Title: Analysis of organisation and activity of mitochondrial respiratory chain complexes in primary fibroblasts using blue native PAGE

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Abstract

Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a well-established technique for the isolation and separation of mitochondrial membrane protein complexes in a native conformation with high resolution. In combination with histochemical staining methods, BN-PAGE has been successfully used as clinical diagnostic tool for the detection of oxidative phosphorylation (OXPHOS) defects from small tissue biopsies in mitochondrial disease patients. However, its application to patient-derived primary fibroblasts is difficult due to limited proliferation and high background staining. Here, we describe a rapid and convenient method to analyse the organization and activity of OXPHOS complexes from cultured skin fibroblasts.

Keywords

Oxidative phosphorylation, supercomplex, in-gel activity, primary fibroblasts, mitochondria

Introduction

BN-PAGE is a convenient and inexpensive separation method that allows the isolation and determination of the oligomeric state of membrane protein complexes in its native state[1]. In combination with histochemical staining and immunodetection, it allows rapid identification of enzymatic and assembly defect in mitochondrial respiratory chain (MRC) complexes, and thus has led to the development of this techniques as a clinical diagnostic tool for studying the OXPHOS defects in patients with monogenic mitochondrial disorder[2, 3].

The functional study of the OXPHOS system using BN-PAGE has provided important insight into the organization of individual complexes within the inner mitochondrial membrane (IMM). In mammals, the four multimeric enzyme complexes of MRC- complex I, II, III and IV (CI, CII, CIII and CIV) are organized into higher-order structures known as supercomplexes (SCs) or respirasomes[4]. The formation of SCs by different stoichiometric combination of

MRC complexes requires specific assembly factors. For example, Cox7a2l (renamed as supercomplex assembly factor I (SCAFI)) acts as the primary factor for SC formation by mediating the stable interaction between CIII and CIV[5]. Although, it is widely accepted that OXPHOS system is organized into both individual MRC complexes and SCs such that both organization coexists within the IMM, the mechanism of SCs formation and its overall role in regulating mitochondrial function is not well understood[6]. Recently, the functional studies of SCs have demonstrated that the supramolecular organization of MRC complexes mediates fine tuning of cell growth in response to different metabolic cues through the formation of different SCs, confer structural stability to CI, limits ROS generation and facilitates electron channelling[7, 8].

This protocol describes a rapid and convenient method to analyse the organization and activity of SCs from primary fibroblast with high resolution. BN-PAGE has been used to identify OXPHOS defects from tissue biopsies such as liver and skeletal muscle however, its application to primary fibroblast is limited[3]. Primary fibroblasts derived from skin biopsies, routinely used as in vitro disease model are characterized by non-exponential growth in culture. Therefore, a minimum cell density of 40 x 10^6 cells is required to achieve a reasonably pure yield of mitochondrial preparation from cultured cells[9]. Secondly, the resolution of SCs and individual complexes is hindered due to the high background staining which occur as a result of high levels of co-migrating proteins[3]. Here, we provide an optimized protocol for the visualization and analysis of MRC complexes from as low as 5x10^6 cells with good resolution.

The basic principles of the BN-PAGE remain unchanged. Non-ionic detergents are used for the solubilization of isolated mitochondrial membrane protein complexes. Mild detergents with low delipidating properties such as digitonin, preserves the supramolecular association of MRC complexes. After solubilization of mitochondria and centrifugation, the anionic dye, Coomassie G-250 is added to the supernatant which binds to all membrane proteins owing to its hydrophobic properties. Dye binding imposes a charge shift that causes all proteins to migrate to the anode at pH 7.5 during electrophoresis. The negatively charged protein surfaces repel each other and prevent the formation of membrane protein aggregates. Furthermore, the negatively charges dye-associated membrane proteins become soluble in detergent-free solution which minimizes the risk of denaturation of detergent-sensitive protein complexes. However, the proteins are not resolved based on the charge/mass ratio but according to the pore size of the acrylamide gradient gels which reduces protein migration velocity as a function of protein mass and can completely stop protein migration at a mass-specific pore size limit[10].

We simplify our method in three major steps: Step 1 to 8 describes mitochondria isolation from the primary fibroblasts, Step 9 to 15 describes solubilization and BN-PAGE run, following that one can opt perform either the in-gel activity staining for different mitochondrial complexes- step 16 to 21 or immunoblotting- step 22-30.

Materials

Primary fibroblast culture: Five days before the performing the experiment, seed approximately 3 x 10^6 cells in culture medium (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and penicillin–streptomycin 50 U/ml and 50 µg/ml, respectively) per 150 cm^2 tissue culture dish. Replenish the culture medium on the third day. **Note 1**

Use Milli-Q-purified water or equivalent in all buffer recipes and protocol steps.

Mitochondria isolation buffer (MIB): 1 M sucrose-Dissolve 342.3 g of sucrose in 1 liter of distilled water; prepare 40 ml aliquots; store at -20 C. 0.1 M Tris/MOPS Dissolve 12.1 g of Tris in 500 ml of distilled water, adjust pH to 7.4 using MOPS powder, bring the solution to 1 liter and store at 4 C. 0.1 M EGTA/Tris Dissolve 38.1 g of EGTA in 500 ml of distilled water, adjust pH to 7.4 using Tris powder, bring the solution to 1 liter and store at 4 C.

Prepare 25 ml of MIB by adding 2.5 ml of 0.1 M Tris–MOPS and 250 µl of EGTA/Tris to 5 ml of 1 M sucrose. Bring the volume to 25 ml with distilled water. Adjust pH to 7.4. Add 1X protease inhibitor cocktail (Roche, 11697498001) fresh, prior to mitochondrial isolation.

Dulbecco's phosphate-buffered saline (DPBS; Gibco, 14190094), 0.25% Trypsin-EDTA (Thermo Fisher Scientific, 25200056), Pierce BCA Protein Assay Kit (Thermo Scientific, 23225).

Gel electrophoresis: 5% digitonin (Thermo Fisher Scientific, BN2006), 4X NativePAGE™ sample buffer (Thermo Fisher Scientific, BN20032), NativePAGE™ 5% G-250 sample additive (Thermo Fisher Scientific, BN2004), 20X NativePAGE™ running buffer (Thermo Fisher Scientific, BN2001), Native PAGE™ Cathode Buffer Additive (20X), NativePAGE™ Novex 3% to 12% Bis-Tris protein gels (1.0 mm, 10 well, Thermo Fisher Scientific, BN2011BX10), NativeMark Unstained Protein Standard (Thermo Fisher Scientific, LC0725), Colloidal blue staining kit (Thermo Fisher Scientific, LC6025).

Anode buffer- Freshly prepare 1000 mL 1X NativePAGE™ Anode Buffer with water.

Cathode buffer: (i) Dark blue cathode buffer- Add 10 ml of NativePAGE™ Cathode Buffer Additive (20X) and 10 ml NativePAGE™ Running Buffer (20X) to 180 ml anode buffer and mix well. (ii) Light blue cathode buffer- Add 1 ml of NativePAGE™ Cathode Buffer Additive (20X) and 10 ml NativePAGE™ Running Buffer (20X) to 189 ml anode buffer and mix well. Prepare fresh each time for immediate use.

Substrates for CI, CII, and CIV activity: For CI activity-Freshly prepare 20 ml of 2 mM Tris/HCl buffer (pH 7.4) from 1M Tris/HCl containing 2.5 mg/ml Nitrotetrazolium Blue chloride (NBT) and 0.1 mg/ml NADH. For CII- Freshly prepare 5 ml of 0.5 mM Tris/HCl buffer (pH 7.4) containing 25 NBT, add 200 µl 1 M sodium succinate, 8 µl 250 mM phenazine methosulfate (prepare 250 mM stock in DMSO) and bring the solution to 10 ml with water. For CIV-Freshly-prepare 20 ml solution containing 50 mM potassium phosphate (pH 7.4), 5 mg of 3,3'-diaminobenzidine tetrachloride (DAB), and 10 mg of cytochrome c.

Immunoblotting: 20X NuPAGE transfer buffer (Thermo Fisher Scientific, NP0006-1)- Freshly prepare 200 ml of 1X NuPAGE transfer buffer with 20% (v/v) methanol in water, 10x Tris Buffered Saline, TBS) (Biorad, 1706435)- Freshly prepare 1X TBS with 0.1% (v/v) Tween-20 (Sigma, P9416) (TBS-T), 5% (w/v) skim milk (Millipore, 1153630500) in TBS-T, Immun-Blot PVDF membrane (Bio-Rad, 1620177), OxPhos complex kit antibody cocktail (Thermo Fisher Scientific, 45-7999)- Prepare 10 ml primary antibody solution (1:1000) in 5% skim milk in TBS-T, Horseradish peroxidase (HRP)–conjugated IgG secondary antibody (Sigma, A9044)- Prepare 10 ml secondary antibody solution (1:5000) in 5% skim milk in TBS-T and Chemiluminescent reagent (e.g., Luminata Forte Western HRP substrate, Merck Millipore, WBLUF0100).

Additional materials and equipment: 1.5-ml Eppendorf tubes, 1 ml syringe (e.g., BD Luer-Lok. 309628), 25-gauge 1 inch needle (e.g., BD Microlance 3), centrifuge, Protein gel electrophoresis chamber system (XCell SureLock Mini-Cell, Thermo Fisher, EI0001), Power supply (e.g., PowerPac HC High-Current Power Supply, Bio-Rad, 1645052), Mini-gel wet-transfer system (XCell II™ Blot Module, Thermo Fisher, EI9051), Blot imaging system (e.g., ChemiDoc XRS+ System with Image Lab Software, Bio-Rad, 1708265) and ImageJ2 software (National Institutes of Health)

Methods

Isolation of mitochondria from primary fibroblasts-

The entire procedure should be conducted on ice, using refrigerated equipment maintained at 4°C.

1. Remove the medium from the cells and wash the cells once with DPBS and detach the cells using trypsin solution at room temperature (RT). Add DMEM to stop trypsinization and transfer the cell suspension to 15 ml Falcon tube.

2. Centrifuge cells at 600g for 5 min at 4 °C. Discard the supernatant and resuspend the cells in ice cold DPBS. Centrifuge cell suspension again at 600g for 5 min at 4°C.

3. Discard the supernatant and resuspend cells in 800 µl of ice-cold MIB and transfer in a 1.5 ml Eppendorf tube. Incubate the cell suspension for 5 min on ice.

4. Homogenize the cells by drawing the solution into a 1 ml syringe fitted with a 25-gauge needle expel it back into the 1.5 ml tube on ice. Take care to expel the solution against the inside wall of the tube as to utilize that force for cell membrane disruption.

5. Repeat the homogenization steps for a total of five times and centrifuge at 600g for 10 min at 4°C. **Note 2**

6. Transfer supernatant (700 µl, avoiding the supernatant close to the pellet containing cell and nuclear debris) to a 1.5-ml tube and centrifuge at 8000g for 10 min at 4°C. Discard supernatant and resuspend pellet containing mitochondria in 800 µl cold MIB. Centrifuge again at 8000g for 10 min at 4°C. **Note 3**

7. Discard the supernatant and resuspend the pellet in 100 µl of cold MIB. **Note 4**

8. Measure mitochondrial protein concentration using the BCA assay and aliquot 100 µg of mitochondrial protein into 1.5 ml tube, centrifuge at 10000g for 10 min at 4°C. Discard supernatant and keep the pellet on ice for performing BN-PAGE. **Note 5**

Solubilization of mitochondrial protein and BN-PAGE:

9. Solubilize the mitochondrial membrane by adding 20 µl sample buffer cocktail (Table 1) to 100 µg mitochondrial protein. Gently mix the pellet without the formation of bubbles and incubate solubilized mitochondria on ice for 20 min. **Note 6**

10. Centrifuge at 20,000g for 20 min at 4°C then collect 20 µl of supernatant into a new 1.5 ml tubes. Add 1.5 µl of Coomassie G-250 sample additive to the above supernatant. **Note 7**

11. Set up electrophoresis system by placing the NativePAGE 3% to 12% gradient gel in the XCell SureLock™ Mini-Cell apparatus. **Note 8**

12. Add 50 ml of dark blue cathode buffer in the inner chamber and check for any leakage. Also, wash the wells with 1 ml dark blue cathode buffer.

13. Fill the inner chamber with remaining cathode buffer and gently load 20 µl sample and protein standards (5 µl) into the wells using a P10 tip. Fill the outer chamber with 600 ml running buffer.

14. Set power supply at 100 V and continue electrophoresis for 30 min until the sample has entered the gel.

15. After the blue running front has moved about one-third of the desired total running distance, remove cathode buffer using a 10-ml pipet or suction tube and fill inner chamber with 200 ml light blue cathode buffer. Continue the run at 200 V for 60 min or until the blue running front reaches gel end. **Note 9**

In-gel activity staining for CI, CII and CIV:

16. Carefully remove gel after completion of the run. If the samples are limiting, the gel can be cut into strips using a Gel Knife and washed with either Tris/HCl or phosphate buffer (pH 7.4). **Note 10**

17. For CI activity: Incubate the gel strip in 20 ml of complex I substrate solution at RT. Appearance of violet bands within 15-30 min of incubation is indicative of CI activity (Fig. 1A). **Note 11**

18. For CII activity: Incubate the gel strip in 10 ml complex II substrate solution for 1-2 h at RT. Appearance of violet band is indicative of CII activity (Fig. 2A).

19. For CIV activity: Incubate the gel strip in 10 ml complex IV substrate solution for 1-2 h at RT. Appearance of brown bands is indicative of CIV activity (Fig. 3A). **Note 12**

20. For CIV + CI activity: Incubate the gel strip first in complex IV substrate solution. After the appearance of appropriate brown signal, wash the gel with Tris/HCl buffer (pH 7.4) and incubate in complex I substrate solution.

21. Stop reaction with 10% acetic acid when the appropriate signal is observed. Wash the gel with water and document the gel using a scanner.

Immunoblotting.

As mentioned above, if the samples are limiting, the gel can be cut in half after the run and used for in-gel activity and immunoblotting.

22. Activate PVDF membrane with methanol and transfer it to 1X NuPAGE transfer buffer.

23. Set up the blotting apparatus using the XCell II™ Blot Module as per the manufacturer's instructions. Prepare the gel/membrane assembly and blotting pads in the cathode core. Remove any trapped air bubbles between the gel and membrane ensuring complete contact of all components.

24. Slide the anode core on top of the pads and fill the blot module with transfer buffer until the gel/membrane assembly is covered.

25. Fill the outer chamber with 600 ml of deionized water and transfer for 2 h at 20 V.

26. Following transfer, incubate the membrane in methanol for 5 min for background destaining. **Note 13**

27. After complete destaining, incubate the membrane in 5% skim milk in TBS-T for 1 h to block the membrane.

28. Incubate membrane with primary antibody solution overnight at 4°C.

29. Wash the blot three times with TBS-T for 5 min each followed by the incubation in primary antibody solution for 1 hr at room temperature. **Note 14**

30. Wash the blot again with TBS-T and visualize the expression levels of SCs and individual complexes using a chemiluminescent reagent and a blot imaging system (Fig. 2A). **Note 15**

Alternatively, the expression levels of SCs and individual complexes can also be visualized using the colloidal blue staining kit as per the manufacturer's instructions (Fig. 2B). The expression levels of individual bands can be determined using densitometry analysis in ImageJ2 software.

Notes

1. Ensure that cells reach 100% confluency. The total cell number should be at least 5 x 10^6 to obtain a minimum yield of 100 µg of mitochondrial pellet.

2. Do not exceed more than 5 strokes. This will introduce the nuclear DNA contamination into the mitochondrial preparation and hinder sample run during BN-PAGE.

3. Minimise the excess pipetting during the transfer of supernatant. Use a wide bore pipette tip (cut tip end with sharp blade) or transfer by tilting to another 1.5 ml tube.

4. Avoid diluting mitochondria with buffer. Mitochondria retain their functionality when stored in a concentrated form.

5. The mitochondrial pellet from step 5 can be stored at −80°C for several months. Divide the mitochondrial in appropriate aliquots before storage to avoid the freeze-thaw cycles.

6. The detergent/protein ratio is very critical for solubilizing the mitochondrial membrane proteins. For 100 µg of mitochondrial protein, the digitonin/protein ratio is 6 g/g. The concentration of digitonin should be adjusted according to the protein concentration, which is given by the expression:

$$Digitonin\ stock\ \left(\frac{w}{v}\right) \times V1 = \frac{Digitonin\ (g)}{Protein(g)} = 6$$

7. The detergent/dye ratio is also very critical for a good resolution of SCs and individual complexes as well as in reducing the background staining during electrophoresis. The concentration of dye should be adjusted to give a detergent/dye ratio of 8 g/g.

8. The wells should be left empty as this majorly distorts the gel. Load 20 µL of 1X sample buffer to any unused lanes.

9. For a better separation of the bands, the gel can be run at low power, 150 V for 2 h.

10. To remove the gradient gel using the Gel Knife by handling it from the bottom, which is stronger and therefore chances of breakage are minimized.

11. Avoid the prolong incubation of gel strip in CI substrate solution as this will increase the background staining. Once the appropriate violet signal starts appearing after 15-30 min of incubation stop the reaction.

12. Due to the common substrates of CIII and CIV it is difficult to detect in-gel activity of CIII. This protocol does not describe the method for in-gel activity of CIII.

13. Properly destain the PVDF membrane with methanol as coomassie dye binds to membranes and thereby reduces protein binding capacity. Do not use nitrocellulose membranes, since these membranes cannot be destained under the conditions described above.

14. Alternatively, the membrane can be reprobed with different antibody for each mitochondrial complexes.

15. Take multiple exposures of the blot to capture faint signal from the SCs as well as the strong signal for individual complexes.

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References

[1] H. Schagger, W.A. Cramer, G. von Jagow, Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis, Anal Biochem 217(2) (1994) 220-30.

[2] R.D. Pitceathly, S. Rahman, Y. Wedatilake, J.M. Polke, S. Cirak, A.R. Foley, A. Sailer, M.E. Hurles, J. Stalker, I. Hargreaves, C.E. Woodward, M.G. Sweeney, F. Muntoni, H. Houlden, J.W. Taanman, M.G. Hanna, U.K. Consortium, NDUFA4 mutations underlie dysfunction of a cytochrome c oxidase subunit linked to human neurological disease, Cell Rep 3(6) (2013) 1795-805.

[3] R. Van Coster, J. Smet, E. George, L. De Meirleir, S. Seneca, J. Van Hove, G. Sebire, H. Verhelst, J. De Bleecker, B. Van Vlem, P. Verloo, J. Leroy, Blue native polyacrylamide gel

electrophoresis: a powerful tool in diagnosis of oxidative phosphorylation defects, Pediatr Res 50(5) (2001) 658-65.

[4] R. Acin-Perez, P. Fernandez-Silva, M.L. Peleato, A. Perez-Martos, J.A. Enriquez, Respiratory active mitochondrial supercomplexes, Mol Cell 32(4) (2008) 529-39.

[5] R. Perez-Perez, T. Lobo-Jarne, D. Milenkovic, A. Mourier, A. Bratic, A. Garcia-Bartolome, E. Fernandez-Vizarra, S. Cadenas, A. Delmiro, I. Garcia-Consuegra, J. Arenas, M.A. Martin, N.G. Larsson, C. Ugalde, COX7A2L Is a Mitochondrial Complex III Binding Protein that Stabilizes the III2+IV Supercomplex without Affecting Respirasome Formation, Cell Rep 16(9) (2016) 2387-98.

[6] J.A. Enriquez, Supramolecular Organization of Respiratory Complexes, Annu Rev Physiol 78 (2016) 533-61.

[7] E. Lapuente-Brun, R. Moreno-Loshuertos, R. Acin-Perez, A. Latorre-Pellicer, C. Colas, E. Balsa, E. Perales-Clemente, P.M. Quiros, E. Calvo, M.A. Rodriguez-Hernandez, P. Navas, R. Cruz, A. Carracedo, C. Lopez-Otin, A. Perez-Martos, P. Fernandez-Silva, E. Fernandez-Vizarra, J.A. Enriquez, Supercomplex assembly determines electron flux in the mitochondrial electron transport chain, Science 340(6140) (2013) 1567-70.

[8] E.A. Shoubridge, Supersizing the mitochondrial respiratory chain, Cell Metab 15(3) (2012) 271-2.

[9] E. Fernandez-Vizarra, G. Ferrin, A. Perez-Martos, P. Fernandez-Silva, M. Zeviani, J.A. Enriquez, Isolation of mitochondria for biogenetical studies: An update, Mitochondrion 10(3) (2010) 253-62.

[10] I. Wittig, H.P. Braun, H. Schagger, Blue native PAGE, Nat Protoc 1(1) (2006) 418-28.

Table 1:

Preparation of sample buffer cocktail and quantity of detergent and dye required for 100 µg protein.

Protein	Digitonin/ protein ratio (g/g)	4× sample buffer (µl)	5% Digitonin (µl)	Water (µl)	Final volume (µl)	Digitonin/ dye ratio (g/g)	5% Coomassie G-250 (µl)
100 µg	6	5	12	3	20	8	1.5

Figure legends:

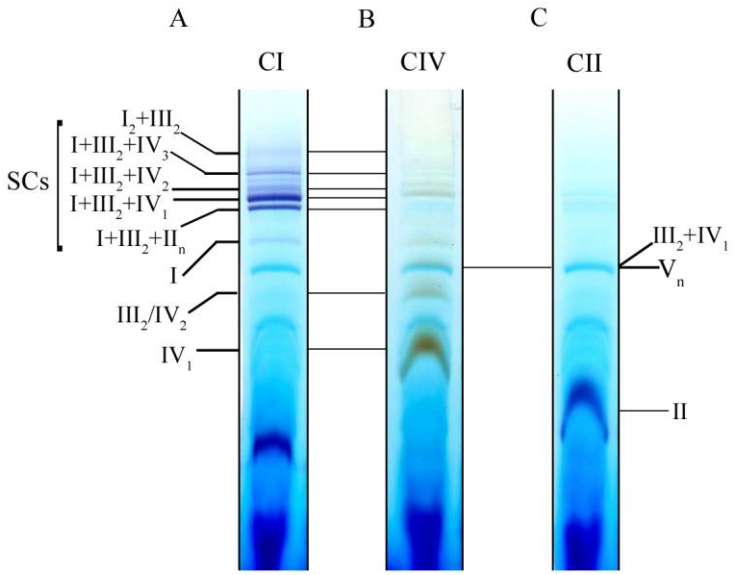

Figure 1: BN-PAGE, followed by in-gel activity assay to visualize the SCs and individual complexes from isolated mitochondria of primary fibroblasts. 100 µg of mitochondrial protein (6 g/g digitonin/protein ratio and 8 g/g digitonin/dye ratio) was used for BN-PAGE and in-gel activity for mitochondrial complexes were performed. (A) CI activity is shown in violet (20 min incubation). (B) CIV in brown (1 h incubation). (C) CII activity is shown in violet (1 h incubation).

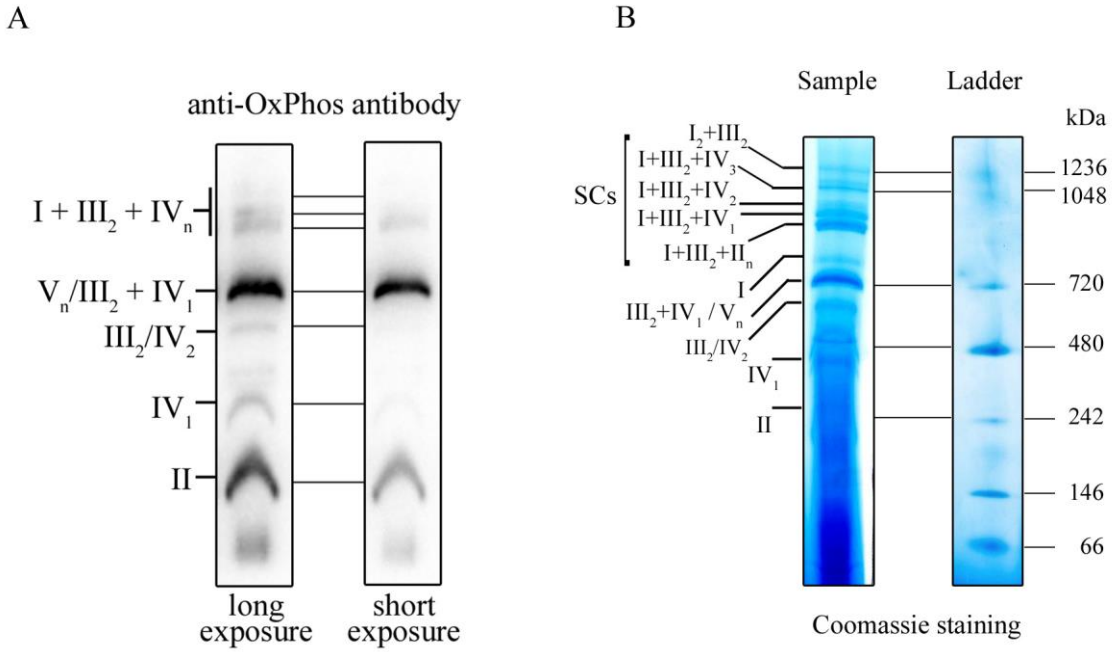

Figure 2: BN-PAGE, followed by immunoblotting or Coomassie staining to visualize the SCs and individual complexes. 100 μg of mitochondrial protein (6 g/g digitonin/protein ratio and 8 g/g digitonin/dye ratio) was used for BN-PAGE and the expression levels of SCs and individual complexes was detected by either (A) immunoblotting using an OxPhos antibody cocktail or (B) coomassie staining and destaining.