Shirihai Lab Protocol for the study of oxygen consumption in isolated mitochondria

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This protocol has been established thanks to important contributions of Dr. Alvaro Elorza, Dr. George W. Rogers, Dr. Martin Brand, Dr. David Ferrick, Dr. David Nicholls and Dr. Anne N. Murphy. Dr. Rogers from Seahorse can be contacted by email: GRogers@seahorsebio.com or at the company website: www.seahorsebio.com.

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1. Mitochondrial isolation.

Hearts were incubated and minced in ice-cold fiber relaxation buffer (during approximately 10 minutes; KCl 100 mM, EGTA 5 mM, HEPES 5 mM adjusted with KOH to pH 7.0; in order to facilitate the release of intermyofibrillar mitochondria) and they were homogenized in 2 ml of HES buffer (HEPES 5 mM, EDTA 1 mM, Sucrose 0.25M, pH 7.4 adjusted with KOH 1M) using a glass dounce homogenizer (20 strokes with loose pestle, 20 strokes tight pestle). The homogenate was centrifuged at 500xg for 10 minutes at 4 °C (pellet discarded and supernatant re-centrifuged at 500xg). The supernatant was centrifuged at 9000xg for 15 minutes at 4 °C and the crude mitochondrial pellet was re-suspended in 100-200 µl of HES buffer with 0.2% of BSA FFA-free (an additional centrifugation step for washing the pellet can be performed). Protein was quantified using BCA (Pierce) and the value of protein measured in HES-BSA 0.2% buffer alone was subtracted.

Livers were homogenized after washing the blood with PBS and using a Potter-Elvehjem (teflon-glass) homogenizer instead, performing 15 strokes in ice-cold isolation buffer (250 mM mannitol, 75 mM sucrose, 100 µM K-EDTA, 10 mM K-HEPES, pH 7.4) supplemented with 500 µM K-EGTA (pH 7.4). Homogenates

were centrifuged at 1000×g for 10 min. Supernatants were removed and centrifuged at 10,000×g for 15 min. Pellets were washed three times in isolation buffer supplemented with 0.5%BSA FFA-free (Sigma A-6003). The first wash buffer was also supplemented with 500 µM EGTA. The final mitochondrial pellet was re-suspended in HES buffer with 0.2% of BSA FFA-free for respiration (and without BSA for complexes activity). This isolation protocol was developed by Kristal, B. S. & Brown, A. M. J Biol Chem 274, 23169-75 (1999).

2. Mitochondrial oxygen consumption measurements using XF24.

2.1 V7 plate loading

The amount of mitochondria was titrated, as the absolute amount of protein loaded is highly dependent on the mitochondrial isolation protocol, the tissue and species and the loading methodology used.

Titration is required in order to avoid O_2 depletion in the well. As a guideline, State II **o**xygen **c**onsumption **r**ates (**OCR**) should be ~100-200 pmol/min/well and State 3 or uncoupled rates should not exceed 1600-1800 pmol/min/well.

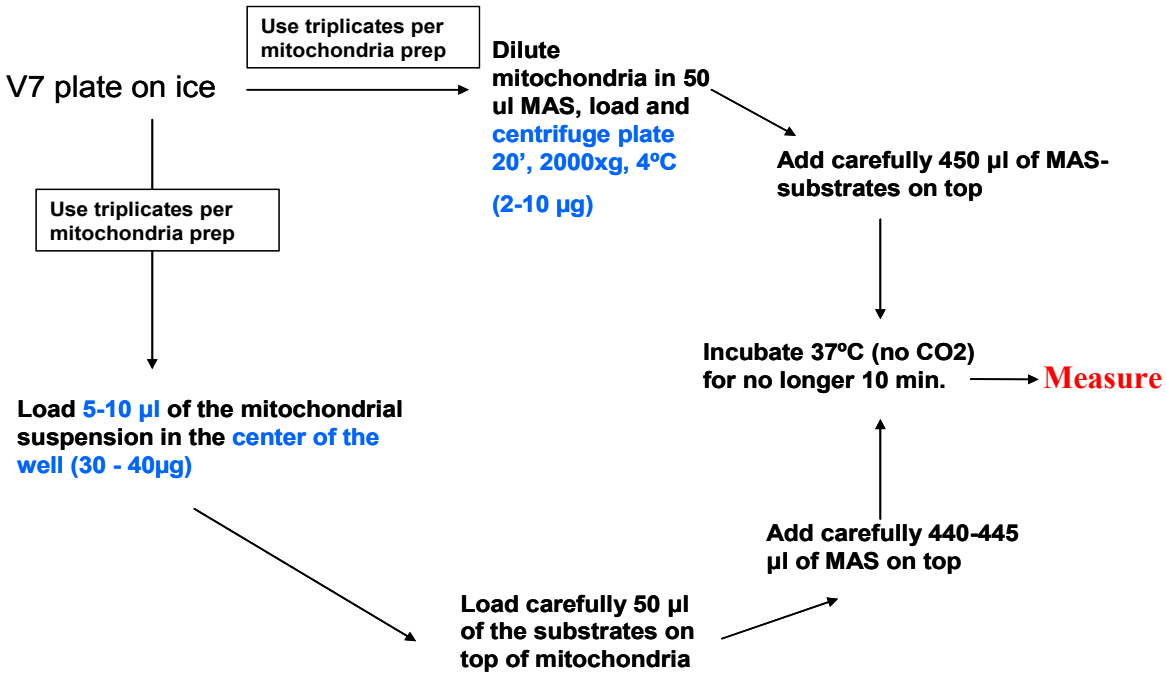
In the present manuscript, isolated mitochondria (30-40 µg in HES-BSA 0.2% buffer per well, n=3-4 replicates per mouse) were loaded in the center of the well (using 5-10 µl, low volume that facilitates the contact and adhesion of mitochondria to the bottom of the well) of a V7 plate on ice and 440-445 µl of ice cold **M**itochondrial **A**ssay **S**olution:

MAS: Sucrose 70 mM, Mannitol 220 mM, KH_2PO_4 5 mM, $MgCl_2$ 5 mM, HEPES 2 mM, EGTA 1 mM, BSA fatty acid-free 0.2 %, pH 7.4 adjusted with KOH 1 M + 50 µl of MAS buffer with 10X substrates (complex II driven respiration: succinate 50 mM + rotenone 20 µM; complex I: pyruvate + malate, 50 mM; or glutamate + malate, 50 mM each) were added on top (final concentration of substrates is 5 mM each and 2 µM for rotenone).

Alternatively, the mitochondrial suspension can be diluted in 1X MAS to 0.04-0.2 mg/ml and 50 µl loaded into the V7 plate wells (2-10 ug per well, depending on the species/tissue/substrates used). After loading, centrifugation of the V7 plate (20 minutes at 2000 x g at 4ºC) is performed to attach the mitochondria at the bottom of the plate. After centrifugation, 450 µl of MAS + 1X substrates (ice cold, 10 mM substrate(s), 2 µM rotenone for complex II) are gently added to each well. This step was not used in the present manuscript, but was successfully developed by Dr. George W. Rogers, Dr. Alvaro Elorza and Dr. Anne N. Murphy (personal communication)

The loaded V7 plate was incubated for 7-8 minutes at 37°C (no CO_2) before loading it into the XF24. Mitochondria attachment to the bottom of the plate can be observed using a microscope (20x) before and after the measurements. The initial consumption rate of oxygen measured before the first injection (port A) is state II (no ADP present, only respiration due to proton leak and contaminant ADP, also known as pseudo-state 4; see below).

Scheme plate loading:



2.2 Loading the cartridge

The dilutions of ADP and the different mitochondrial chemicals are freshly prepared the day of the experiment from concentrated stocks.

The four sequential injection ports of the Seahorse cartridge contained (in 1X MAS solution and adjusted to pH 7.4):

A (first port injected): 50 µl 10X substrate and ADP 2.5 mM;

B: 55 µl Oligomycin 20 µM (ATP synthase inhibitor);

C: 60 µl 2,4-dinitrophenol (DNP; uncoupler) 1 mM;

D: 65 µl Antimycin A 40 µM (complex III inhibitor).

Therefore the final concentrations are ADP 250 µM, Oligomycin 2 µM, DNP 100 µM and Antimycin 4 µM.

Oxygen consumption rates (pmols oxygen/min) were monitored in real time after the injection. State III was determined after port **A** injection, State IV after port **B** and uncoupled respiration rates after port **C**. Antimycin A was used as a control, as it blocks mitochondrial oxygen consumption linked to the electron transport chain. The ratio between state III and state IV is used as a control for the quality of the mitochondrial preparation and it is known as RCR (respiratory control ratio). For complex IV measurements, TMPD 1mM and Ascorbate 100 mM (in 1X MAS, which allow delivery of electrons to complex IV independently of the other complexes) were loaded and injected to the well after inhibition of respiration by Antimycin A injection. The final concentration in the well was TMPD 100 µM and Ascorbate 10 mM.

2.3 Measurement protocol (performed at 37 ºC)

Total XF Assay Time is ~ 65 min.

-Load Cartridge (29 min calibration)

-Load V7 plate.

1) Equilibration (3 cycles of 2 min Mix, 2 min Wait).
2) State II (2 cycles of 25 sec Mix, 4 min Measure).
3) Injection port A.
4) State III (1 cycle of 25 sec Mix, 4 min Measure, 30-60 sec Mix*).
5) Injection port B.
6) State IV_o (1 cycle of 25 sec Mix, 4 min Measure, 30-60 sec Mix*).
7) Injection port C.
8) Uncoupled (1 cycle of 25 sec Mix, 4 min Measure, 30-60 sec Mix*).
9) Injection port D.
10) Antimycin A (1 cycle of 25 sec Mix, 4 min Measure).

* Adjust the time to ensure optimal replenishment of oxygen in the sensor.

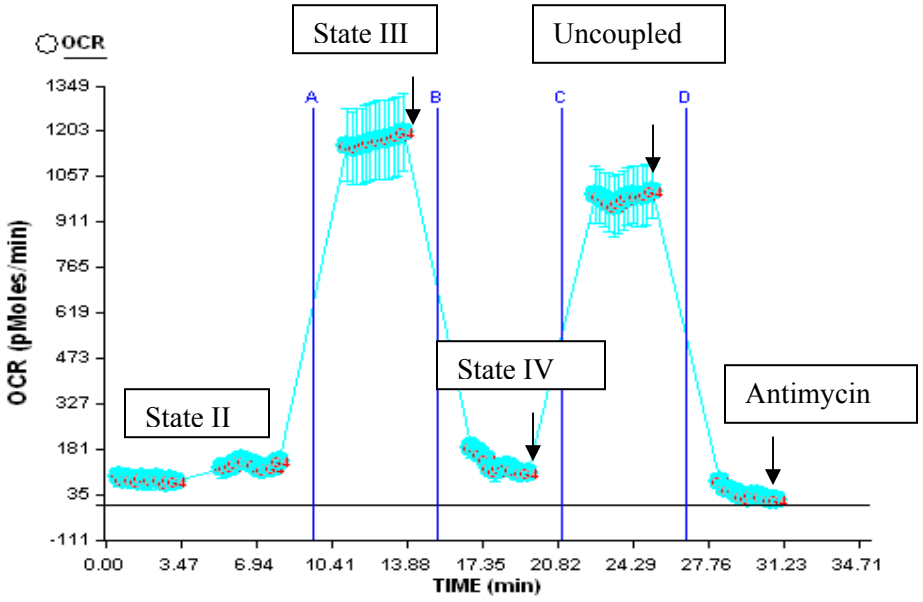
Below you will find a table with all the commands of the measurement protocol summarized above:

Command	Time	Port
Calibration	29 min	-
Mix	1 min	
Wait	3 min	
Mix	1 min	
Wait	3 min	
Mix	30 sec	
Measure	3 min	
Mix	1 min	
Measure	3 min	
Mix†	30 - 60 sec	
Inject	-	A
Mix	1 min	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		B
Mix	1 min	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		C
Mix	1 min	
Measure	3 min	
Mix†	30 - 60 sec	
Inject		D
Mix	1 min	
Measure	3 min	
† The mixing commands AFTER measurement commands are optional and facilitate the sensors returning to ambient O2 concentration. These steps are useful if the basal respiration rate (OCR) is above 200 pmol/min.		

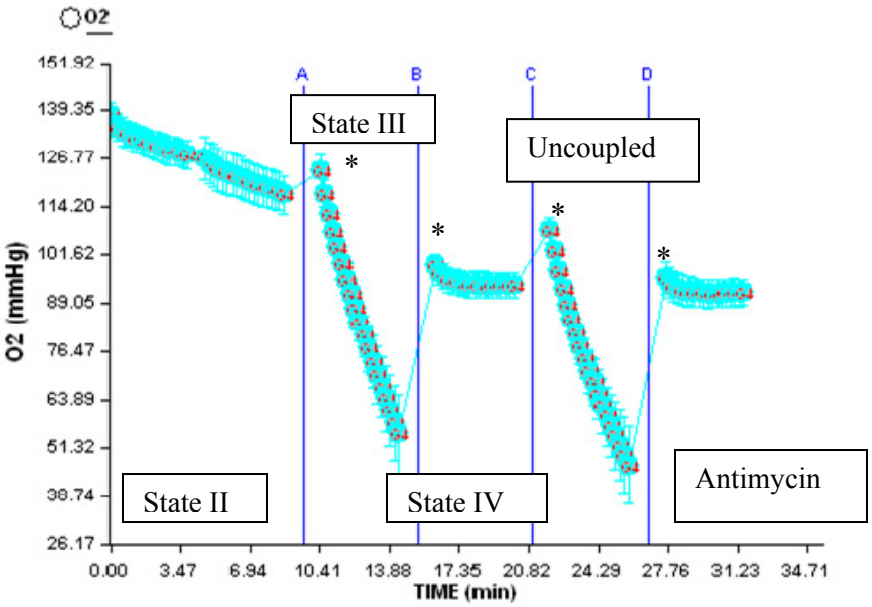
3. Representative OCR tracings.

For state III and DNP (uncoupled respiration), the higher point to point value of **OCR** (**o**xygen **c**onsumption **r**ates, pmols oxygen consumed per minute, calculated using the AKOS algorithm (which is a component of the XF Reader Software) for each sample was selected. For state IVo and antimycin A, the

lower point to point OCR values were selected. The values selected in this particular trace are highlighted with arrows. In this representative tracing, we show isolated mitochondria from heart (20 µg protein/well, n=4 replicates ± SEM) using 5 mM pyruvate and 5 mM malate. State III/State IV, the RCR > 4 (10.09), showing that it is a good quality mitochondrial preparation, together with the inhibition with antimycin A. Increase of respiration with the uncoupler (port C) must be close to state III or higher (also known as state IIIu).



(see absolute O_2 mmHg values in the next page)



*The mixing time after injection determines the optimal replenishment of O_2 after injection (it should be close to ambient O_2, i.e. 90 mmHg minimum). In addition, O_2 should never reach 0 mmHg