Mitochondrial oxygen consumption

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Cage Studies

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EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Aw WC, Towarnicki SG, Melvin RG, Youngson NA, Garvin MR, Hu Y, Nielsen S, Thomas T, Pickford R, Bustamante S, Vila-Sanjurjo A, Smyth GK, Ballard JWO (2018) Genotype to phenotype: Diet-by-mitochondrial DNA haplotype interactions drive metabolic flexibility and organismal fitness. PLoS Genet 14(11): e1007735. doi: 10.1371/journal.pgen.1007735

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PROTOCOL STATUS

Working

1 Isolation buffer: 154 mM KCl, 1 mM EDTA, pH 7.4

2 Mitochondrial Assay Solution (MAS): 115 mM KCl (Sigma P9333), 10 mM KH2PO4 (Sigma P9791), 2 mM MgCl2 (Sigma M1028), 3 mM HEPES (Sigma H0887), 1mM EGTA (Sigma E4378), FA-free BSA 0.2% (A7511), adjust solution to pH 7.2 using 5N KOH.CAUTIONS: The presence of BSA in the MAS solution preserves mitochondrial coupling. It is essential to have a concentration of 0.2% BSA in the MAS solution.

3 Injection compounds: Port A: 2.5 â€“ 20 mM ADP (Sigma A2754); Port B: 40 ÂµM Oligomycin (Sigma O4876); Port C: 2.5 - 320 ÂµM BAM 15(Tim Tec ST056388)/ 40 - 160 ÂµM FCCP (Sigma C2920); Port D: 20 ÂµM Antimycin A (Sigma A8674) and Rotenone (Sigma R8875).TIPS: ADP can be prepared as 100mM stock in MAS solution and adjusted to pH 7.2. Other injection compounds can be prepared at 1000X in DMSO and diluted to 10X with MAS for port loading. The final concentration in the well after injection is 1X concentration.

4 Complex I Assay Media: 11 mM Pyruvate (Sigma P2256), 11 mM malate (Sigma M1000), 11 mM L-proline (Sigma P0380), pH 7.2.TIPS: Pyruvate, malate and L- proline can be prepared as 100 mM stock in MAS solution and adjusted to pH 7.2. The substrates can then be diluted to the desired concentration (11 mM) with MAS.EQUIPMENT

5 Collect 10 whole wandering third instar larvae and wash them in water and 70% ethanol (for this optimization we included males and females). Place them in 100 ÂµL of ice-cold mitochondrial isolation buffer in 1.5 mL microcentrifuge tubes.

6 Gently homogenize the third instar larvae or thoraces with 80 strokes up and down with a plastic microtube pestle.CAUTIONS: Press dounce straight up and down without grinding. If a pellet forms during homogenization, release it with a spatula or pipette tip and continue the process.

7 Transfer the homogenate into a 1-cc syringe containing 200 ÂµL of isolation buffer.

8 Force the homogenate through the gauze filter and collect in a new 1.5 mL microcentrifuge tube.

9 Centrifuge the filtered homogenate at 1500 x g at 4oC for 8 minutes.CAUTIONS: Centrifugation at a higher speed should be avoided as increasing the spin to 5000 x g results in mitochondria with low respiration rates.

10 Remove and discard the cloudy supernatant, careful do not disturb the pellet.

11 Wash the pellet with 200 ÂµL of mitochondrial isolation buffer and then resuspend in 20 ÂµL of isolation buffer.

12 Perform the Bradford assay according to the manufacturerâ€™s instructions (Sigma B9616).TIPS: We suggest 2 ÂµL of mitochondrial stock be diluted with 10 ÂµL of mitochondrial isolation buffer (1 to 6 dilutions) to ensure the readings are within the standard curve range for Bradford assay.

13 Prepare the XF sensor cartridge

14 The day before the assay, hydrate the XF sensor cartridge at 23oC overnight in XF calibration buffer.NOTES: The assay was performed at 23oC and temperature is subjective to change based on the study design.

15 On the day of assay, load the XF sensor cartridge injections port with injections compounds.

16 Volume of compounds loaded in each port: Port A: 50 ÂµL; Port B: 55 ÂµL; Port C: 60 ÂµL; Port D: 65 ÂµL.

17 Calibrate the sensor cartridge by following the XF instrument protocol (Table 1).

18 Preparation of Mitochondrial Complex I Assay Plate

19 Dilute the mitochondrial stock solution (obtained from step 1.7) with MAS to yield a final concentration of 0.02 - 0.4 Âµg/ÂµL.TIPS: Due to the high concentration of mitochondria in the suspension, it is recommended that the mitochondria be first mixed by gently pipetting, and then performing 1 to 6 dilutions with MAS to form a final volume of ~100ÂµL (This additional step is subject to the mitochondrial stock concentration. E.g. a lower concentration of mitochondria may require 1 to 3 dilutions). This is then added to a larger volume to form the desired concentration.

20 Pipette 50 ÂµL of the diluted mitochondria into each well of XF cell culture microplate. E.g., 50 ÂµL of diluted mitochondria with 0.1, 0.2 and 0.4 Âµg/uL concentration will results in 5, 10 and 20 Âµg of total mitochondria per well.

21 Transfer 50ÂµL of MAS into each background correction wells.

22 Centrifuge the XF cell culture microplate at 2254 x g at 4oC for 20 minutes.

23 After centrifugation, visualize the mitochondria under the microscope using 20X magnification. Make note of any wells that do not have a â€œmonolayerâ€ of mitochondria adhered to the well bottom.

24 Add 450 ÂµL of complex I assay media to each well and incubate the cell culture microplate at 23oC for 10 minutes.NOTES: The assay was performed at 23oC and temperature is subjective to change based on the study design.

25 Subsequently, exchange the calibration plate with the cell culture microplate and follow the instructions on the instrument controller to continue with XF instrument protocolÂ