Analyzing Microgram Quantities of Isolated Mitochondria in the Agilent Seahorse XFe/XF24 Analyzer

Application Note

Introduction

Enhanced appreciation of the role of altered mitochondrial function in tumorigenesis, aging, neurodegenerative, metabolic, and cardiovascular diseases has stimulated the development of a variety of new approaches for assessing mitochondrial function and dysfunction. As the field moves towards the discovery of mitochondrial-related, molecular mechanisms that underlie disease, and drugs to prevent or reverse disease development, the demand for higher throughput and more sensitive assay methods increases. The importance of drug candidate screens for mitochondrial toxicity is also recognized.

Measurement of the oxygen consumption rate (OCR) of mitochondria is valuable, since electron transport and oxidative phosphorylation consume oxygen, and reflect the mitochondrial and nuclear genomes' expression of functional components of the electron transport chain.

Agilent Technologies

A high-throughput assay in which both energy demand and substrate availability can be tightly controlled for mechanistic studies has been developed for the Agilent Seahorse XFe/XF24 Analyzer using isolated mitochondria[1]. Figure 1 illustrates the general work flow for this experiment. The optimized conditions enable sensitive measurement of the OCR and proton extrusion or extracellular acidification (ECAR), by isolated mitochondria attached to the bottom of an Agilent Seahorse XF Cell Culture Microplate. This approach is uniquely capable of analyzing small quantities of material (1–10 µg of mitochondrial protein per well). Sequential measurement of Basal, State 3, State 4, and uncoupler-stimulated respiration or any other combination, can be made through additions of reagents from the drug injection ports of the XF assay cartridge. This technique can be particularly useful for mechanistic studies, drug screening, and for enabling respiratory data to be obtained on small mitochondrial samples.

Reagents

Mitochondrial isolation buffer (MSHE+BSA)

The following reagents were used: 70 mM sucrose, 210 mM mannitol, 5.0 mM HEPES, 1.0 mM EGTA, and 0.5 % (w/v) fatty acid free BSA, pH 7.2.

Mitochondrial assay solution (MAS, 1X)

The following solutions were used: 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1.0 mM EGTA and 0.2 % (w/v) fatty acid-free BSA, pH 7.2 at 37 °C. 3x stock MAS is needed for dilution of substrates, ADP, and respiration reagents.

ADP and substrate stocks

The following substrate stocks were used: 0.5 M succinic acid, 0.5 M malic acid, 0.5 M glutamic acid, 0.5 M pyruvic acid, and 1 M ADP, all dissolved in H_2O and adjusted to pH 7.2 with potassium hydroxide. Pyruvate should be made fresh the day of the assay.

Respiration reagent stocks

The following reagent stocks were used: 10 mM FCCP, 2 mM rotenone, 5 mg/mL oligomycin, and 40 mM antimycin A in 95 % ethanol. Do not use 100 % ethanol as it contains traces of benzene, which is detrimental to mitochondrial function. Combine 1.0 M ascorbate in H_2O, pH 7.2. 10 mM N1,N1,N1,N1-tetramethyl-1,4-phenylene diamine (TMPD) in H_2O, pH 7.2, with an equimolar concentration (10 mM) ascorbate to ensure TMPD remains reduced.

All reagents were stored at –20 °C, except pyruvate, which was prepared fresh on the day of each experiment.

Fatty-acid-free BSA was purchased from EMD Biosciences. Purified H_2O was purchased from Thermo Scientific and used for respiratory media and reagents. All other chemicals were purchased from Sigma-Aldrich.

Isolation of Mouse Liver Mitochondria

Mice were euthanized using isofluorane in accordance with IACUC procedures. Mitochondria were isolated by a method similar to Schnaitman and Greenawalt[2]. The liver was extracted and minced in ~10 volumes of MSHE+BSA at 4 °C, and all subsequent steps of the preparation were performed on ice. The material was rinsed several times to remove blood. The tissue was then disrupted using a drill-driven Teflon dounce homogenizer with 2–3 strokes. Homogenate was centrifuged at 800 g for 10 minutes at 4 °C. Following centrifugation, fat/lipid was carefully aspirated, and the remaining supernatant was decanted through two layers of cheesecloth to a separate tube and centrifuged at 8,000 g for 10 minutes at 4 °C. After removal of the light layer, the pellet was resuspended and the centrifugation repeated. The final pellet was resuspended in a minimal volume of MSHE+BSA. Total protein (mg/mL) was determined using Bradford Assay reagent (Bio-Rad).

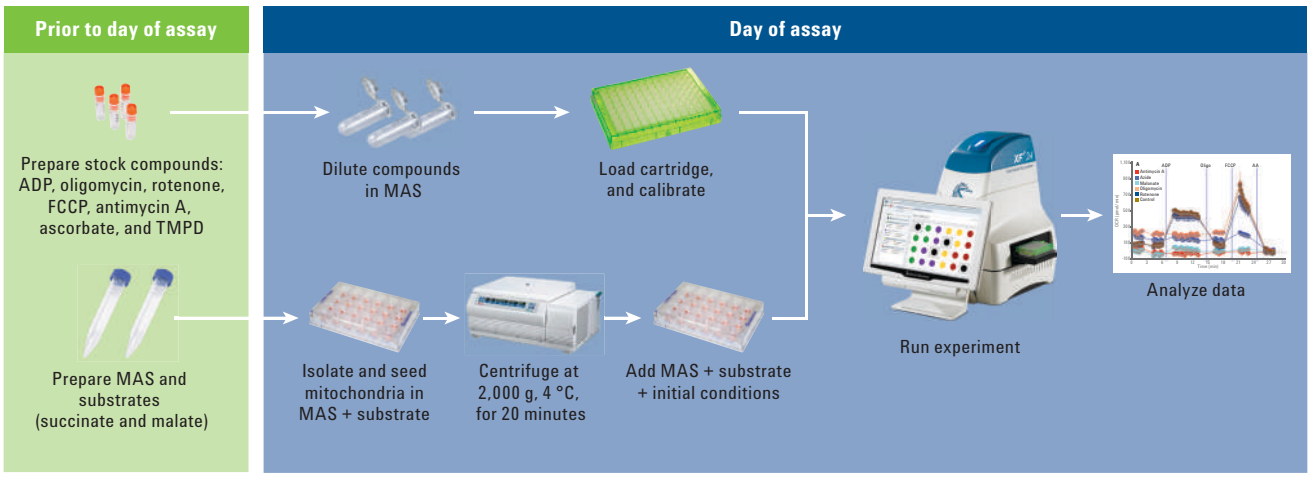

Figure 1. Flow chart of the XF assay.

XF Assay Preparation

To minimize variability between wells, mitochondria were first diluted 10x in cold 1x MAS + substrate, then subsequently diluted to the concentration required for plating. Note that substrate is included in the initial dilution, and is present during the centrifugation step. Next, while the plate was on ice, 50 µL of mitochondrial suspension was delivered to each well (except for background correction wells). The XF24 cell culture microplate was then transferred to a centrifuge equipped with a swinging bucket microplate adaptor, and spun at 2,000 g for 20 minutes at 4 °C.

After centrifugation, 450 µL of prewarmed (37 °C) 1x MAS + substrate + initial conditions (if applicable) was added to each well. The mitochondria were viewed briefly under a microscope at 20x to ensure consistent adherence to the well. The plate was then transferred to the Seahorse XFe/XF24 Analyzer, and the experiment initiated.

Coupling Assay

This experiment examines the degree of coupling between the electron transport chain (ETC), and the oxidative phosphorylation machinery (OXPHOS), and can distinguish between ETC and OXPHOS with respect to mitochondrial function/dysfunction. Mitochondria (5 µg/well mouse liver mitochondria or 2–3 µg rat heart mitochondria work well in the Seahorse XFe/XF24, respectively for succinate) begin in a coupled state with substrate present, in this case, succinate (10 mM), and rotenone (2 µm) (State 2). State 3 initiated with ADP, State 4 induced with the addition of oligomycin (State 4o), and FCCP-induced maximal uncoupler-stimulated respiration (State 3u) were sequentially measured, allowing respiratory control ratios (RCR: State 3/State 4o, or State 3u/State 4o) to be assessed as previously described[4,5]. Injections were as follows: port A, 50 µL of 40 mM ADP (4 mM final); port B, 55 µL of 25 µg/mL oligomycin (2.5 µg/mL final); port C, 60 µL of 40 µm FCCP (4 µm final); and port D, 65 µL of 40 µm antimycin A (4 µM final). (The final volume of the assay will be 730 µL, which translates to ~ 10x dilutions for ports A, B, C, and D, respectively, Figure 2A.)

Electron Flow Assay

This experiment examines sequential electron flow through different complexes of the electron transport chain, which can identify the mechanism of mitochondrial dysfunction or modulation (Figure 2B).

With the initial presence of 5 µg mitochondria per well, 10 mM pyruvate, 2 mM malate and 4 µm FCCP, injections were made as follows: port A, 50 µL of 20 µM rotenone (2 µm final); port B, 55 µL of 100 mM succinate (10 mM final); port C, 60 µL of 40 µm antimycin A (4 µm final); port D, 65 µL of 100 mM ascorbate plus 1 mM TMPD (10 mM and 100 µm final, respectively). Typical mix and measurement cycle times for the assays are illustrated in Table 1, and are common to all experiments presented unless otherwise noted.

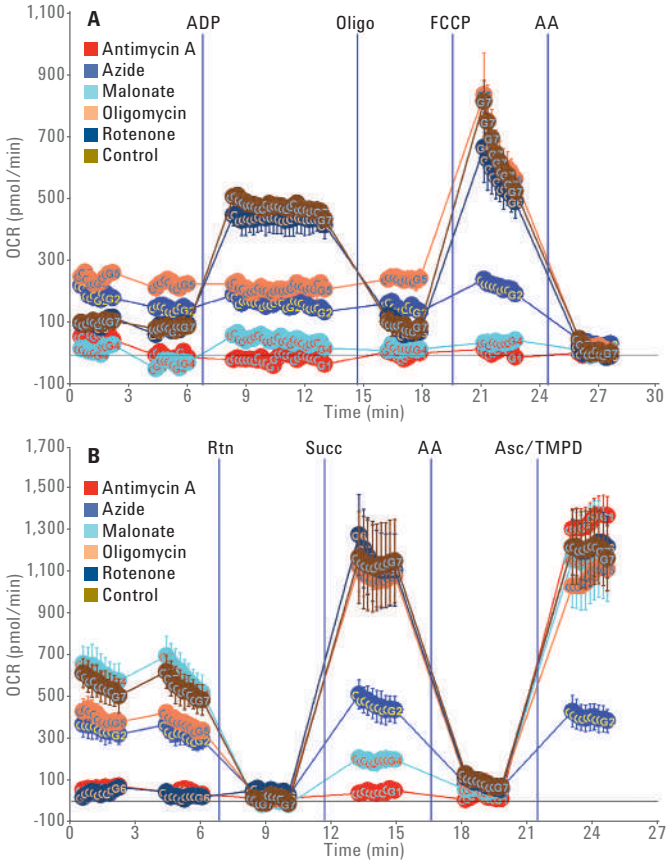

Figure 2. Determining mechanism of altered mitochondrial function using the coupling and electron flow assays in tandem to elucidate mechanistic activity of agents that perturb mitochondrial function. Coupling (A) and electron flow experiments (B) were performed as described in Methods. Initial conditions are as follows: Controls (no additives), 4 µm antimycin-A, or 20 mM sodium azide, or 10 mM malonate, or 2.5 µg/mL oligomycin, or 2 µm rotenone.

Data Analysis

All data were analyzed using the XF software, or XFe Wave, and displayed as either point-to-point oxygen consumption rates (pmol/min/well), or absolute oxygen tension in mm Hg O_2 versus time. Data is presented as the average of 3–5 replicate wells ±SEM.

Interpretation of Results

By using the two assays described, coupling and electron flow, it becomes possible to pinpoint the mechanism of unknown compounds, or genetic manipulation, on mitochondrial function (Figure 2). To illustrate the method, five well-described compounds that affect mitochondrial function were supplemented as initial conditions.

Inclusion of rotenone in the initial conditions showed no effect on the OCR values in the coupling experiment, as rotenone is already present and respiration is being driven by Complex II-IV activity. However, it was observed that in the electron flow experiment that pyruvate/malate-dependent respiration is inhibited at the beginning of the assay in contrast to the control, in which robust respiration is present. The fact that the control and the rotenone group show identical responses upon subsequent injections illustrates that the remainder of the electron transport chain is functioning properly.

Malonate is a competitive inhibitor of succinate dehydrogenase, a component of complex II, and antimycin inhibits complex III. For malonate, all the respiratory rates are inhibited as anticipated except for Complex I driven respiration at the start of the electron flow experiment, and the ascorbate/TMPD-driven respiration mediated by complex IV. As with malonate, the effects of antimycin A on the coupling portion show complete inhibition and no response to ADP, oligomycin, or FCCP. However, unlike malonate, antimycin A prevents both Complex I and II mediated respiration due to inhibition of Complex III, resulting in loss of activity throughout the electron flow portion of the assay until the addition of ascorbate and TMPD, indicating that complex IV remains active.

Azide inhibition of Complex IV is demonstrated in both the coupling and electron flow experiments, with reduced respiration throughout the assay. Most instructive is the fact that addition of ascorbate and TMPD could not increase electron flow (and thus O_2 consumption) at Complex IV. Finally, oligomycin, an inhibitor of the ATP synthase (Complex V) prevents only ATP synthesis (the ADP-stimulated rate) in the coupling experiment, but does not affect electron flow among the complexes.

Assay Optimization with other Mitochondria Sources

When adapting this method to mitochondria isolated from other species/tissues, it is critical to ensure that an optimal amount of mitochondria are used per well. First, titrate the quantity of mitochondria to be used per well to ensure OCR values are within the linear range of response of the mitochondria, and within the dynamic range of the instrument (Figure 3A). State 2 rates should be kept between 100–200 pmol/min/well. Typically, 1–10 µg of isolated mitochondria is a good range for optimizing of the assay, but more may be required depending on mitochondrial activity/quality and purity.

If OCR results show high State 2 rates or poor response to ADP or other compounds (40 µg, Figure 3A), it is likely that too much mitochondria is being used per well. In all cases, it is imperative to review the absolute O_2 tension data. The corresponding O_2 tension data in Figure 3B illustrates the result of well overloading, and shows complete depletion with mouse liver mitochondria samples of ≥10 µg/well (O_2 tension approaches or reaches zero). Sensors do not have adequate time to return to ambient O_2 tension before the next measurement cycle, as evidenced by the steady decline in O_2 tension for each successive measurement cycle. These two factors prevent accurate measurement of OCR, with State 3 being underestimated and apparent poor response of the mitochondria to oligomycin and FCCP.

Use saturating concentrations of ADP to ensure the maximal State 3 rate is obtained for the duration of the measurement cycle (that is, the State 3 rate does not decline as judged by point-to-point data). Typically, 2–4 mM ADP is sufficient for saturation (Figure 4). Note that relatively high concentrations of ADP (1.0–4.0 mM) are needed to sustain stable State 3 respiration due to the very small microchamber formed during the measurement period. Using lower concentrations (0.125–1.0 mM) will result in depletion of ADP during this time period. By obtaining stable OCR values for State 3, followed by determination of State 4o respiration, a respiratory control ratio (RCR value) may be calculated as 3/4o (in this example, (1,075 pmol/min)/(233 pmol/min) or ~4.6).

Assay Optimization Hints

- While this Application Note uses actions of known compounds to demonstrate the utility of the coupling and electron flow assays, the same strategy may be applied to compounds of unknown function or tissue subject to genetic manipulations.
- This method does not employ the use of plate coating. Adapting these methods to use mitochondria from different tissues/species, relative purity level (for example, percoll gradient isolation), or assay solutions containing KCl may require the use of polyethyleneimine (Gerenscer; *et al*. 2009)[3] or other coating to ensure adequate adhesion to the well plate.
- Centrifugation in larger volumes of mitochondrial suspension results in lower maximal respiratory rates, likely due to loss of mitochondria to the sides of the wells. Seeding 50 µL/well is recommended. It is also recommended that a coupling experiment be performed in which all wells are treated identically to ensure consistency of mitochondrial adherence across the plate.

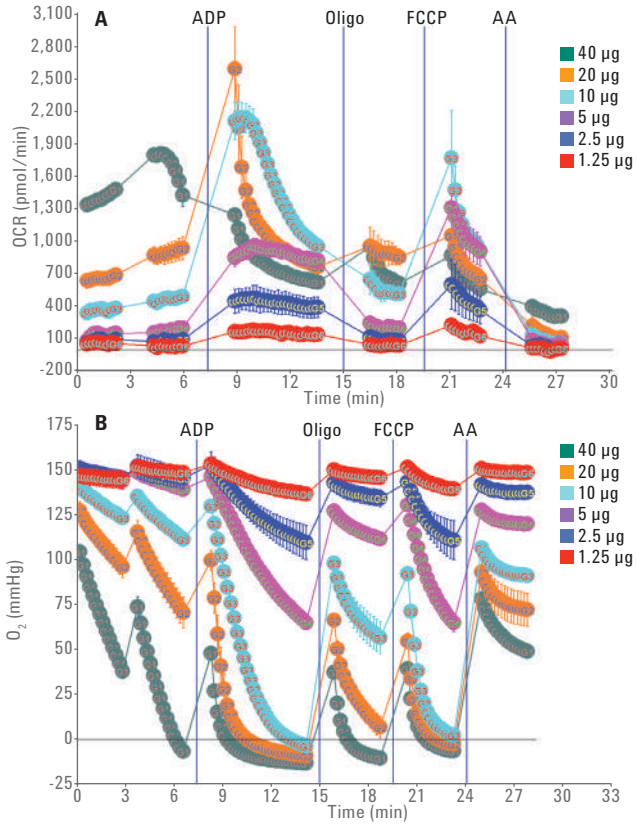

Figure 3. Determining optimal quantity of mitochondria. Using 1.25–40 µg of mouse liver mitochondria attached to an XF24 Microplate, the coupling experiment was performed in the presence of succinate/rotenone, as described in Methods. Blue vertical lines denote injections of indicated compounds. A) OCR for 1.25–40 µg samples. B) the absolute O_2 tension (in mm Hg) in the microchamber for 1.25–40 µg samples. Note that samples at 10 µg and above show unstable State 3 rates for OCR and depletion of O_2 in the microchamber.

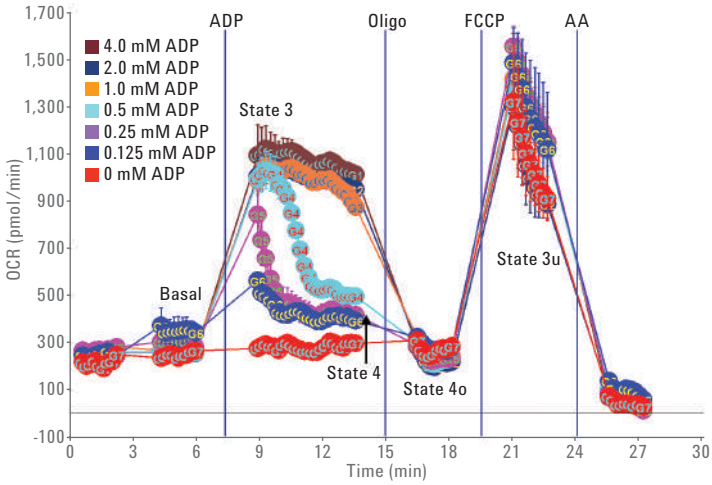

Figure 4. Determining optimal ADP concentration. Titration of ADP using 5 µg mouse liver mitochondria/well. ADP (0-4 mM) was injected via port A to initiate State 3 respiration and the measurement time was extended to 6 minutes. Note that 2-4 ADP mM is sufficient to maintain a relatively stable rate of State 3 respiration rate for the duration of the measurement period, while lower concentrations show exhaustion of ADP and transition to State 4 respiration.

- In this Application Note, *substrate* refers to the carbon source provided for oxidation by mitochondria, including (but not limited to): succinate* (10 mM), glutamate/malate (5–10 mM each), pyruvate/malate (10/2 mM each), or palmitoyl carnitine/malate (40–80 µm and 0.1–0.5 mM, respectively). It is imperative that substrate be included in the dilution and subsequent adherence of mitochondria to the XF microplate. This is to prevent or reduce any damage or loss of respiratory activity due to a potential mitochondrial permeability transition event. *Initial conditions* refer to any additional compounds that may affect mitochondrial function, and are included at 1.1x final concentration in the 450 µL of MAS that is added to each well after the adherence step, but before the microplate is inserted into the Seahorse XFe/XF24 instrument.

  * It is customary practice to include rotenone with succinate to prevent any Complex I driven respiration.

- Extending the measurement time for State 3 or lowering the concentration of ADP used, will show exhaustion of ADP, as illustrated by the point-to-point rate data in Figure 4 (0.5–1.0 mM ADP and a measurement time of 6 minutes) The method provides the ability to record State 3 respiration followed by exhaustion of ADP to State 4.

- The assays presented have been designed for the shortest duration possible, but note that more than one measurement cycle may be used after each injection to ensure kinetic equilibrium.

- Certain types or preparations of mitochondria can exhibit a run-down effect in respiration across the time frame of the coupling assay. Experiments have demonstrated that this effect can often be reduced/eliminated by initiating the coupling assay in the presence of ADP (State 3 respiration) due to the protective effects of adenine nucleotides on mitochondrial function. (that is, include ADP in the 450 µL of assay buffer added to each well after the mitochondria are adhered to the plate).

References

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4. Chance, B.; Williams, G. R. The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.* **1956**, *17*, 65-134.

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Table 1. Instrument run protocol.

Start protocol		
Command	**Time (min)**	**Port**
Calibrate		
Wait	10	
Mix	1	
Wait	3	
Mix	1	
Wait	3	
Mix	1	
Measure	3	
Mix	1	
Measure	3	
Mix	1	
Inject		A
Mix	1	
Measure	3-6	
Mix	1	
Inject		B
Mix	1	
Measure	3	
Mix	1	
Inject		C
Mix	1	
Measure	3	
Mix	1	
Inject		D
Mix	1	
Measure	3	
End protocol		

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