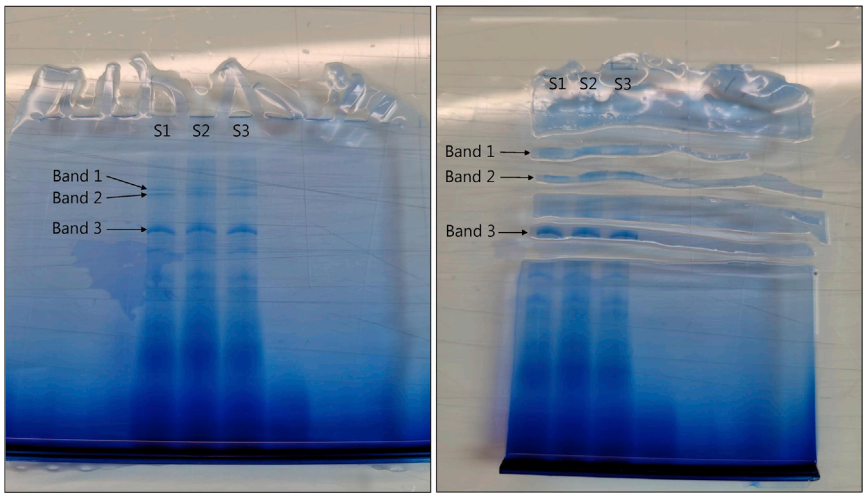


Figure 5. Gel imaging. Each lane of the gel corresponds to a different animal, from left to right: S1, S2, and S3

The three most visible bands were selected for in-gel digestion and were numbered from top to bottom: band 1, band 2 and band 3. The selected band numbers do not reflect the bands selected in Gonzalez-Franquesa et al. (2021) and are used only as a technical demonstration of the presented protocol.

In-gel protein digestion

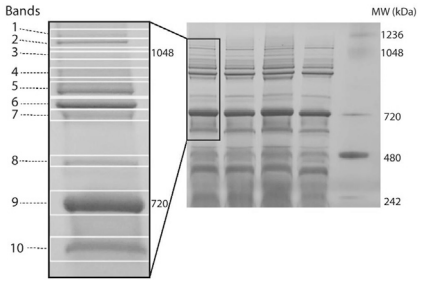
Timing: 2 h (day 1) + overnight incubation + 2.5 h (day 2)

Day 1

In this step, previously identified supercomplex-containing bands are selected (Jha et al., 2016; Gonzalez-Franquesa et al., 2021), excised from the gel and inserted into different tubes (Figure 6). Then, the gel bands are prepared for LC-MS/MS-based proteomics and the extracted proteins are digested into peptides (Shevchenko et al., 2006) (Figure 7).

Note: Special care should be taken to avoid contamination by keratins in the gel pieces which could interfere with the downstream proteomics analysis. Therefore, it is encouraged to use gloves during the following steps.

18. Prepare the 2 mL tubes for the different bands and experimental conditions and place the gel on a cutting board.



Band	Complexes
1	I+II+III+IV+V
2	I+II+III+IV+V
3	I+II+III+IV+V
4	I+II+III+IV+V
5	I+II+III+IV+V
6	I+III+IV+V
7	I+II+III+IV+V
8	I+II+IV+V
9	II+IV+V
10	II+III+IV+V

Figure 6. Respiratory complexes composition of BN-PAGE gel bands

Gel image depicting the bands selected and analyzed in Gonzalez-Franquesa et al. (2021). On the left, images of the gel with the sample lanes, band numbers and a molecular weight standard. On the right, respiratory complexes composition of each band. We have been granted permission from the author to include this figure.

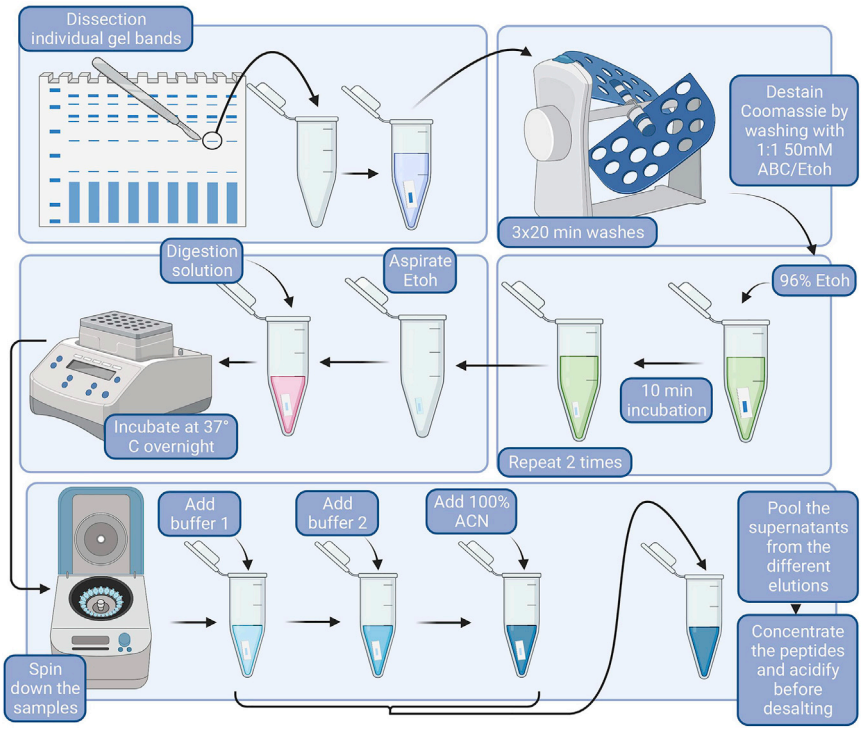


Figure 7. In-gel digestion schematic workflow
Created with BioRender.com.

Note: Using round bottom tubes helps during this step as they allow for an easier resuspension and mixing of the gel pieces.

19. Using a scalpel cut the selected bands of interest into a long slice by performing a horizontal cut from end to end of the gel (Figure 5).

Note: In order to guide the users on which bands to select for analysis, we have included the respiratory complexes composition of the bands analyzed in Gonzalez-Franquesa et al. (2021) in Figure 6.

20. Excise individual bands as seen in Figure 5, separating the gel slice using fine forceps and a scalpel before transferring the bands into the tubes.
21. De-stain the Coomassie from the gel bands by washing with 1 mL of 50 mM ammonium bicarbonate (ABC)/ethanol (1:1 volume). Incubate at RT in a rotating wheel for 20 min.
    a. Repeat step 21 for a total of 3 de-staining washes.

Note: Incubate until Coomassie staining has been completely removed from the sample. If necessary, perform more washes (Figure 8).

22. Remove the ABC/ethanol buffer using a pipette and add 1 mL of 96% ethanol. Incubate at RT in a rotating wheel for 10 min.
    a. Repeat this step for a total of 2 incubations with 96% ethanol.

Note: After this step, the gel bands should shrink and turn white.

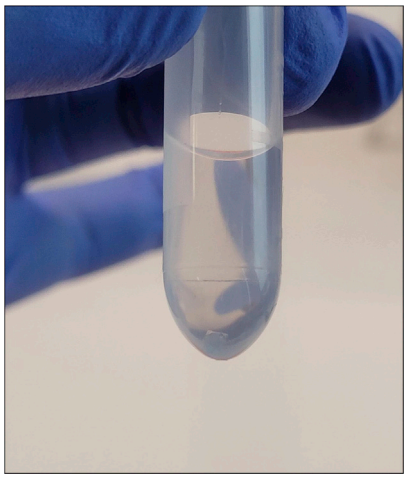


Figure 8. Single gel band during in-gel digestion

23. Aspirate as much of the ethanol as possible using a pipette. Care should be taken to avoid touching the gel bands. Let the cap of the tube open for the rest of the ethanol to evaporate, but do not allow for the gel bands to dry completely.

Note: It is important to remove the ethanol from the tube as it might inhibit the enzymatic activity of trypsin during protein digestion.

24. Add 100 µL of Digestion buffer to cover the gel bands and incubate at 37°C, 700 rpm for 5 min in a thermomixer using the 2 mL SmartBlock.

Note: Ensure that the gel bands expand in contact with the solution and lose the white color.

25. Cover the gel bands by adding 250 µL of 50 mM ABC and place the tubes back in the thermomixer at 37°C, 700 rpm.
    a. Seal the tubes to avoid evaporation and incubate the mixture overnight (between 18 and 12 h).

Note: To save time, prepare the stage tips that will be later used (step 38) during the incubation steps.

Day 2

26. The next day, spin the tubes to ensure the gel bands are in the liquid.
27. Start extracting the peptides from the gel bands by adding 35 µL (1:10 volume) of Buffer 1 into the tubes.
    a. Incubate the gel bands in the buffer for 20 min with gentle shaking in a thermomixer set at RT using the 2mL Smart Block.
    b. Transfer the supernatant into a new 1.5 mL tube using gel-loading tips to avoid gel pieces.
28. Add 100 µL of Buffer 1 to cover the gel pieces.
    a. Incubate as indicated in step 27a.
    b. Transfer the supernatant into the same tube used in step 27b.
29. Now, dispense 100 µL of Buffer 2 into the tubes containing the gel bands.
    a. Again, incubate as indicated in step 27a.
    b. Collect the supernatant into the same tube used in step 28b.
30. Proceed to add 100 µL of 100% ACN to cover the gel bands.

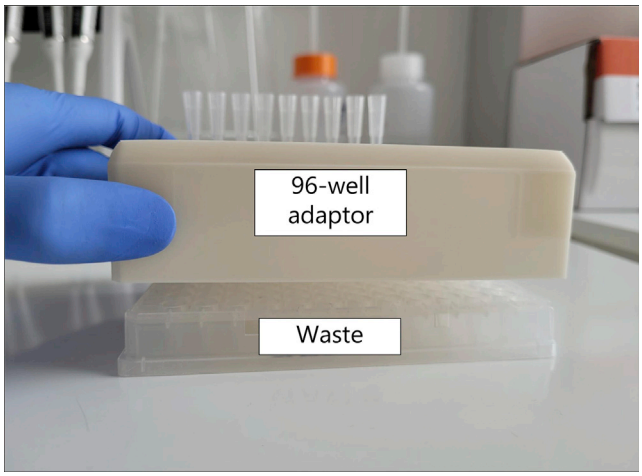


Figure 9. Setup used for peptide desalting
Stage-tips being held in a 96 well adaptor on top of a waste microtiter plate.

a. Incubate as in step 27a.
b. Transfer the supernatant to the tube used in step 29b.

31. Place the tubes in a centrifuge concentrator (with tube rotor) set at 45°C in alcoholic volumes (V-AL) mode and evaporate the buffer from the samples leaving 100–150 μL left (approximately 45 min).

Note: If the buffer from the samples does not evaporate after 45 min, increase the temperature of the centrifuge concentrator to 60°C and resume evaporation until samples have the desired volume.

32. Check the pH of the peptide solution using band strips and acidify the peptides by gradually adding 1% TFA solution until the pH is 2.5.

Pause point: The acidified peptides can be stored at 4°C for a week.

Peptide desalting

Timing: 2 h

At this point, peptides are desalted prior loading into LC-MS/MS instrumentation using in-house crafted C-18 filters (stage-tips) as previously described (Rappsilber et al., 2007). Stage-tips are placed in an in-house 3D printed centrifugation-proof tip-holder rack that allows for the elution of loaded stage-tips into the 96 well microtiter plate underneath (Figure 9). Alternatively, Evosep tip boxes (EV2001, Evosep, Denmark) are also suitable for this procedure (Bache et al., 2018).

33. Activate the C18 stage-tip filters:
    a. Load 20–50 μL of 100% MeOH and centrifuge (500g, 3 min).

Note: To avoid the stage-tip from drying completely always ensure there is a small volume on top of the C18 tips.

    b. Wash with 50 μL of Buffer 2 (500g, 3 min).
    c. Wash two times with 50 μL of Buffer 3 (500g, 3 min).

Note: The stage-tips are now activated and are ready to bind the peptides.

34. Centrifuge the peptide-containing tubes at maximum speed for 2 min to precipitate any particles present in the solution.

35. Transfer the supernatant to the activated stage-tip.

Note: Leave around 10–15 μL in the bottom of the tube to avoid any particles that could interfere with stage-tip peptide desalting.

    a. Perform cycles of centrifugation of 500g for 3 min until almost all volume has passed through the stage-tip and discard the flow through in each cycle.

36. Load 50 μL of stage-tip Buffer 3 and run cycles of centrifugation as indicated in step 35a.
37. Repeat step 36 for a total of two washes.
38. Change the stage-tip adaptor to a new and clean microtiter plate.
39. Elute peptides from the stage tips in a single step:
    a. Load 40 μL of Elution buffer
    b. Centrifuge at 500g for 3 min.
40. Place the microtiter plate containing the eluted peptides in a preheated (45°C) centrifuge concentrator (with the plate rotor) in vacuum-aqueous mode until the volume has been completely evaporated (approximately 30 min).
41. Resuspend the peptides:
    a. Load 10 μL of A* buffer into the sample-containing wells.
    b. Shake the plate at 1,400 rpm for 10 min in a thermomixer.
    c. Spin the plate down in a centrifuge for 30 s at 750g.
42. Measure peptide concentration by assessing the A260/A280 ratio using a spectrophotometer. Adjust peptide concentration to 0.1 μg/μL in 10 μL of A* buffer.

Note: Use A* buffer as blank during the spectrophotometry measurement.

Pause point: The plate containing the desalted peptides can be sealed to avoid sample evaporation and stored at −20°C indefinitely.

Note: Even though the plates are sealed and stored at −20°C, evaporation can still occur. Therefore, peptides should be resuspended by shaking in a thermomixer set at 1.500 rpm for 10 min followed by a quick spin in a multifuge centrifuge for 1 minute before MS injection.

Mass spectrometry

Timing: approximately 100 min per sample

The liquid chromatography tandem mass spectrometry (LC-MS/MS) setup used in Gonzalez-Franquesa et al. (2021) consisted of a combination of an EASY-nLC nano-flow UHPLC system coupled with a LTQ Q-Exactive HFX Orbitrap mass spectrometer (both from Thermo Fisher Scientific, Germany). Peptides from in-gel digested samples were injected on a 15 cm column packed with C-18 beads (Dr. Maisch GmbH, Germany), washed with 0.5% formic acid and separated using a 65 min linear gradient ranging from 5 to 40% of a 80% ACN, 0.5% formic acid buffer at a flow rate of 250 nL/min. Mass spectra were acquired in a data-dependent manner, with automatic switching between MS and MS/MS using a top-12 method. MS spectra were acquired in the Orbitrap analyzer with a mass range of 300–1750 m/z and 60,000 resolutions at m/z 200. HCD peptide fragments

Table 1. HPLC gradient and MS method parameters

Liquid chromatography gradient	
Time	Mixture (%B)
0	5
40	25
50	40
55	80
65	80
Mass spectrometry setup	
Setting	Value
Instrument	
Polarity	Positive
Full MS	
Microscans	1
Resolution	60,000
Automatic gain control target	3×10^6 ion counts
Maximum ion time	120 ms
Scan range	300–1750 m/z
Data dependent-MS^2	
Microscans	1
Resolution	15,000
Automatic gain control target	1×10^5 ion counts
Maximum ion time	60 ms
Loop count	12
Isolation window	1.3 m/z
Isolation offset	0
Fixed first mass	100 m/z
Normalized collision energy	28
Data dependent settings	
Minimum automatic gain control target	6×10^3 ion counts
Apex trigger	–
Charge exclusion	Unassigned, 1
Peptide match	–
Exclude isotopes	On
Dynamic exclusion	30.0 s

acquired at 28 normalized collision energy were analyzed at high resolution in the Orbitrap analyzer.

Table 1 contains MS method parameters used in Gonzalez-Franquesa et al. (2021). This MS method is intended to serve as an example. When using other LC or MS instruments, users are recommended to adjust the MS method to their setup.

Alternatives: Other HPLC and mass spectrometry instrumentation represent valid options for this analysis. In particular, Evosep One's predefined short chromatographic gradients (e.g. 21 min) could substantially reduce the measurement time per sample although it might reduce the high resolution peptide separation achieved by an EASY-nLC (Bache et al., 2018; Krieger et al., 2019). Different mass spectrometers could also be considered for the MS analysis, such as other Orbitrap (e.g. Thermo Fisher Orbitrap Exploris 480) or Time of Flight (e.g. Bruker timsTOF) instruments (Meier et al., 2018; Bekker-Jensen et al., 2020). The user should carefully consider their LC-MS/MS set-up, the choice of which may be influenced by measurement time, number of samples, resolution, protein depth, and often machine availability. We refer

Table 2. Expected peptide concentration

	Concentration (μg/μL)	A260/A280	Volume (μL)	Peptide amount (μg)	Average of peptide amount	Standard deviation
Band 1						
Sample 1	0.069	1.46	10	0.69	0.78	0.07348
Sample 2	0.087	1.472	10	0.87		
Sample 3	0.078	1.476	10	0.78		
Band 2						
Sample 1	0.127	1.254	10	1.27	1.21667	0.0411
Sample 2	0.121	1.231	10	1.21		
Sample 3	0.117	1.25	10	1.17		
Band 3						
Sample 1	0.121	1.325	10	1.21	1.12	0.06976
Sample 2	0.104	1.341	10	1.04		
Sample 3	0.111	1.303	10	1.11		

any inexperienced mass spectrometry user to this introductory guide to mass spectrometry-based proteomics (Sinha and Mann, 2020).

EXPECTED OUTCOMES

Successful application of this protocol results in desalted and purified tryptic peptides from the selected BN-PAGE gel bands that correspond to mitochondrial supercomplexes. When measuring peptide yield and purity in a spectrophotometer, the peptide yield should be between 0.5 and 1.5 μg of peptides (Table 2), depending on the thickness of the selected band. Before loading in LC-MS/MS, adjust the peptide concentration to 0.1 μg/μL if possible.

Table displaying the measurements of peptide concentration of each band from the three different animal samples. All peptides were resuspended in 10 μL of A* buffer. The average of peptide yield and standard deviation were calculated between replicates. The ratio A260/A280 of each band provides a measurement of the expected purity (A260/A280 of ~1.0 is considered optimal).

LIMITATIONS

Excision and MS analysis of specific BN-PAGE gel bands allows for a targeted analysis of previously described mitochondrial supercomplexes which can be visualized by Coomassie blue staining. For the purpose of mitochondrial complexes, we have focused this protocol in the study of previously identified complexes and visible bands. However, this protocol can technically be used for any gel bands that can be visualized by Coomassie blue staining. One limitation of the protocol described is the reduced resolution by molecular mass in comparison to the analysis of a greater number of equally-sized and continuous gel slices (Greggio et al., 2017). However, this method drastically reduces the MS analysis complexity and time, which makes it easily adaptable and applicable to larger scale studies. As this protocol uses label-free quantification for its MS-based proteomic analysis, it does not allow for absolute quantification of proteins, thus subunit stoichiometry quantification is based on relative changes.

TROUBLESHOOTING

Problem 1

Electrophoresis sudden stop

The low amperage used during the running of the gel can cause the power supply to stop suddenly due to a high resistance and stop the BN-PAGE run (step 12).

Potential solution

To avoid this, it is important to use a PowerPac™ Basic Power Supply that supports low amperages (mA) and to maintain a low temperature during the gel run by using the aforementioned ice bucket and cold chamber. Keeping the power supply in the cold chamber the night before the experiment also reduces the temperature rise during the gel run. If the power supply keeps stopping, exchange the buffers in both inner and outer chambers for fresh cold buffers, and/or change to a more powerful power supply.

Problem 2

Gel pieces do not expand and/or remain white in contact with digestion buffer

Lack of band expansion and color change might cause an impaired protein digestion due to a deficient diffusion of digestion buffer into the gel band (step 24).

Potential solution

Occasionally, the absorption of digestion buffer is not immediate and requires an incubation step. Ensure that the gel bands are covered in digestion buffer and incubate at 4°C for up to 30 min. If the problem persists, repeat the incubation step for another 30 mins.

Problem 3

Samples and/or wash solutions do not flow through the stage-tip during desalting

If fragments of gel are transferred to the stage-tips by mistake, it is possible for them to block, therefore interrupting the sample flow during loading and subsequent washes (step 35).

Potential solution

If the stage-tip blockage is a partial blockage, separate the blocked stage-tip from the rest and follow the same steps described in the protocol, although the number of centrifugation cycles needed might increase.

In case the stage-tip is completely blocked, transfer the sample to a new stage-tip using gel-loading tips and re-start peptide desalting.

Problem 4

Low peptide yield

When measuring the peptide yield resulted from the application of this protocol and it is lower than 0.4 μg (expected outcomes).

Potential solution

Low peptide concentration upon measurement with the spectrophotometer might be due to some peptides still adhered to the walls of the microtiter plate. To resuspend remaining peptides, shake and spin the MS plate again as noted under step 42.

In case the problem persists, the bands selected for in-gel digestion might be faint and contain a lower protein concentration. Increasing the amount of isolated mitochondrial sample material to 100 μg/lane would increase the protein concentration in the faint bands of the gel.

Another cause of sample loss during the protocol might be an incomplete protein digestion. The enzyme/protein ratio is important during protein digestion. If the samples were analyzed by LC-MS/MS, the performance of the protein digestion can be evaluated using the percentage of missed cleavages. A value higher than 30% of missed cleavages is indicative of an incomplete protein digestion. If that is the case, run a titration to optimize the concentration of trypsin.

Problem 5

Low sample purity

If the ratio A260/A280 of the samples is between 1.8 and 2, the desalted peptides are not pure enough and might result in poor protein identification/quantification during MS analysis (expected outcomes).

Potential solution

One potential explanation is that one round of C18 peptide desalting was not sufficient to purify the peptides present in the samples. In that case, either a second round of C18 peptide desalting or an orthogonal peptide desalting step using Styrenedivinylbenzene-reverse phase sulfonate (SDB-RPS) stage-tips could be performed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Atul S. Deshmukh (atul.deshmukh@sund.ku.dk , Blegdamsvej 3B (07-7) - 2200 Copenhagen, Denmark).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new data or code.

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AUTHOR CONTRIBUTIONS

A.S.D. supervised this work. R.M.-J., A.G.-F., and B.S. performed experiments and wrote the manuscript. A.S.D. edited the manuscript. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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