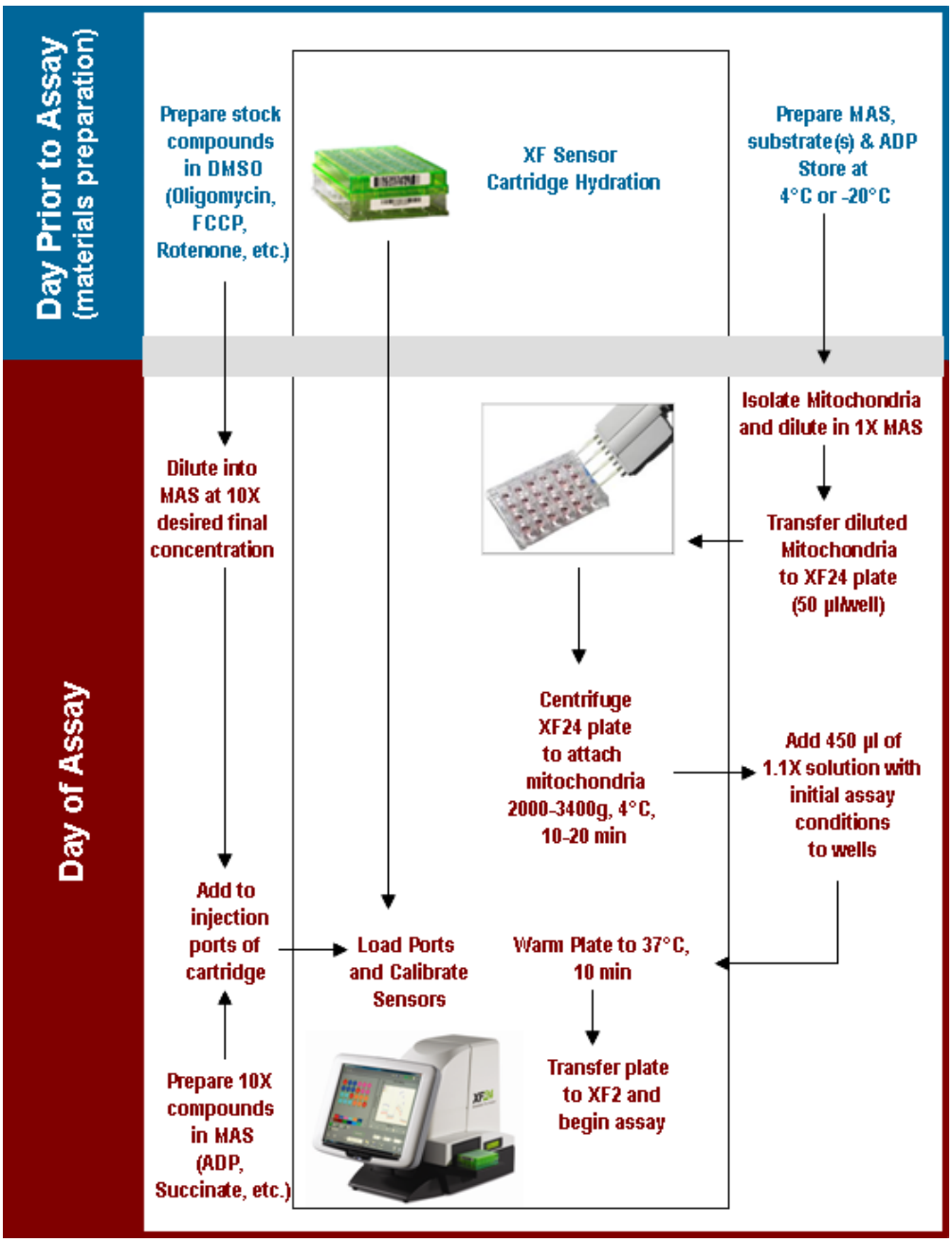
Isolated Mitochondria Assay using the XF24 Analyzer (revision 091104)

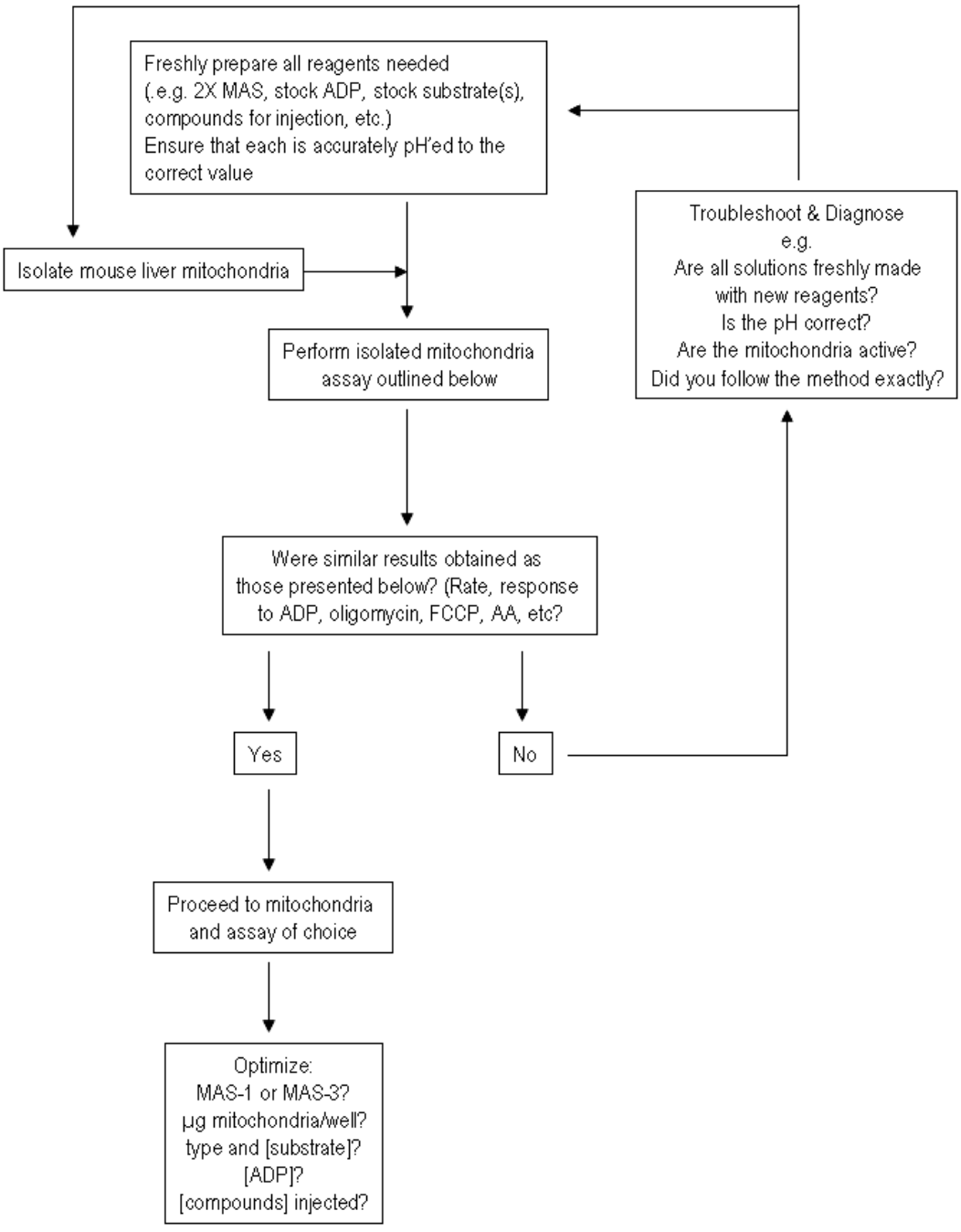
The XF Analyzer instruments were originally designed for use with intact cells attached to a tissue culture plate. Since many researchers routinely utilize isolated mitochondria to answer fundamental questions about mitochondrial function, bioenergetics and metabolism, Seahorse Bioscience (in conjunction with collaborators) has developed a protocol that expands the application of the XF24 analyzer to include isolated mitochondria. The advantages over traditional methods (e.g. Clarke Electrode Apparatus) include higher throughput (20 samples per assay) and smaller amounts of isolated mitochondria required (as low as 5 µg total mitochondrial protein per well).

Isolated Mitochondria Assay Flow Chart



Suggested Workflow for optimizing conditions using isolated mitochondria in the XF24

The methods presented on the following pages have been optimized for mitochondria isolated from mouse liver. Even if you are an experienced user of isolated mitochondria, Seahorse Bioscience strongly suggests taking a bit of time to reproduce the results shown in this protocol. This will ensure that all reagents prepared and used are working properly and provides the user with practice of the methodology on the XF24. Once the user has become familiar with this process and has validated the quality of all reagents used, then these methods can be translated to perform the desired experiments with the isolated mitochondria of choice. *Keep in mind that the protocol n the following pages provides a starting point only, and that users will be required to optimize the conditions for their specific application and experimental design.*



I. Reagents, Materials and Preparation of MAS and Injected Compounds

Two different Mitochondrial Assay Solutions (MAS) are provided below. Please use the buffer that will be optimal for your mitochondrial system of choice and your intended research goals.

MAS-1: Sucrose/Mannitol based MAS: Seahorse Bioscience has used this buffer with success for mitochondria purified from mouse liver and rat liver. Due to its high content of sucrose/mannitol, this buffer tends to be more protective of isolated mitochondria.

MAS-3: KCl based MAS: Seahorse Bioscience has used this buffer with success for mitochondria purified from mouse liver and rat liver. This buffer is more representative of the physiologic conditions *in vivo*.

Experiments performed by Seahorse Bioscience have shown that comparable results have been obtained using either MAS with mitochondria isolated from mouse liver.

Note that there are a number of Mitochondrial Assay Solutions reported in the literature that can vary in both the concentration and identity of components (e.g. some solutions include EDTA). Seahorse Bioscience recommends freshly preparing, testing and optimizing your solution of choice if different than those listed below.

I.1. Components/Formulation of Mitochondrial Assay Solution-1 (MAS-1)

Compound	Brand	Catalog Number	MW or Molar Concentration	Final Concentration (1X)	Grams or ml for 500 ml of 1X MAS	Grams or ml for 250 ml of 2X MAS
Sucrose	Sigma	S9378	342.30	70 mM	11.98 g	11.98 g
Mannitol	Sigma	M9647	182.2	220 mM	20.04 g	20.04 g
KH_2PO_4	Sigma	P9791	136.09	5 mM	0.34 g	0.34 g
$MgCl_2$	Sigma	M1028	1.0 M	5 mM	2.5 ml	2.5 ml
HEPES	Sigma	H0887	1.0 M	2 mM	1.0 ml	1.0 ml
EGTA*	Sigma	E4378	100 mM	1 mM	5.0 ml	5.0 ml
FA-free BSA	Sigma	A7511	66,430	0.2%	1.0 g	1.0 g
* Please make a 100 mM stock solution of EGTA and ensure the pH is ~ 7.2				MAS-1 may be aliquoted and stored at -20°C. Smaller volumes may be made by scaling down reagents.		

Dissolve components listed in table above into ~350 ml dH_2O for a 1X solution or ~150 ml dH_2O for a 2X solution. Warm solution to 37°C. Adjust solution to pH 7.2 using KOH. Add dH_2O to the appropriate final volume (500 ml or 250 ml for 1X and 2X solutions, respectively.)

I.2. Components/Formulation of Mitochondrial Assay Solution-3 (MAS-3)

Compound	Brand	Catalog Number	MW or Molar Concentration	Final Concentration	Grams or ml for 500 ml of 1X KHE	Grams or ml for 250 ml of 2X MAS-3
KCl	Sigma	P9333	74.56	115 mM	4.29 g	4.29 g
KH_2PO_4	Sigma	P9791	136.09	10 mM	0.68 g	0.68 g
$MgCl_2$	Sigma	M1028	203.31	2 mM	0.204	0.204
HEPES	Sigma	H0887	1.0 M	3 mM	1.5 ml	1.5 ml
EGTA*	Sigma	E4378	100 mM	1 mM	5.0 ml	5.0 ml
FA-free BSA	Sigma	A7511	66,430	0.2%	1.0 g	1.0 g
* Please make a 100 mM stock solution of EGTA and ensure the pH is ~ 7.2				MAS-3 may be aliquoted and stored at -20°C. Smaller volumes may be made by scaling down reagents.		

Dissolve components listed in table above into ~400 ml dH_2O for a 1X solution or ~200 ml dH_2O for a 2X solution. Warm solution to 37°C. Adjust solution to pH 7.2 using KOH.

Add dH_2O to the appropriate final volume (500 ml or 250 ml for 1X and 2X solutions, respectively.)

I.3. Components/Formulation of compounds to affect mitochondrial function

It is recommended that all compounds to be added or injected are diluted with the appropriate MAS. It is convenient and suggested to prepare a 2X MAS to use for dilution of compounds and substrates.

Compound	Brand	Catalog Number	Final Concentration	Dissolve in:
Glutamate	Sigma	G8415	5 mM	50 mM stock, MAS
Malate	Sigma	M6413	5 mM	50 mM stock, MAS
Succinate	Sigma	S2378	5 mM	50 mM stock, MAS
ADP	Sigma	A2754	0.25 – 2.0 mM	2.5 - 20 mM stock, MAS
Rotenone	Sigma	R8875	2 µM	Stock 1000X (1 mM) in DMSO. Dilute to 10X in MAS
Oligomycin	Sigma	O4876	2 µM	Stock 10000X (20 mM) in DMSO. Dilute to 10X in MAS
FCCP	Sigma	C2920	0.4 to 4 µM	Stock 10000X (4-40 mM) in DMSO Dilute to 10X in MAS
Antimycin A	Sigma	A8674	4 µM/1.5 µg/ml	Stock 1000X (40 mM/1.5 mg/ml) in DMSO. Dilute to 10X in MAS
Note: ADP can be made as a 100 mM stock in dH_20, pH to 7.0. Succinate, malate and glutamate may be prepared from either their respective free acids or sodium salt derivatives. All may be prepared as 500 mM stocks in dH_20, pH to 7.0. Be aware that sodium ions can influence isolated mitochondria function depending on the species and organ used for preparation. ADP and substrates may be stored at -20°C. Oligomycin, FCCP and Antimycin A should be freshly diluted in MAS for each experiment. Stock solutions in DMSO or ethanol (95%) may be stored at -20°C.				

I.4. Other items needed

XF24 Biosensor Cartridge
XF24 Tissue Culture Plate
Calibration buffer (Seahorse Bioscience)
Multi-channel pipettes and tips, centrifuge with plate adaptors, Eppendorf and Falcon tubes

II. Preparation of XF Assay Templates, XF Cartridges and Mitochondria

II.1. Prepare an XF assay template (via the Assay Wizard) using the XF24 operation manual as a guide and incorporating proper experimental design. Upload the assay template to the XF24 Analyzer before starting the assay. The experiment outlined below is an example of how to obtain the various mitochondrial respiration states using the XF24.

Use the following table as a guide to program the Mix, Wait, Measure and Injection protocol.

Command	Time	Port
Calibration	-	-
Equilibrate	12 min*	
Mix	25 sec	
Measure	4 min	
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject	-	A
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		B
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		C
Mix	25 sec	
Measure	4 min	
Mix†	30 - 60 sec	
Inject		D
Mix	25 sec	
Measure	4 min	

* Default Equilibrate command consists of 2 min Mix, 2 min Wait repeated 3X.

† 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.

Note that the Measure times indicated are guidelines only. Typical Measurement times are 3-5 minutes, and can be longer to allow for ADP exhaustion be depleted from the transient micro-chamber. It is advised to empirically determine the optimal Mix and Measure times for your desired application and experiment.

II.2 Prepare the XF sensor cartridge

II.2.1. Hydrate the XF sensor overnight in XF Calibration Buffer at 37°C, no CO_2.

II.2.2. Before calibration, load the XF sensor cartridge injection ports with following compounds listed in the table below.

Injection Ports	Volume	Concentration in Port	Final Concentration in Well
A: ADP	50 µl	2.5 – 20 mM	0.25 – 2 mM
B: Oligomycin	55 µl	20 µM	2 µM
C: FCCP	60 µl	40 µM	4 µM
D: Antimycin A	65 µl	40 µM	4 µM
Note: Vigorous mixing of the stock 20 uM oligomycin is required to prevent precipitation			
Note: Other substrates (e.g. glutamate and/or malate) may be substituted for succinate. Please consider the goals of your experiment and the information to be derived from the assay to choose appropriately.			

II.2.3 Prepare isolated mitochondria by the standard protocol(s) used in your laboratory. Typical isolated mitochondrial suspensions yield ~ 20-100 mg/ml mitochondrial protein as measured by a Bradford or BCA Assay.

III. Mitochondria attachment to the bottom of the plate well

III.1 Calibrate the sensor cartridge (loaded with desired compounds) as described in the XF manual.

III.2. Once the calibration has been started, dilute an appropriate volume of isolated mitochondria in MAS to yield a final concentration of 200 µg of mitochondrial protein/ml (= 10 µg/50 µl). For example, if the isolated mitochondria are 60 mg/ml, then dilute 5 µl of mitochondria in 1.5 ml of MAS (200 µg/ml final). Note that due to large dilution factors and the mitochondria being a suspension, it is recommended that the mitochondria be first mixed by gentle pipetting, then diluted into a small volume (~100 µl) of 1X MAS. This is then added to the larger volume (1.4 ml) and pipetted gently several times to mix thoroughly.

III.3. Transfer 50 µl (10 µg total*) of the diluted mitochondria into each well of a V7 XF24 Tissue Culture Plate. For background correction wells (A1, B4, C3 and D6) use 50 µl of MAS *(no mitochondria!).*

** Note: The final amount of total protein/well will depend from which tissue the mitochondria were obtained, e.g. mitochondrial rich tissues will contain more mitochondria per µg total protein than less mitochondrial rich tissues. Further, the amount of mitochondria per µg total protein will also be dependent on the overall purity of the mitochondrial preparation.* ***It is strongly suggested to perform a titration experiment with the mitochondria to determine the optimal amount to use.***

III.4. Spin down mitochondria at 4°C for 10-20 minutes at 2000-3600g. *The time and speed may need to be tested for optimal mitochondrial activity*. When finished, visualize the mitochondria on the well surface using a 20X or 40X lens. Note any wells that do not appear to have a consistent "monolayer" of mitochondria adhered to the well bottom. Add 450 µl of 1.1X intitial media conditions (if applicable) to each well (see IV below). Place the plate at 37°C (no CO_2) for 8-10 minutes to warm (do not allow warming to go longer than 10 minutes).

IV. Preparing initial buffer conditions for the assay

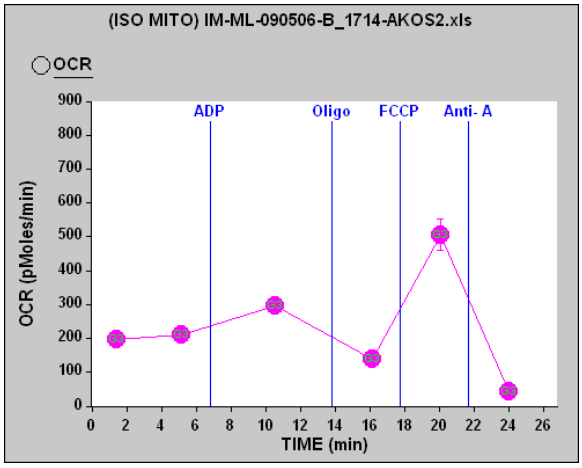
IV.1 During the time of centrifugation, prepare a 1.1X solution of succinate (5.5 mM) and Rotenone (2.2 µM) in 1X MAS.

IV.2. BEFORE the 10 minute warm up period, add 450 µl of 5.5 mM succinate and 2.2 µM rotenone in 1X MAS to each well (final volume is 500 µl, final concentrations of succinate and rotenone are 5 mM and 2 µM, respectively).

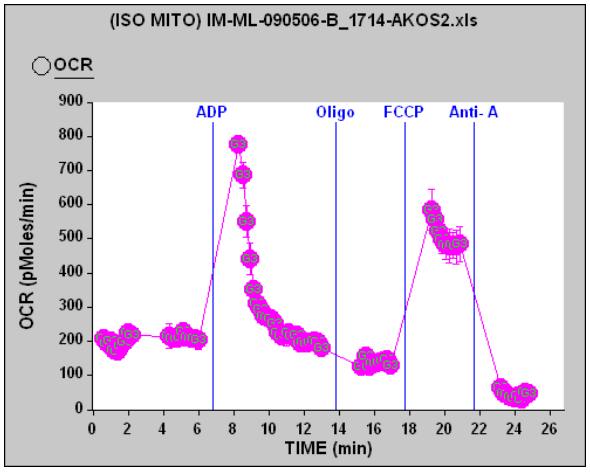
IV.3 After calibration is finished, follow the directions on the instrument controller and when prompted, exchange the calibration plate for the plate containing the mitochondria and continue to follow the directions on the instrument controller.

V. Examples of results and data analysis

The data shown below are typical results obtained using the methods outlined above.



OCR vs. time (2 wells), middle point mode

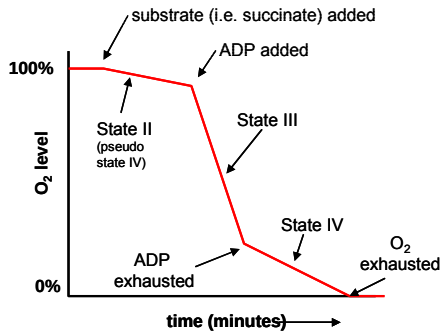


OCR vs. time (2 wells), point to point mode

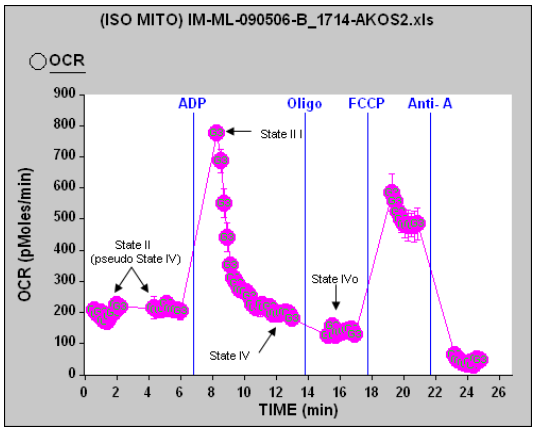
Conditions: MAS-1, 10 µg mouse liver mitochondria, intial assay conditions: 5 mM succinate, 2 uM rotenone, [ADP] = 0.25 mM, [Oligomycin] = 2 uM, [FCCP] = 4 uM, [Antimycin A] (AA) = 2 uM.

Note that the middle point mode (left) under-estimates the State III rate (Succ. + ADP) since the respiration rate changes rapidly during the course of this measurement period, which is evident in the point to point mode (right). The middle point mode accurately represents the other rates as these are relatively stable during the course of the measurement period.

The figure below shows examples of theoretical respiration states (if using a Clarke Electrode type apparatus, left) and actual respiration states obtained with the XF24 analyzer (right). The left panel shows [O_2] vs. time, while the right panel shows OCR (pmol/min) vs. time (point to point mode). Note that near the end of measurement 3 (after the injection of ADP), ADP exhaustion is apparent in the panel on the right as evidenced by the decrease in OCR during the course of the measurement.

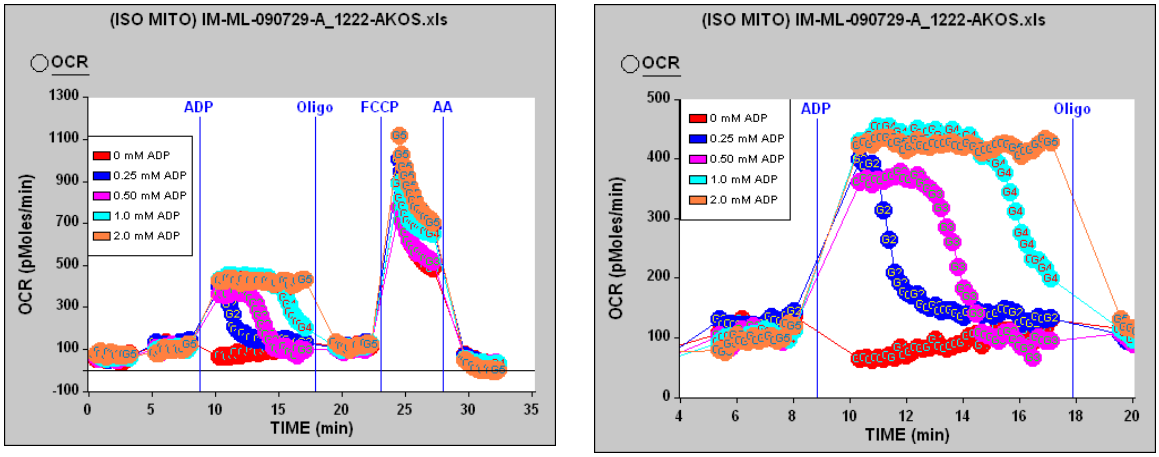


Theoretical respiration states
(Clarke Electrode apparatus)



Respiration states obtained with the XF24

VI. Example of ADP titration using mitochondria isolated from mouse liver



Example of ADP titration
Entire Assay Period (left) and zoom of period immediately after ADP injection (right).
(Error bars have been omitted for clarity)

Conditions: MAS-1, 10 µg mouse liver mitochondria, intial assay conditions: 5 mM succinate, 2 µM rotenone, [ADP] = 0 – 2 mM, Oligomycin = 2 µM, FCCP = 4 µM, Antimycin A (AA) = 2 µM.

Note that the concentration of ADP correlates directly with the length of time the mitochondria respire at State III.

VII. Obtaining State III and State IV respiration values and RCR values.

State III respiration is defined as ADP-stimulated respiration in the presence of saturating substrate. Isolated mitochondria are put into State III when ADP is added to intact mitochondria in the presence of excess substrate (typically glutamate + malate or succinate). This is the value of the slope of [O_2] vs. time, and is transformed by the XF software algorithm to OCR in units of pmol/min.

State IV respiration is defined as oxygen consumption by isolated mitochondria on a particular substrate, in the absence of ADP or any metabolic inhibitors. State IV respiration may be measured when the added ADP is exhausted (converted to ATP). Again, this is the value of the slope [O_2] vs. time, and is transformed by the XF software algorithm to OCR in units of pmol/min.

State IV may also be obtained by adding oligomycin to the mitochondria (State IVo), which inhibits Complex V and thus prevents the conversion from ADP to ATP. This method is commonly used by researchers to determine State IV. This is because an accurate State IV is difficult to achieve since there may be contaminating ATPases in the crude mitochondrial preparation which convert newly formed ATP back to ADP, and thus prevent a true "ADP exhausted" state. As with State III and IV, this is the value of the slope of [O_2] vs. time, and is transformed by the XF software algorithm to OCR in units of pmol/min.

Often, researchers also make a distinction between respiration rates with respect to the O_2 consumption rate before any ADP is added (substrate only) and after the added ADP is exhausted (converted to ATP). The former is referred to as *State II respiration or Resting Respiration*, and sometimes used as a "pseudo" State IV measurement.

The ratio of the State III rate to the State IV rate is called the *Respiratory Control Rate* (RCR) and indicates the tightness of the coupling between respiration and phosphorylation. With isolated mitochondria the coupling is not perfect, probably as a result of mechanical damage during the isolation procedure.

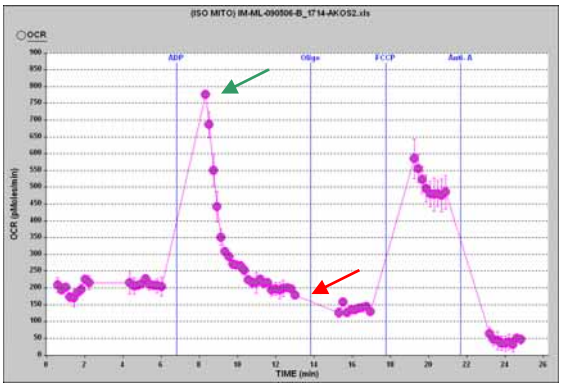
Note there are several methods to calculating an RCR value:

1) State III/State IV (using ADP exhaustion to determine the State IV rate)
2) State III/State II (State II is used as a "pseudo State IV" measurement)
3) State III/State IVo (State IV is induced by adding oligomycin to inhibit Complex V)

Please choose the method that is most suitable for your experiments and research goals. Examples of the 3 methods to calculate RCR values using XF data are illustrated below.

Method 1: State III/State IV (using ADP exhaustion to determine the State IV rate)

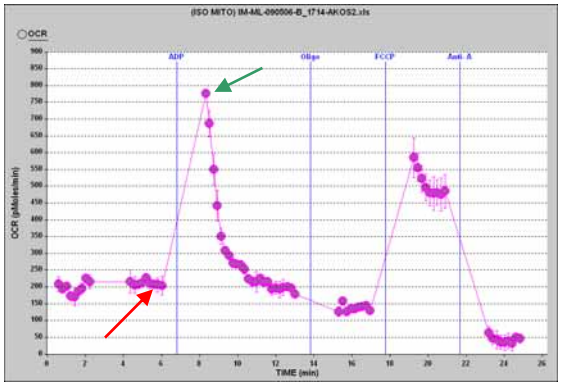
One can quickly estimate the RCR value from the graph by looking at the maximal and minimal rates obtained immediately after adding Succinate + ADP (green and red arrows, respectively). In this example, the State III rate is ~ 775 and the State IV rate is ~ 200, and thus the RCR is ~3.9.



Respiration states obtained with the XF

Method 2: State III/State II (State II is used as a "pseudo State IV" measurement)

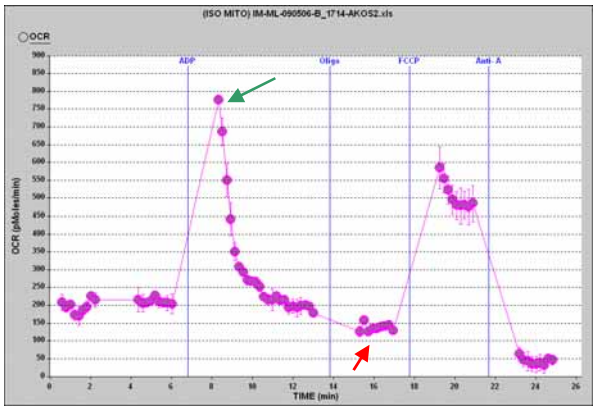
As above, one may estimate the RCR value from the graph by looking at the rate before succinate and ADP is added (State II, red arrow) and the maximal rate obtained immediately after adding Succinate + ADP (green arrow). In this example, the State III rate is ~ 775 and the State II rate is ~ 210, and thus the RCR is ~3.7.



Respiration states obtained with the XF

Method 3: State III/State IV (State IV is induced by adding oligomycin to inhibit Complex V)

Alternatively, one may estimate the RCR value from the graph by looking at the maximal rate obtained immediately after adding Succinate + ADP (green arrow), and the rate after oligomycin is added (State IVo, red arrow). In this example, the State III rate is ~ 775 and the State IVo rate is ~ 135, and thus the RCR is ~5.7.



Respiration states obtained with the XF

One may obtain exact rate values by viewing the group boxes in plate layout in the XF software if using the middle point mode (i.e. the rate is constant across the measurement period). Rate values from either the middle point or point to point graph modes may be obtained by right clicking on the graph in the XF software and choosing the "Save Graph Data…" function. This will generate a new tab in the excel worksheet which will display all the individual rate values (and associated error) of the data shown on the graph in a columnar format.

VI. Notes, Suggestions and Comments

The methods described above have been used successfully with isolated mouse liver mitochondria. Isolated mitochondria from other tissues and species can be used, however, the tissue, species (including age and sex), and method of isolation will contribute to the overall activity, degree of coupling and other variables associated with the isolated mitochondria.

Starting values, ranges, and optimization: it is recommended that the following parameters be explored and optimized depending on the overall goal(s) of the experiment and research topic.

- amount of mitochondria used (will be dependent on tissue type and purification method)
- the concentration of substrates and compounds injected
- Mix and Measure times
- MAS buffer type (1 or 3)

VII. References:

Please see Seahorse Bioscience's XF24 Training Course Workbook for a complete guide to operating and analyzing data using the Seahorse XF24 Flux Analyzer Instrument

For methods on isolating rat liver mitochondria and a introduction to State III and State IV respiration, please see: http://www.ruf.rice.edu/~bioslabs/studies/mitochondria/mitoprep.html

For a review of OXPHOS, Bioenergetics, and measuring respiration with isolated mitochondria, please see: http://www.bmb.leeds.ac.uk/illingworth/oxphos/

Please see the series of classical papers by B. Chance and G.R. Williams on the Respiratory Chain and Respiratory Enzymes in Oxidative Phosphorylation (1955-1956, various journals).

For an overall review of metabolism, including glycolysis, the TCA cycle, B-oxidation, the respiratory chain, oxidative phosphorylation, and much more, please see: "Metabolism at a Glance" 3rd Edition, by J.G. Salway, 2004, Blackwell Publishing, Ltd.