



**Measurement and Analysis of Mitochondrial Function *In Situ*
in Permeabilized Muscle Fibers**

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The Author(s) of the SOP	Dr. Sean Ng, Magda Lensinski
Working Group Members	Dr. Sean Ng, Magda Lensinski
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1 Objective

To utilize the Oroboros O2k high-resolution respirometry system for precise measurement and analysis of cellular oxygen consumption, mitochondrial function, and bioenergetic efficiency in biological samples, namely in dystrophic skeletal muscle. This system enables the assessment of mitochondrial respiration, oxidative phosphorylation, and metabolic flexibility, providing critical insights into cellular metabolism and bioenergetics in health and disease states.

2 Applicability/Scope

This SOP is intended for researchers who are interested in examining mitochondrial physiology in the context of metabolic disorders, neurodegenerative diseases, cancer metabolism, and aging. High-resolution oxygen consumption measurements can be conducted on various sample types, such as isolated mitochondria, permeabilized muscle fiber bundles, permeabilized cells, intact cells, and tissue homogenates. For this SOP, permeabilized muscle fiber bundles from dystrophic skeletal muscle will be reviewed.

3 Summary of the Procedure

The Oroboros O2k high-resolution respirometer is a modular oxygraph system used to measure cellular and mitochondrial respiratory activity of biological or biochemical samples. Combined with oxygen consumption, the O2k provides the basis to measure reactive oxygen species generation, mitochondrial membrane potential, ATP production, Ca^{2+} , and pH.

4 Definitions

- BIOPS - biopsy preservation solution
- MiR05 - mitochondrial respiration medium
- ddH₂O - double distilled water

5 Materials and Equipment Required

- Oroboros O2k high-resolution respirometer
- Gas-tight Hamilton syringes (10–500 μL)
- DatLab software
- Fine forceps (Dumont #5 or #55)
- Micro scissors
- Dissection microscope
- Ice bucket + chilled BIOPS
- Filter paper or weighing boats
- Analytical microbalance (0.01 mg resolution)

Consumable buffers, substrates, and reagents – see Attachments/Checklists section.

6 Step-by-Step Procedure

Pre-Tissue Collection

1. Thaw BIOPS and MiR05 buffers (stored at -20°C, see “Buffer and Substrate Preparation”) by placing Eppendorf tubes in cool beaker of water (~30 minutes). Keep buffers on ice once majority of buffer has thawed.
2. Label 1.5 mL Eppendorf tubes, “BIOPS” (3 tubes), “1.5 mL BIOPS+Saponin” (2 tubes), and “MiR05” (2 tubes).
3. Turn on PC
4. Turn on Oroboros O2k by flipping switch on rear of the instrument
5. Open DatLab program
6. Remove stoppers from the O2k chambers and place with top down on top of O2k
7. Turn on left stirrer (F11 on keyboard), right stirrer (F12), and lights (F13)
8. Aspirate ethanol from each chamber, then wash:
 - a. NOTE: To prevent damaging the O₂ sensors, keep aspirator pointed away from membrane inside chamber (i.e., point aspirator towards midline of instrument) and refill chambers immediately after aspiration.
 - b. Fill chamber with ddH₂O and insert stopper halfway into chamber. Incubate for 5 minutes. Repeat three times.
9. Volume Calibration (if needed)
 - a. Remove stopper and aspirate ddH₂O from Chamber A
 - b. Add 2.1 mL of MiR05 to Chamber A
 - c. Loosen the collar on the stopper with the Allen wrench in the O2k accessory kit, and push collar to the top of stopper
 - d. Slowly lower stopper into chamber until the first bit of liquid emerges out of capillary on top of stopper.
 - i. There should not be any air bubbles inside the chamber. If bubbles are present, repeat steps 9b-d.
 - e. Push collar all the way to the bottom of stopper and tighten with Allen wrench.
 - f. Repeat steps 9a-e for Chamber B
 - g. NOTE: Do not interchange stoppers between chambers nor O2k instruments. If mismatched, minor imperfections on bottom of stoppers will alter volume inside chamber when stopper is fully depressed.
10. Air Calibration
 - a. Open chamber slightly by elevating stopper ~2 cm above fully depressed position
 - b. Leave stir bars on for at least 30 minutes
 - i. NOTE: During step 10b wait time, you can prepare saponin (see “Buffer and Substrate Preparation”) and then proceed with subsequent steps (i.e., leave for study visit/tissue collection).
 - c. When steady state has been achieved (red line) and O₂ level (blue line) is between 180-210, click on the blue Y₁ box in top right corner of Chamber A.

- d. Hold shift, left click, and drag your mouse across the steady O₂ line to create a mark for each chamber.
- e. Select the chamber of interest and click F5 to access calibration settings.
- f. Click the dropdown box under “**Select Mark**” for the “Air Calibration”. Select the number that corresponds to the mark you made in step 10d.
- g. Ensure “Slope uncorrected (pmol/(s.ml))” is between -1 and +1.
- h. Click “Calibrate and Copy to Dashboard” Repeat steps 10c-10h for all chambers.
 - i. Steps 10c-h can be completed during muscle incubation with BIOPS and MiR05 washes (see section “Permeabilized Fiber Bundle Preparation”)
 - ii. See section “Chamber Calibration” under “Quality Assurance and Quality Control” for information regarding appropriate calibration selection.
- i. Pipette 1.5 mL BIOPS into labeled 1.5 mL Eppendorf tubes and keep on ice. Bring these to tissue collection.

Permeabilized Fiber Bundle Preparation

1. Place muscle sample (~10 mg) in 1.5 mL Eppendorf with BIOPS immediately upon collection. Keep on ice.
 - a. NOTE: For murine sample, the entire muscle (e.g., EDL, TA, etc.) is placed in BIOPS on ice.
2. Transfer sample to cell culture dish containing BIOPS (entire sample should be covered) and place dish on pre-cooled (liquid nitrogen) metal block
3. Under the microscope, separate fibers quickly and carefully (Figure 1). Ensure you rid sample of blood, connective tissue, and fat
 - a. NOTE: Re-cool metal block frequently (every 15-20 minutes) with liquid nitrogen so buffer does not warm up



Figure 1. Rat soleus muscle fiber preparation. Muscle was teased into larger muscle strips and then prepared into fiber bundles. Fat and connective tissue was removed from muscle during process. Image from (Kuznetov et al., 2008, Nature Protocols)

4. Transfer muscle fiber bundles to 1.5 mL Eppendorf containing 1.5 mL BIOPS and saponin stock solution: 15 µL for human skeletal muscle (30 µg/mL) or 20 µL for rodent skeletal muscle (40 µg/mL). Saponin should be prepared fresh each day, see “Buffer and Substrate Preparation”)
5. Place sample (in BIOPS+saponin) on rotating nutator in fridge for 30 minutes



- a. NOTE: Ensure solution is mixing by observing air bubble movement. If no air bubble movement, invert manually to allow movement and return sample to nutator
 - b. Make sure that the muscle sample is not stuck between the Eppendorf lid and the side of the tube.
 - c. During 30-minute nutator incubation, thaw all substrates on ice and prepare pyruvate (see “Buffer and Substrate Preparation”)
6. After 30-minutes, transfer bundles to 1.5 mL Eppendorf tubes containing 1.5 mL of MiR05. Place Eppendorf tubes on rotating nutator in fridge for 15 minutes
 - a. NOTE: If air calibration is not yet complete, perform steps 10c-h
 - b. Collect fresh oxygen during incubation

Tissue Wet Weight

7. Dab muscle fiber bundle on Kim wipe to remove excess liquid
8. Place bundle on edge of weigh boat and record stable weight
9. Weight is taken twice to confirm accurate measurement
10. Immediately place bundle back in MiR05

Running an Experiment

11. Remove and place stoppers on top of O2k
12. Press F11 and F12 to turn off the left and right stirrers, respectively
13. Add blebbistatin to chambers (see “Buffer and Substrate Preparation”). Press F11 and F12 to turn on the left and right stirrers and allow mixing for 1 minute.
 - a. Press F13 after to turn off chamber lights as blebbistatin is light sensitive
14. Press F11 and F12 to turn off the left and right stirrers, respectively
15. Add fiber bundles to designated chamber and ensure all fibers are in solution (i.e., not stuck on edge of chamber above solution) and press F11 and F12 to turn on the left and right stirrers, respectively
16. Position stoppers in chamber until they are half-way down
17. Slowly inject a small amount of fresh oxygen through capillary in the stopper. Fully depress stopper when O₂ level (blue line) reaches ~350, and ensure there are no air bubbles in the chamber
 - a. If air bubble present in chamber: remove stopper, add MiR05, and repeat step 16
18. Wait for background (red line) to reach steady state (~15 minutes).
 - a. NOTE: It is critical that you wait for steady state to be achieved before the addition of every substrate.

For the remainder of the experiment, record every occasion when you add a substrate to the chamber by clicking F4 (or CTRL + left-click spot on graph that event occurred).

1. Begin experiment:

- a. Note: You may find it easier to follow the printable version of this protocol when working in the lab (see Appendix A: Printable OXPHOS Protocol)
2. Add 5 mM pyruvate (5 μ L of 2M pyruvate stock) and 2 mM malate (5 μ L of 800 mM malate stock). Label Event: "PM"
3. ADP titration
 - a. Add 25 μ M ADP (1 μ L of 50 mM ADP stock; Label Event: D 0.025)
 - b. Add 50 μ M ADP (1 μ L of 50 mM ADP stock; Label Event: D 0.05)
 - c. Add 100 μ M ADP (2 μ L of 50 mM ADP stock; Label Event: D 0.1)
 - d. Add 250 μ M ADP (6 μ L of 50 mM ADP stock; Label Event: D 0.25)
 - e. Add 500 μ M ADP (10 μ L of 50 mM ADP stock; Label Event: D 0.5)
 - f. Add 1000 μ M ADP (20 μ L of 50 mM ADP stock; Label Event: D1)
 - g. Add 2000 μ M ADP (4 μ L of 500 mM ADP stock; Label Event: D2)
 - h. Add 4000 μ M ADP (8 μ L of 500 mM ADP stock; Label Event: D4)
 - i. Add 6000 μ M ADP (8 μ L of 500 mM ADP stock; Label Event: D6)
4. Add 10 mM glutamate (10 μ L of 2M glutamate stock; Label Event: G)
5. Add 10 μ M cytochrome-c (5 μ L of 4 mM cytochrome-c stock; Label Event: Cyt-C)
6. Add 10 mM succinate (20 μ L of 1M succinate stock; Label Event: S)
7. Upon completion, close the software – this will automatically save your data
8. Clean the chambers
 - a. 2 x 2-minute washes with ddH₂O
 - b. 3 x 5-minute washes with 70% ethanol
 - c. 1 x 10-minute wash with 100% ethanol
 - d. Leave 70% ethanol in chamber until next use
 - e. Rinse stoppers with ddH₂O and 70% ethanol in sink
9. Place in chambers (do not completely depress) and turn off O2k

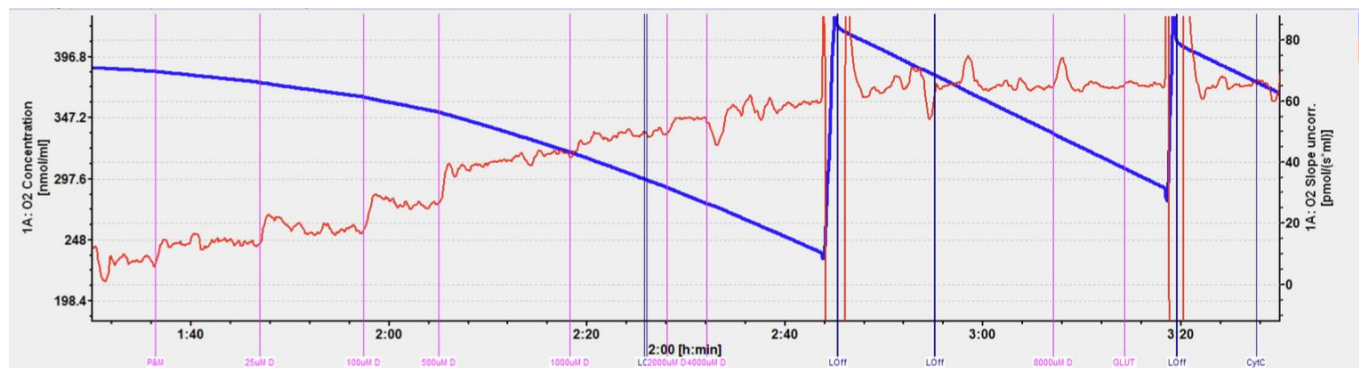


Figure 2. Sample O2k tracing of ADP titration of skeletal muscle fiber bundle from DM1 patient biopsy



Analyzing data

1. Open the file you wish to analyze and click "F3"
2. In the top right corner of Chamber A, click on Y_2 so the red box is highlighted
3. Using the technique to "make a mark" like you did for air calibration (see step 10. f), make marks for each of your steady states following a substrate addition as well as your background steady state.
 - a. If you make a mark and wish to delete it, hold shift and right click your mouse and drag over the mark you wish to delete, then let go and the mark will disappear
4. Once all marks are made, click "layout" at the top of the screen and select "O5 Flux per Volume Corrected"
 - a. Your data is now corrected using the background curve you imported
5. Click F2 and select copy to clipboard, your data can now be pasted into excel
6. Repeat steps 2-5 for Chamber B

7 Quality Assurance and Quality Control

Steady States

It is very important to make sure a steady state is achieved before each addition in order to get accurate data. When waiting for background steady state (after adding fiber but before adding any substrates), wait until respiration has leveled out ABOVE zero. If the background is below zero respiration will be a negative value and all data will be corrected for a negative rate. This may take 10+ minutes to achieve proper steady state. After adding Glutamate/Pyruvate/Malate respiration often tends to slowly increase, again, make sure you have an accurate steady state so you do not end up overestimating this rate and getting a lower rate with your first substrate addition (may need to wait 5+ minutes). If data is very noisy, make a large mark over steady state as opposed to trying to find a small flat line. Taking a larger area average will give a more accurate reading.

Expected readings

- Air calibration
 - Prior to the addition of experimental samples, substrates, or oxygen, O_2 levels (blue line) should range between 180-210 when the slope (red line) is plateaued around 0. If basal oxygen levels are elevated or if the slope fails to stabilize, the sensor may require maintenance.
- Leak respiration
 - After the sample and oxygen are added to the chamber, but prior to the addition of substrates, the slope (red line) should be between 0-20. Higher values could be indicative of higher leak respiration, however if a chamber consistently shows elevated values, the sensor may require maintenance

Chamber Calibration

Prior to experimentation, each chamber requires calibration to ensure accurate oxygen concentration readings. This is required each time a polarographic oxygen sensor is cleaned and membranes are replaced. The sensors are calibrated by a two-point calibration, with the determination of the constant signal recorded at 100% and 0% air saturation (achieved with sequential additions of Na-dithionite after oxygenation) in the experimental medium (typically MiR05). The most recent calibration file should be selected when running experimental protocols. More details can be found at

[https://www.bioblast.at/index.php/MiPNet19.18D_O2k-Series G and DatLab 6: Calibration](https://www.bioblast.at/index.php/MiPNet19.18D_O2k-Series_G_and_DatLab_6:_Calibration)

8 Safety Precautions

All personnel who perform activities with this SOP must have the basics training in laboratory safety procedure and Oroboros O2k equipment.

9 References

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10 Attachments/Checklists

Protocol Checklist:

<u>Outcome</u>	<u>Event</u>	<u>Substrate [Stock]</u>	<u>Addition to Chamber (μL)</u>	<u>Complete?</u>
Myosin II ATPase activity inhibition	Blebbistatin	10 mM	1	-
LEAK	Pyruvate	2 M	5	-
	Malate	800 mM	5	-
ADP Sensitivity	25 μM ADP	50 mM	1	-
	50 μM ADP	50 mM	1	-
	100 μM ADP	50 mM	2	-
	250 μM ADP	50 mM	6	-
	500 μM ADP	50 mM	10	-
	1000 μM ADP	50 mM	20	-
	2000 μM ADP	500 mM	4	-
	4000 μM ADP	500 mM	8	-
Max OXPHOS	6000 μM ADP	500 mM	8	-
Max CI	Glut	2 M	10	-
Membrane Integrity	Cyt-C	4 mM	5	-
Max CI+CII	Succ	1 M	20	-

BIOPS buffer

Preparation of BIOPS

1. Add approximately 500mL of ddH₂O to 1000mL beaker
2. While constantly stirring, add stock solutions of CaK₂EGTA** and K₂EGTA*
3. Weigh and add all powder chemicals
4. Adjust pH to 7.1 using KOH pellets
5. Using graduated cylinder, bring total volume to 1000mL
6. Aliquot into 15mL falcon tubes
7. Store at -20 °C.

http://wiki.oroboros.at/images/3/3c/MiPNet03.02_Chemicals-Media.pdf

Chemical	Final Concentration	Molecular Weight	Stock Solution	Addition to 1 Litre final volume	Source and product code
CaK₂EGTA**	2.77mM		100mM	27.7mL	
K₂EGTA*	7.23mM		100mM	72.3mL	
Na₂ATP	5.77mM	551.1		3.180g	Sigma A 2383, - 20°C
MgCl₂ • 6H₂O	6.56mM	203.3		1.334g	Scharlau MA 0036, RT
Taurine	20mM	125.1		2.502g	Sigma T 0625, RT
Na₂Phospho creatine	15mM	255.1		3.827g	Sigma P 7936, -20°C
Imidazole	20mM	68.1		1.362g	Fluka 56750, RT
Dithiothreitol (DTT)	0.5mM	154.2		0.077g	Sigma D 0632, 4°C
MES Hydrate	50mM	195.2		9.76g	Sigma M8250, RT

BIOPS contains the following ion concentrations:	
Ca²⁺ Free	0.1 <u>uM</u>
Mg²⁺ Free	1mM
MgATP	5mM
Ionic Strength	160mM

****CaK₂EGTA:** Dissolve 2.002g CaCO₃ (Sigma, C 4830; 100g) in 100mM hot (80°C) solution of EGTA (7.608g of EGTA-Sigma E4378 in 200mL ddH₂O). Add 2.3g of KOH and adjust pH to 7.0 using KOH (Sigma, P 1767). Freeze unused portions.

***K₂EGTA:** Mix 100 mM EGTA and 200 mM KOH (dissolve 7.608g EGTA and 2.3g KOH in 200mL ddH₂O). Adjust pH to 7.0 using KOH. Freeze unused portions

MiR05 buffer

Preparation of MiR05

1. Add approximately 500mL of ddH₂O to 1000mL beaker
2. While constantly stirring, add stock solution of K-Lactobionate*
3. Weigh and add all powder chemicals
4. Adjust pH to 7.1 using KOH pellets
5. Using graduated cylinder, bring total volume to 1000mL
6. Filter and then aliquot into 10mL falcon tubes
7. Store at -20 °C.

http://wiki.oroboros.at/images/d/d9/MiPNet14.13_Medium-MiR06.pdf



Chemical	Final Concentration	Molecular Weight	Addition to 1 Litre final volume	Source and Storage temp
EGTA	0.5mM	380.4	0.190g	Sigma E4378 (25g), RT
MgCl ₂ • 6H ₂ O	3mM	203.3	0.610g	Scharlau MA 0036 (250g), RT
K-Lactobionate*	60mM	358.3 free acid	120mL of 0.5M	Aldrich 153516 (100g), RT
Taurine	20mM	125.1	2.502g	Sigma T 0625 (25g), RT
KH ₂ PO ₄	10mM	136.1	1.361g	Merk 104873 (1kg), RT
HEPES	20mM	238.3	4.77g	Sigma H 7523 (250g), RT
Sucrose	110mM	342.3	37.65g	Roth 4621.1 or Sigma 84097(1kg), RT
BSA	1g/L	154.2	1g	Sigma A 6003 fraction V (25g), 4°C

MIRO5 contains the following ion concentrations:	
Ca ²⁺ Free	0.0 <u>uM</u>
Mg ²⁺ Free	2.1mM
K ⁺	90mM
Na ⁺	0
EGTA free	0.46mM
Osmolarity	330 <u>mOsm</u>
Ionic Strength	95mM

***K-Lactobionate:** Dissolve 35.83g lactobionic acid in 100mL ddH₂O, adjust pH to 7.0 with KOH, adjust final volume to 200mL with ddH₂O

Saponin

Preparation of stock solution – **Prepare fresh every day**

1. Weigh 6 mg of Saponin and add to 2 mL of BIOPS
2. Keep on ice.

P: Pyruvate

Pyruvic acid, sodium salt, C₃H₃O₃Na; Sigma P 2256, 25 g, store at 4 °C; FW = 110.0

Preparation of 2 M stock solution (dissolved in H₂O) – **Prepare fresh every day**

1. Weigh 44 mg of pyruvic acid directly into a 0.5 mL Eppendorf tube.
2. Add 0.2 mL H₂O.
3. Adjust pH to 7.0.
4. Keep on ice.

M: Malate



L-Malic acid, C₄H₆O₅; Sigma M 1000, 100 g, store at RT; FW = 134.1

Preparation of 800 (or 400) mM stock solution (dissolved in H₂O):

1. Weigh 536.4 (or 268.2) mg of L-malic acid.
2. Add 3 mL H₂O.
3. Neutralize with 5 M KOH (approx. 350 µL).
4. Adjust final volume to 5 mL (in 5 mL volumetric glass flask).
5. Divide into 0.5 mL portions.
6. Store frozen at -20 °C.

G: Glutamate

L-Glutamic acid, monosodium salt hydrate, C₅H₈NO₄Na; contains 1 mol H₂O /mol; Sigma G 1626, 100 g, store at R.T.; FW = 169.1

Preparation of 2 M stock solution (dissolved in H₂O)

1. Weigh 1.691 g L-Glutamic acid, monosodium salt hydrate, and transfer to a 5 ml volumetric glass flask.
2. Add 3 ml H₂O.
3. Adjust pH with 5 M KOH to pH 7.0.
4. Adjust final volume to 5 ml and divide into 0.5 ml portions.
5. Store at -20 °C.

D: 500 mM ADP

Adenosine 5'diphosphate potassium salt, C₁₀H₁₅N₅O₁₀P₂K; Merck 117105-1GM (Calbiochem) FW = 501.3; store at 4 °C. Recommended due to lower ATP impurities, we use 'ADP-Mg' in OROBOROS Lab. Alternative source: Sigma A 5285, 1 g, store at -20 °C; FW = 501.3.

Preparation of 500 mM ADP stock solution (dissolved in H₂O):

1. Weigh 501.3 mg of ADP = 1 mmol ADP
2. Add 1.2 ml H₂O (ADP is not dissolved at this stage).
3. Neutralize with 5 M KOH (approx. 450 µl). ADP will dissolve after addition of KOH.
4. Check pH and adjust to 7 with 5 M KOH if necessary.
5. Adjust final volume to 2 ml and divide into 0.2 ml portions.
6. Store at -80 °C.

D: 50 mM ADP (from 500 mM ADP – above)

1. Pipette 10 µL of 500 mM ADP into 600 µL Eppendorf tube.
2. Add 90 µL of ddH₂O.



3. Store at -80°C.

C: Cytochrome c

From equine heart; Sigma C 7752, 50 mg, store at -20 °C; FW = 12384 Da.

Preparation of 4 mM cytochrome c solution (dissolved in H₂O):

1. Weigh 50 mg cytochrome c into a small glass beaker. Difficult to weigh, since the powder is electromagnetic.
2. Add 1 ml H₂O; dissolves easily.
3. Divide into 0.2 ml portions.
4. Store at -20 °C.

S: Succinate

Succinate disodium salt, hexahydrate, C₄H₄O₄Na₂ X 6H₂O; Sigma P 2378, RT, FW=270.1

Preparation of 1M succinate stock (dissolved in H₂O):

1. Weigh 2.701 g succinate into volumetric glass beaker.
2. Add 6 ml H₂O
3. Check pH and adjust if necessary to 7.0 with 1M HCl (~60-120 µL typically needed).
4. Adjust final volume to 10mL and divide into 0.5mL portions.
5. Store at -20 °C.

11 Examples & Tips

Buffers

Buffers must be thawed fresh each day for actual data collection. When thawed, write the date of thawing on the tube and place any leftover buffer in the fridge. For practice, buffers in the fridge can be reused. If it was thawed <2 days prior, it is fine to use as is. If it was thawed 3 days prior or more (up to 1 week. Throw out buffers that are more than a week old) it must be topped up with EGTA. To do this, pour buffer into graduated cylinder to get accurate volume. Thaw a 0.5M EGTA aliquot from the freezer and add corresponding volume to get the following final concentrations: BIOPS: 10mM EGTA; MiRO: 0.5mM EGTA.

Substrates

When making substrates, adjust the pH to 7.1 and aliquot into small volumes, enough for 6 chambers for 1 protocol. When running experiments, thawed substrates cannot be put back in the freezer, they must be placed in the fridge and can be used for up to 1 week after thawing (except for FCCP, oligomycin, and Rotenone, see below).



**** ADP must be thawed fresh each day, do not reuse for actual data collection (ok for practice)**

Pyruvate must be made fresh each day, make 2M solution in a 1.5mL eppendorf by combining powder and water (see above), throw out pyruvate at the end of each day.

Syringes and Beakers

Each protocol being followed should have its own separate syringe and beaker filled with water. Syringes should be rinsed with water prior to use and should be wiped with a kimwipe after being put in the chamber to remove residual buffer. When switching substrates, syringes should be rinsed thoroughly with water.

Oxygen Levels

Do not let oxygen levels drop below 150. If oxygen is below 200, make next addition and wait for steady state. Once respiration has achieved steady state, loosen stopper and re-oxygenate up to 350. Push stopper back in and wait for steady state. Often when reoxygenating, the respiration rate's steady state will be higher than before reoxygenation. If this happens, take an average of the rate before reoxygenation and after reoxygenation and then proceed with adding next addition.