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.**