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Dear Senior Review Editor Alisha DSouza,

Thank you for sending the manuscript titled "**Analysis of mitochondrial respiratory chain complexes in cultured human cells by blue native polyacrylamide gel electrophoresis**" by Konovalova for peer-review. I greatly appreciated the rapid handling of the manuscript by The Journal of Visualized Experiments. I have now modified the manuscript as recommended by the editor and reviewers.

The detailed responses to the reviewers' comments are listed separately. I have carefully considered the reviewers' comments, and hope that the changes I made are acceptable and that the manuscript is now suitable for publication in The Journal of Visualized Experiments.

Yours sincerely,

Svetlana Konovalova
Postdoctoral researcher
University of Helsinki

TITLE:

Analysis of Mitochondrial Respiratory Chain Complexes in Cultured Human Cells Using Blue Native Polyacrylamide Gel Electrophoresis and Immunoblotting

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KEYWORDS:

Mitochondria, OXPHOS complexes, blue native PAGE, electrophoresis, respiratory chain complexes, mitochondrial proteostasis

SHORT ABSTRACT:

This protocol analyzes mitochondrial respiratory chain complexes by blue native polyacrylamide gel electrophoresis. Here the method applied to cultured human cells is described.

LONG ABSTRACT:

Mitochondrial respiration is performed by oxidative phosphorylation (OXPHOS) complexes within mitochondria. Internal and environmental factors can perturb the assembly and stability of OXPHOS complexes. This protocol describes the analysis of mitochondrial respiratory chain complexes by blue native polyacrylamide gel electrophoresis (BN-PAGE) in application to cultured human cells. First, mitochondria are extracted from the cells using digitonin, then using lauryl maltoside, the intact OXPHOS complexes are isolated from the mitochondrial membranes. The OXPHOS complexes are then resolved by gradient gel electrophoresis in the presence of the negatively charged dye, Coomassie blue, which prevents protein aggregation and ensures electrophoretic mobility of protein complexes towards the cathode. Finally, the OXPHOS complexes are detected by standard immunoblotting. Thus, BN-PAGE is a convenient and inexpensive technique that can be used to evaluate the assembly of entire OXPHOS complexes, in contrast to the basic SDS-PAGE allowing the study of only individual OXPHOS complex subunits.

INTRODUCTION:

Mitochondria are multifunctional organelles playing an important role in energy production, regulation of cellular metabolism, signaling, apoptosis, aging, etc.[1-3]. The energy production in mitochondria relies on the oxidative phosphorylation function that couples respiration with ATP synthesis. In human cells, the mitochondrial oxidative phosphorylation system (OXPHOS) is composed of five complexes. Complexes I-IV create an electrochemical proton gradient in the mitochondrial intermembrane space that is used by complex V to produce ATP. Each OXPHOS complex is multimeric and, except complex II, composed of the subunits encoded by both nuclear and mitochondrial genes. Any defects in the core components of the OXPHOS complexes caused by mutations or environmental stress can perturb the assembly and functionality of the oxidative phosphorylation system. In addition, the proper assembly of functional OXPHOS complexes requires a large number of assembly factors[4-6].

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a fundamental technique enabling analysis of intact protein complexes and can be used to study the assembly of OXPHOS complexes. First, mitochondria are isolated from the cells by digitonin, which is a mild detergent that permeabilizes the plasma membrane of the cells. Then, by using lauryl maltoside, OXPHOS complexes are released from the mitochondrial membranes. By using gradient gel electrophoresis, the OXPHOS complexes are separated according to their mass. Coomassie blue G-250 (not R-250) added to the sample buffer and the blue cathode buffer dissociates detergent-labile associations but preserves individual respiratory chain complexes intact[8]. During the electrophoresis, the blue cathode buffer containing dye is replaced by the cathode buffer without dye, which ensures efficient transfer of OXPHOS complexes to the PVDF membrane[8]. To visualize OXPHOS complexes, the PVDF membrane is sequentially incubated with antibodies corresponding to the selected subunits of the five OXPHOS complexes.

The method described here with some modifications can be applied to any cultured cells. In addition, this method can be used for analysis of OXPHOS complexes in mitochondria isolated from tissue samples[9]. BN-PAGE requires at least 5-10 µg of mitochondria for each sample per run. Using the method described here, 500,000 cultured cells such as HEK293, SH-SY5Y or 143B cells can yield approximately 10 µg of mitochondria. However, a sufficient amount of the cells for the BN analysis depends on the specific cell type.

The most common method to study mitochondrial OXPHOS proteins is SDS-PAGE and western blotting. However, SDS-PAGE allows studying only individual OXPHOS subunits and, in contrast to BN-PAGE, cannot be used to evaluate the assembly of entire OXPHOS complexes. Clear native-PAGE separates protein complexes in native conditions without the presence of negatively charged dye and has a significantly lower resolution compared to BN-PAGE. However, clear native-PAGE is milder than BN-PAGE so it can retain labile supramolecular assemblies of protein complexes such as OXPHOS supercomplexes that are dissociated under the BN-PAGE conditions[10]. In this protocol, the gradient gel is used to separate complexes; however, alternatively, non-gradient separation can be used if the relatively expensive gradient maker is not available[11].

Important, the method described here allows analyzing the assembly of OXPHOS complexes, while the functionality of the complexes is not assessed. A high-resolution BN-PAGE followed by in-gel activity assay[11] as well as spectrophotometric enzymatic activity assay of the mitochondrial complexes[12] are efficient techniques for the functional analysis of the OXPHOS complexes. However, both of these methods do not assay the assembly of OXPHOS complexes.

Thus, BN-PAGE is the optimal method to investigate the assembly of individual OXPHOS complexes. For example, some mitochondrial disorders, such as Leber hereditary optic neuropathy (LHON), mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), are associated with altered assembly of one or more components of the OXPHOS system[5]. By using the BN-PAGE method described here, the molecular mechanisms of mitochondrial diseases can be studied.

PROTOCOL:

NOTE: This protocol is based on an associated publication by Hilander et al.[13] .

1. Preparation of buffers and solutions

NOTE: All the buffers and solutions used in the protocol are summarized in **Table 1**. The given volumes of the buffers are sufficient to prepare and run 10 samples. All the buffers can be stored at +4 °C for up to one year.

1.1. Prepare 20 mL of 3x gel buffer containing 1.5 M aminocaproic acid, 150 mM Bis-tris in distilled water (dH_2O). Adjust pH to 7.0.

1.2. Prepare 10 mL of 2 M aminocaproic acid in dH_2O.

1.3. Prepare 1 mL of the mitochondrial buffer by combining the following: 0.5 mL of 3x gel buffer, 0.5 mL of 2 M aminocaproic acid and 4 µL of 500 mM EDTA.

1.4 Prepare 1,000 mL of cathode buffer containing 15 mM of Bis-tris and 50 mM of tricine in dH_2O. Adjust pH to 7.0.

1.4.1 Prepare 200 mL of blue cathode buffer by adding 0.04 g of Coomassie blue G-250 to 200 mL of cathode buffer.

1.5. Prepare 1,000 mL of anode buffer containing 50 mM Bis-tris in dH_2O. Adjust pH to 7.0.

1.6. Prepare 2.5 mL of sample buffer containing 750 mM aminocaproic acid and 5% Coomassie blue G-250 in dH_2O.

2. Preparation of mitochondrial lysates

2.1. Plate the cells the day before the collection. For HEK293 or 143B cells, use Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% L-glutamine, 100 mg/mL penicillin and 100 mg/mL streptomycin. Grow the cells in a cell culture incubator at +37 °C in a 5% CO_2 humidified atmosphere. Ensure that there are at least 500,000 cells for each sample on the day of collection.

2.2. Gently wash the cells once with ice-cold PBS. Avoid detaching the cells from the plate. Scrape the cells and pellet them at 750 x *g* for 10 min at +4 °C.

2.3. Wash the cell pellet twice with ice-cold PBS, and centrifuge as in step 2.2. Measure the protein concentration with the Bradford method using a commercial kit. Pellet the cells at 750 x *g* for 10 min at +4 °C.

NOTE: After the cell collection, perform all steps at +4 °C and do not vortex.

2.4. Prepare 20 mL of PBS with protease inhibitor by adding 200 µL of 100x protease inhibitor to 20 mL of PBS. Keep on ice. Resuspend the cells in PBS with protease inhibitor to a final protein concentration of 5 mg/mL.

2.4.1. To calculate the volume of PBS with protease inhibitor needed to resuspend the cells at 5 mg/mL, calculate the protein amount in each sample. For this calculation, use the protein concentration measured in step 2.3 and the volume of PBS used to resuspend the cells after washing in step 2.3.

2.5. Prepare 3.3 mM digitonin in PBS with protease inhibitor. Add 3.3 mM digitonin to a final concentration of 1.65 mM. Mix well and incubate on ice for 5 min.

2.5.1. To prepare 3.3 mM digitonin in PBS with protease inhibitor, dissolve 4 mg/mL digitonin in PBS at 100 °C until no precipitate is visible and cool on ice immediately. Add 10 µL of 100x protease inhibitor to 1 mL of digitonin solution.

NOTE: Use only a fresh solution of digitonin.

CAUTION: Digitonin is toxic! Use a face mask, gloves and a lab coat.

2.6. Add PBS with proteinase inhibitor (prepared in step 2.4) to the final volume of 1.5 mL. Centrifuge at 10,000 x *g* for 10 min at +4 °C. Remove the supernatant. In this step, mitochondria are pelleted.

2.7. Resuspend the mitochondrial pellet in mitochondrial buffer. The volume of mitochondrial buffer is half of the volume of PBS in step 2.4.

2.8. Prepare fresh 10% lauryl maltoside in PBS. 1 mL is sufficient for 10 samples. Add 10% lauryl maltoside to the final concentration of 1%. Incubate on ice for 15 min (this step can be longer up to a couple of hours).

2.9. Centrifuge at 20,000 x *g* for 20 min at +4 °C. Collect the supernatant into a new tube. Measure the protein concentration using the Bradford method using a kit. Add sample buffer, a volume that is half of the volume of lauryl maltoside used in step 2.8. Samples can be stored at -80 °C for up to 6 months.

3. Preparation of gradient gel for BN-PAGE

3.1. Pour the gradient gel for BN-PAGE at room temperature. Place the gradient maker on a stir plate and connect it with flexible tubing to the peristaltic pump. Attach an infusion set with the needle to the tubing. Place a magnetic stirrer into the gradient maker. Wash the tubing with dH_2O at maximum pump speed for 10 min.

3.2. Empty the tubing and the gradient maker. Use a pipet to remove any leftover dH_2O in the channel between chambers of the gradient maker. Close the channel and tubing with the valve.

3.3. Assemble two glass plates in the blue native gel holder, which has a hole on the bottom, and place it on the stand. Make sure that it is more or less on a straight surface with a water balance. Place the needle connected to the tubing between the glass plates.

3.4. Prepare 6% and 15% gel solutions (**Table 2**). Keep on ice. Add APS and TEMED (they start polymerization) last. Mix gently to avoid making air bubbles.

3.5. Load the proximal end of the gradient-gel-mixer tubing chamber with 6% gel and the distal end with 15% gel. The total volume of the gel should be equal to the volume of the separating gel between the glass plates. Thus, use 2.6 mL of 6% gel and 2.1 mL of 15% gel to the gradient gel mixer for one 8.3 cm x 7.3 cm sized gel.

3.6. Switch on the magnetic stirrer, and open the tubing and channel between chambers of the gradient maker. Immediately switch on the peristaltic pump to 5 mL/min. Fill the glass plates, and do not allow bubbles to enter the gel. Remove the needle when there is no gel in the tubing.

3.7. Gently overlay the gel with dH_2O. Keep the gel at room temperature for at least 1 h for polymerization.

3.8. Immediately after pouring the gel, wash the tubing by filling the gradient chambers with dH_2O and using the peristaltic pump at maximal speed. When preparing two and more gels, always clean the system in between.

3.9. Prepare 1x gel buffer by mixing 3 mL of 3x gel buffer and add 6 mL of dH_2O. Remove dH_2O from the surface of the gel with filter paper gently. Wash the surface of the gel with 1x gel buffer. Remove 1x gel buffer with filter paper gently.

3.9.1. Avoid fibers from the filter paper on the gel. To ensure this, cut the paper to small pieces with scissors, but do not tear the paper.

3.10. Place the comb between the glass plates, but do not immerse it fully to be able to pour the stacking gel under the comb. Prepare the 4% stacking gel (**Table 2**). Add ammonium persulphate (APS) and tetramethylethylenediamine (TEMED) last as they start polymerization easily. Mix gently avoiding air bubbles.

3.11. Overlay the stacking gel, avoiding bubbles under the comb, and immerse the comb fully. Let the stacking gel polymerize for at least 30 min. Remove the comb and wash the wells with 1x gel buffer using a pipet.

3.11.1. After polymerization, the gel can be stored at +4 °C for a couple of days. To store the gel, wrap the gel in paper soaked with dH_2O and plastic film to prevent drying of the gel.

4. Blue native gel electrophoresis

NOTE: To prevent degradation of OXPHOS complexes run electrophoresis at +4 °C. Use pre-chilled buffers.

4.1. Load the gel cassette with the blue cathode buffer until the bottom of the wells. Loading of the samples is easier when the cassette is not filled to the top. Wash the wells with the blue cathode buffer and then fill the wells with the blue cathode buffer using pipet.

4.2. Load the samples (5-30 µg of protein) into wells. Gently fill the gel cassette to the top with the blue cathode buffer and the tank with anode buffer.

4.3. Run the gel for 15 min at a constant voltage of 40 V. Then increase the voltage to 80 V (or use a constant current of 6 mA), do not exceed 10 mA. Run the gel until the dye reaches 2/3 of the gel length.

4.4. Replace the blue cathode buffer with cathode buffer and continue electrophoresis until the dye front has run out. In total, electrophoresis takes about 3 hours.

4.5. Retrieve the glass plates and transfer the proteins to the polyvinylidene fluoride (PVDF) membrane by semi-dry blotting. Use a constant voltage of 25 V and a current limited to 1.0 A for 30 min.

4.5.1. Alternatively, use wet blotting to transfer the OXPHOS complexes to a PVDF membrane.

4.6. Continue with blocking of the membrane and antibody incubation according to the standard immunoblotting protocol. Use the primary antibodies against subunits of OXPHOS complexes sequentially.

NOTE: For example, use anti-NDUFA9 antibody (1:2000) for complex I, anti-SDHA antibody (1:2000) for complex II, anti-UQCRC2 antibody (1:1000) for complex III, anti-COX1 antibody (1:2000) for complex IV and anti-ATP5A antibody (1:2000) for complex V. The list of the antibodies is presented in the **Table of Materials**.

REPRESENTATIVE RESULTS:

Using BN-PAGE, defects in the assembly of mitochondrial OXPHOS complexes can be investigated. **Figure 1A** shows the assembly of respiratory chain complexes in human neuroblastoma cells (SH-SY5Y) treated with chloramphenicol, which specifically inhibits translation of mtDNA-encoded OXPHOS subunits. Chloramphenicol depleted the OXPHOS complexes that contained mitochondrially-encoded subunits (complex I, III, IV; **Figure 1A**), while complex II, which is exclusively encoded by nuclear genes, was compensatorily upregulated. Complex V was not immunoblotted here.

Multiple freeze-thaw cycles destroy OXPHOS complexes. **Figure 1B** demonstrates the degradation of OXPHOS complexes by the freeze-thaw cycles. Mitochondrial respiratory complexes in sample 3 that have undergone multiple freeze-thaw cycles have a lower band intensity and are shifted in comparison to the same samples, which were frozen only once. An uncropped western blot image of **Figure 1B** is shown in **Supplementary Figure 1**.

The quality of the gel is important for sharp and clear bands. **Figure 1C** shows an immunoblot from a gel that did not polymerize well. Stripping of the membrane also can affect the detection of OXPHOS complexes. In **Figure 1D**, complex I was detected before or after stripping. The signal to noise ratio is much lower after stripping.

FIGURE AND TABLE LEGENDS:

Figure 1. BN-PAGE immunoblots from successful and sub-optimal experiments.
(A) Depletion of OXPHOS complexes by inhibitor of mitochondrial translation. Human neuroblastoma cells (SH-SY5Y) were treated with chloramphenicol (CAP) for 48 hours. CTRL, control cells without chloramphenicol treatment. The lower panel shows the quantification of the immunoblot. The value of the control is taken as 1 (shown as a dashed line). Data are presented as mean ± SD, n = 3, $^*P < 0.0001$ (as compared to control using unpaired Student's t-tests. **(B)** Disruption of OXPHOS complexes by multiple freeze-thaw cycles. Samples 1-3 were prepared from human osteosarcoma cells (143B) in the same conditions. Samples number 1 and 2 were frozen and melted only once, while the sample number 3 underwent four freeze-thaw cycles. **(C)** BN-PAGE immunoblots of OXPHOS complexes isolated from HEK293 cells. The smearing of the bands is caused by low quality of the gel. **(D)** Effect of stripping on the detection of OXPHOS complexes. Detection of Complex I in human neuroblastoma cells (SH-SY5Y) before and after stripping of the same membrane. OXPHOS complexes were detected using anti-NDUFA9 antibody (complex I), anti-SDHA antibody (complex II), anti-UQCRC2 antibody (complex III) and anti-COX1 antibody (complex IV).

Table 1. Buffers and solutions used for BN-PAGE.

Table 2. Recipes of gel for BN-PAGE.

Supplementary Figure 1. Uncropped western blot image of Figure 1B.

DISCUSSION:

One of the most critical parts of the protocol is how to preserve intact OXPHOS complexes during sample preparation, storage and gel electrophoresis. Thus, mitochondria should be isolated at

+4 °C and the samples should not undergo freeze-thaw cycles. OXPHOS complexes can only tolerate one freeze-thaw cycle during the whole procedure. Multiple freeze-thaw cycles destroy OXPHOS complexes (**Figure 1B**). Control and experimental samples that are to be compared should be prepared in parallel to avoid any differences in storage conditions, which might give misleading results. If it is not possible to perform all the steps of the protocol in parallel with all the samples, we recommend to freeze washed cell pellets at -80 °C (step 1.4 in this protocol) and later carry out the rest of the protocol for all the samples together at least until gel electrophoresis. Special attention should be paid to the cleanliness of the electrophoresis apparatus (tank, cassette). If the same apparatus is used for SDS-PAGE, wash it very well before BN-PAGE. Any residual SDS can cause dissociation of the OXPHOS complexes during electrophoresis.

High-quality gradient gels are another critical part of the protocol. Precast gels for blue native PAGE are commercially available; however, it is not recommended to use them, since the buffers used for commercial gels might have a composition, which is different from the sample buffers. The gradient of the gel used here (6-15%) is optimal for separation of individual OXPHOS complexes. To detect higher-order supramolecular structures of OXPHOS, also known as respiratory chain supercomplexes[14], some optimization is required[11].

For the visualization of OXPHOS complexes use the specific antibodies sequentially, based on their properties. For example, use first the antibody that gives the weakest signal and the antibody with the strongest signal last. This is important since the stripping weakens the detection (**Figure 1D**). The composition of respiratory chain complexes can be investigated if after BN-PAGE the gel is subjected to the second dimension SDS-PAGE[15].

The protocol described here suggests using antibodies against individual OXPHOS complexes sequentially. However, the commercially available OXPHOS antibody cocktail can be used to detect all five OXPHOS complexes simultaneously. Nevertheless, to be able to detect incompletely assembled OXPHOS complexes and define their identity, antibodies against individual OXPHOS complexes should be used sequentially. This step is time-consuming; however, it can be essential for testing new experimental conditions and models.

The concentration of digitonin used for mitochondrial isolation should be optimized for the specific cell type. As a detergent, digitonin permeabilizes cell membranes. The optimal concentration of digitonin efficiently permeabilizes the plasma membrane of the cells leaving the mitochondrial membranes intact. Too low a concentration of digitonin causes high contamination of the mitochondrial extracts while too high a concentration damages the mitochondrial membranes and reduces the total mitochondrial yield. The optimal digitonin/protein ratio (g/g) varies from 0.3 to 1. Western blot analysis of proteins extracted from pellets and supernatants can be used to test the optimal concentration of digitonin[16].

This protocol does not include protein loading control on the immunoblot; therefore, the protein concentration of extracted complexes should be carefully measured at least in triplicates to ensure equal loading. In addition, the samples can be run in BN-PAGE in replicates. If the assembly of selective OXPHOS complexes is impaired, unaffected complexes can serve as loading controls.

The estimation of the molecular mass of the protein complexes in the BN-PAGE is challenging[18]. The current protocol does not include the molecular weight marker; therefore, to estimate the assembly of the OXPHOS complexes, the control samples containing unaffected complexes should always be included in the analysis. The control samples for BN-PAGE are usually untreated or wild type cells.

The method presented here is optimized for the detection of mitochondrial respiratory chain complexes; however, it can also be applied for the evaluation of oligomerization of mitochondrial proteins[19]. In addition, the assembly rate of OXPHOS complexes can be studied by first depleting mitochondrial-encoded subunit containing complexes by chloramphenicol and then following their recovery after removal of chloramphenicol[10]. Thus, BN-PAGE can be used to evaluate steady-state levels and assembly of OXPHOS for different applications including molecular diagnostics of human mitochondrial disorders[9,11].

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DISCLOSURES:

No conflicts of interest declared.

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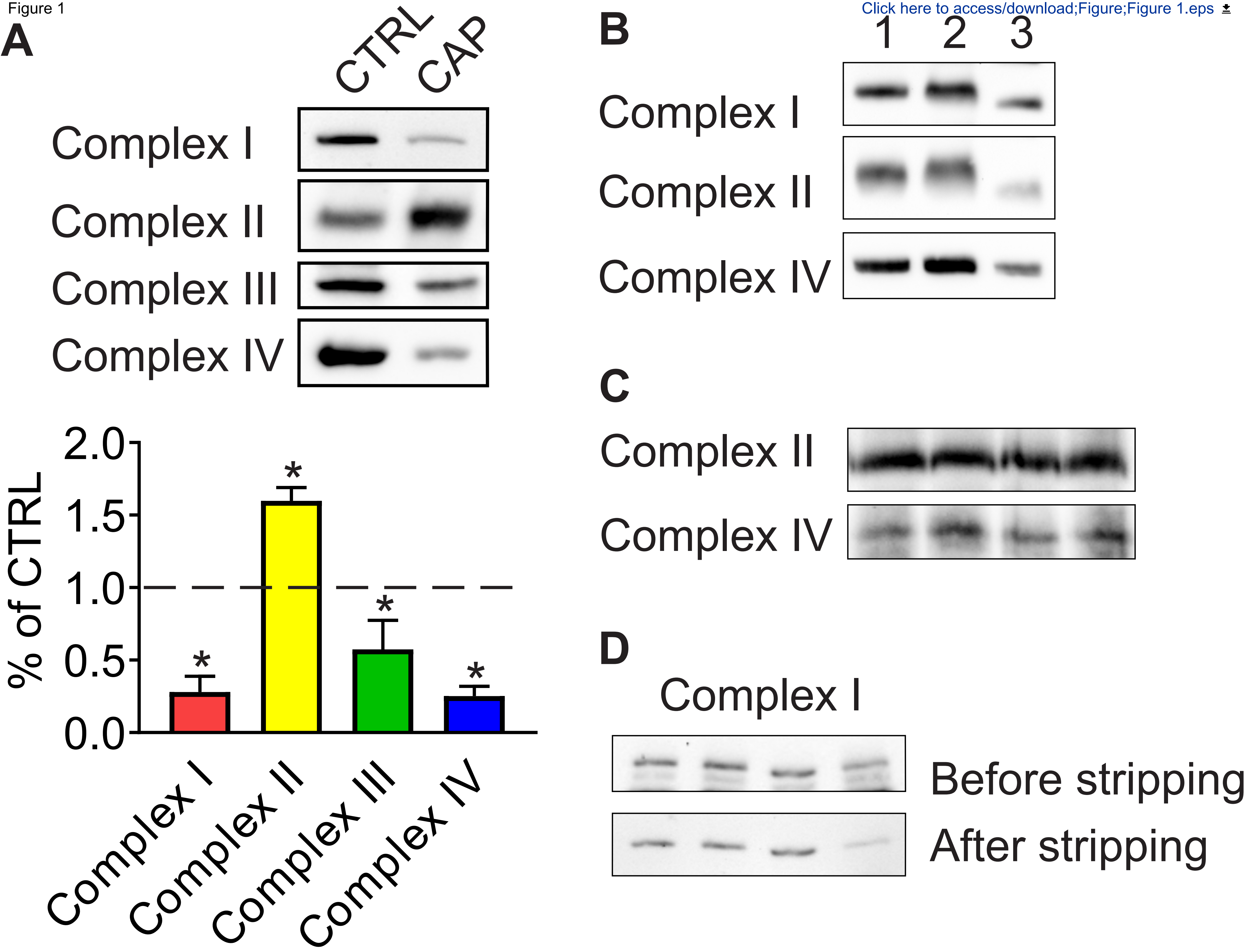
A
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Complex III
Complex IV
% of CTRL
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Complex IV
B
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2
3
Complex I
Complex II
Complex IV
C
Complex II
Complex IV
D
Complex I
Before stripping
After stripping

Table 1

Buffer or solution	Composition	Recipe
3 x gel buffer	1.5 M aminocaproic acid	3.94 g of aminocaproic acid
	150 mM Bis-tris	0.63 g of Bis-tris
	dH_2O	Add dH_2O to 20 mL
	pH 7.0	Adjust pH to 7.0
Cathode buffer	15 mM Bis-tris	3.14 g of Bis-tris
	50 mM tricine	8.96 g of tricine
	dH_2O	Add dH_2O to 1000 mL
	pH 7.0	Adjust pH to 7.0
Blue cathode buffer	0.02% coomassie blue G	0.04 g of Serva blue G
	15 mM Bis-tris	200 mL of Cathode buffer
	50 mM tricine	
	dH_2O	
	pH 7.0	
Anode buffer	50 mM Bis-tris	20.93 g
	dH_2O	Add dH_2O to 2000 mL
	pH 7.0	Adjust pH to 7.0
Mitochondrial buffer	1.75 M aminocaproic acid	0.5 mL of 3 x gel buffer
	75 mM Bis-tris	0.5 mL of 2 M aminocaproic acid
	2 mM EDTA	4 µL of 500 mM EDTA
	dH_2O	-
	pH 7.0	-
Sample buffer	750 mM aminocaproic acid	0.94 mL of 2 M aminocaproic acid
	5% comassie blue G	0.125 g of Serva blue G
	dH_2O	Add dH2O to 2.5 mL
2M aminocaproic acid	2 M aminocaproic acid	2.62 g of aminocaproic acid
	dH_2O	Add dH_2O to 10 mL

Table 2

Blue native gels	Separating 6% gel	Separating 15% gel	Stacking 4% gel
3 x gel buffer	3.3 mL	3.3 mL	1.64 mL
40% Acrylamide/Bis 37.5:1	1.5 mL	3.75 mL	0.5 mL
dH_2O	5.14 mL	0.93 mL	2.77 mL
Glycerol	0	2 mL	0
10% Ammonium persulfate*	60 µL	10 µL	60 µL
TEMED	4 µL	2 µL	6 µL
Total volume	10 mL	10 mL	5 mL

*Make always fresh 10% ammonium persulfate in dH2O

Name of Material/ Equipment	Company	Catalog Number
100 mm cell plates	Thermo Fisher S	130182
40% Acrylamide/Bis 37.5:1	Bio-Rad	161-0148
Aminocaproic acid	Sigma	A2504
Ammonium persulfate	Sigma	A3678
Anti-ATP5A	Abcam	14748
Anti-MTCOI	Abcam	14705
Anti-NDUFA9	Abcam	14713
Anti-SDHA	Abcam	14715
Anti-UQCRC2	Abcam	14745
Bis-tris	Sigma	B7535
Bradford protein assay kit	BioRad	5000006
Cell scrapers	Fisher	11597692
Coomassie blue G250 (Serva Blue G)	Serva	35050
Digitonin	Sigma	D141
DMEM	Lonza	12-614F/12
EDTA	Life Technologie	15575-038
FBS	Life Technologie	10270106
Fetal bovine serum	Life Technologie	10270106
Glycerol	(Sigma	G5516
Gradient maker	Bio-Rad	1654120
Lauryl maltoside (n-Dodecyl β-D-maltoside)	Sigma	D4641
L-glutamine	Life Technologie	25030024
L-glutamine	Gibco	25030
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma	T9281
PBS	Life Technologie	BE17-516F
Penicillin/Streptomycin	Lonza	17-602E
Penicillin/streptomycin	Gibco	15140
Power supply	GE Healthcare	EPS 301
Protease inhibitors	Thermo Scientifi	10137963
Trans-blot turbo mini PVDF	BioRad	1704156
Tricine	Sigma	T9784
Trypsin-EDTA	Gibco	15400054
Vertical electrophoresis apparatus	BioRad	1658004

Comments/Description
Complex V subunit
Complex VI subunit
Complex I subunit
Complex II subunit
Complex III subunit

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Author(s):	Svetlana Konovalova

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Title: Postdoctoral researcher

Signature:
Date: 15.10.2018

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RESPONSES TO EDITORIAL AND PEER REVIEW COMMENTS

The author thanks Editorial board and Reviewers for the comments and for the criticism on some parts of the manuscript.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author: The manuscript now is corrected for grammar and spelling issues.

2. Please revise lines 27-29, 43-44 to avoid previously published text.

Author: The lines are revised.

3. Abstract (line 26): Please do not include references here.

Author: The reference is removed.

4. Please revise the Introduction to include a clear statement of the overall goal of this method, advantages over alternative techniques with applicable references to previous studies, and information to help readers to determine whether the method is appropriate for their application.

Author: The Introduction is revised accordingly.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

6. 1.1: Please specify the cells used in this protocol and indicate culturing conditions.

7. 1.3: Please provide the concentration of trypsin and reaction conditions (temperature and time).

8. 1.8.1: What volume of protease inhibitor is added?

9. 1.12.1: Please list an approximate volume to prepare.

Author: More details are added to the protocol.

10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Author: The shorter steps of the protocol are combined.

11. Please include single-line spaces between all paragraphs, headings, steps, etc.

Author: The spaces are included.

12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Author: The steps for the video are highlighted with yellow color.

15. Tables 1 and 2: Please use the following volume units (mL, µL) instead of ml/µl. Please use the period symbol (.) for the decimal separator (e.g., 7.0, 2.62, etc.). Please use subscripts in chemical formulae to indicate the number of atoms, e.g., H2O.

Author: The units, period symbol, subscripts are corrected.

16. Please do not number the Table of Materials in the manuscript. Please sort the items in alphabetical order according to the name of material/equipment.

Author: The Table of Materials is not numbered and the items now in alphabetical order.

17. Discussion: Please discuss any limitations of the technique.

Author: The limitations of the method now discussed.

18. References: Please do not abbreviate journal titles.

Author: The journal titles are not abbreviated now in the references.

19. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Author: The items in Table of Materials now in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a protocol that provides analysis of mitochondrial complexes using BN-PAGE. Step-by-step instructions is given to ensure a successful performance of the technique presented. The protocol, however, would improve if the following is addressed.

The author thanks Reviewer for the comments and have now modified the manuscript accordingly.

Major Concerns:

1.I think step 1.5 should be performed after step 1.7 but before step 1.8.

Author: By measuring protein concentration at step 1.5. (currently, step 2.3.) we determine the protein amount in each sample. Then, after pelleting, we calculate the volume of PBS that should be used to get protein concentration equal to 5 mg/mL. To make this step clear, the calculation is now described in the Protocol in step 2.4.1.

2. The author should also stress that Commassie blue G-250, not R-250 should be used throughout the process.

Author: Introduction part now stresses that Coomassie G-250 is used.

3. There is a non-gradient BN-PAGE method that is available and should be mentioned in the Introduction or Discussion section.

Author: Non-gradient BN-PAGE method now mentioned as an alternative method in Introduction.

4. In the figures, a whole gel image containing all the resolved mitochondrial complex's bands should be given. Western blot image of individual complex's subunit band is not enough.

Author: In this protocol, the antibodies against individual OXPHOS complexes are used sequentially. The signal that each antibody gives depends on the specific antibody. Therefore, in the whole gel image, some of the OXPHOS complexes are overexposed and some are underexposed. That is why we prefer to present individual bands of the complexes.

How many cells were used for mitochondrial isolation should be given. Mitochondrial yield under such conditions should also be given

Author: The number of cells used for mitochondrial isolation now is given in the Introduction and Protocol part. The yield of mitochondria obtained with this protocol is given in the Introduction part.

Minor Concerns:

Typos should be corrected. For example: "brakes outer membranes" should really be "breaks outer membranes"

Author: The typos are corrected.

Reviewer #2:

Manuscript Summary:

The manuscript is clear and the method, although generally used in the field and previously described in excellent protocols (see for instance references 5 and 10 in the manuscript), must be of value for the readers that would benefit on visualisation of the full protocol.

The author thanks Reviewer for the positive comments and important suggestions.

Major Concerns:

- The title do not correspond with the techniques, there is not visualisation by BN-PAGE but by Western blot of the separated complexes. That point is crucial, if not there is already a protocol in JOVE (ref. 10) that explain the protocol.

Author: The title is modified accordingly.

Minor Concerns:

- In general the manuscript is well written, but it will benefit of a full language revision. In particular the word "brake" is used several times instead of "break"

Author: The typos are corrected.

- A full list of the antibodies to be used to detect the different complexes is essential to ensure the reproducibility of the method. The Figure 1 should clearly indicate the antibodies used in the Western Blots.

Author: The full list of antibodies is presented in Table of Materials. The legend of Figure 1 now indicates the antibodies used for immunoblotting.

Reviewer #3:

Manuscript Summary:

Konolava manuscript describes the use of blue native gel electrophoresis to analyze assembly of mitochondrial oxidative phosphorylation complexes from cultured cells. However important information about respiratory complexes, what are the expected results, how to optimize the solubilization with digitonin and lauryl maltoside and proper controls and molecular weight markers is not included in the experimental design. There are various concerns noted

The author thanks Reviewer for the detailed comments. The author also agrees that more information about respiratory complexes, expected results and optimization of the protocol should be included. The manuscript is modified accordingly.

Major Concerns:

1. Introduction does not include any description on the OXPHOS complexes, and what are the expected results using BN-PAGE to analyze the assembly of the complexes.

Author: The description of OXPHOS complexes is included to the Introduction, the applications of the method are also discussed.

2. Manuscript lacks information on how to control for equal loading in the experimental design. Describe how loading is normalized as commonly done in SDS-PAGE by using actin, tubulin or GAPDH antibodies?

Author: That is a nice suggestion, the information about loading control is now included in the Discussion.

In general, there is no good loading control for blue native blots. Some studies use GAPDH or tubulin as a loading control (for example, Emelyanova et al, Am J Physiol Heart Circ Physiol. 2016); however, these are not mitochondrial proteins and reflect the impurity of mitochondrial fraction. In some cases, Complex II can be used as a loading control when the complexes containing mitochondrially-encoded subunits are affected. However, the loading control should be found for the specific study. Careful measurement of protein concentration and running the samples in replicates is one of the ways to control equal protein loading for BN-PAGE.

3. Lack of incorporation of molecular weight markers in the experimental design. How would you know that you have a fully assembled complex versus an assembly intermediate?

Author: The estimation of the molecular mass of the protein complexes in BN-PAGE is challenging (Wittig et al., Molecular & cellular proteomics 2010). The current protocol does not include molecular weight marker, therefore to estimate the assembly of OXPHOS complexes the control samples containing unaffected complexes should be always included in the analysis. Now, this is described in Discussion.

4. Lack of information on how optimize digitonin and lauryl maltoside solubilization. Author should explain this better. How would you know if the amount of digitonin/lauryl maltoside is optimal or not? Include a range of concentrations to be used for each detergent.

Author: The optimization of digitonin now described in the Discussion, the range of concentrations is also included.

5. Figure 1 blots should include the whole western blot and not cropped images. Molecular weight markers should be included.

Author: In this protocol, the antibodies against individual OXPHOS complexes are used sequentially. The signal that each antibody gives depends on the specific antibody. Therefore, in the whole gel image, some of the OXPHOS complexes are overexposed and some are underexposed. That is why we prefer to present individual bands of the complexes.

Minor Concerns:

1. Line 43: spell out BN-PAGE (first time is used in the text although already defined in the abstract).

Author: The abbreviation of BN-PAGE is disclosed.

2. Procedure to prepare solutions should be described before the step were solution will be used. It is a little confusing describing the preparation of the solution after the step where it is needed particularly for inexperienced readers. Maybe having a separate section before the protocol section describing the preparation of solutions required will be more helpful than the way is presented in the current manuscript or:

Step 1.8.1 should be described before current step 1.8.

Step 1.12.1 should be described before step 1.12.

Step1.15.1 should be described before step 1.15.

Step 2.12.1 should be described before step 2.12.

Step 3.3.1, 3.1.2 and 3.3.1 should be described before step 3.1.

The author thanks Reviewer for this good suggestion. The separate section describing the preparation of solutions and buffers is included in the protocol.

3. All along the text, the authors should include molarities rather than grams of reagents.

Author: The molarities of reagents are used.

4. Indicate storage conditions of buffers.

Author: The storage conditions are indicated.

5. In line 102: please describe which method/reagent was used to measure protein concentration in this step. Authors should mention that the protein determination method needs to be compatible with the presence of detergent in the sample.

Author: The method of protein measurement is specified.

6. Include approximate running times for electrophoresis with the blue cathode buffer (step 3.4 and then for clear cathode buffer (step 3.5).

Author: The approximate running time for electrophoresis is included.

7. In line 192-193: Be more specific on how many times sample #3 underwent freeze-thaw cycles

Author: The number of freeze-thaw cycles is indicated.

8. Describe which controls should be used in the experimental design.

Author: The controls used for the analysis are described in Discussion.

9. In step 3.7: please describe the transfer buffer composition used for semidry transfer of proteins to PVDF membrane. In our experience, semi-dry transfer is not very efficient to transfer very large molecular weight proteins. Wet transfer works much better to transfer completely the very large OXPHOS complexes. Include wet transfer as alternative procedure in the protocol to transfer complexes to PVDF.

Author: In the protocol, Trans-Blot Turbo Mini PVDF Transfer Packs from Bio-Rad is used (indicated in Table of Materials). This pack includes transfer buffer.

Wet transfer is mentioned as an alternative method to transfer OXPHOS complexes to the membrane.

10. What is the purpose of the graph in figure 1A? This is not informative and since there is no statistical analysis the graph does not add anything to the figure.

Author: The author shows this figure to demonstrate one of the ways how the data from BN-PAGE could be presented. Now the figure is modified and includes statistical analysis.

11. In figure 1B. Can you observe the degradation products of sample #3 in the gel? The whole blot should be included in the figure.

Author: We could not see any degradation products in sample #3. Probably, the degradation products do not contain subunits that are detected by antibodies, or there are multiple degradation products that do not form clear bands.

12. Explain Figure 1D? why is membrane stripped and blotted again with same antibody?

Author: In the figure 1D the blot was first used to detect complex II and IV. Then the blot was probed with anti-NDUFA9 antibody to detect complex I. However, the background from the previously used antibody, detecting complex II and IV did not allow to get clear blot. That is why to get clear blot we tried to strip membrane and immunoblot it again.

Supplemental fig 1