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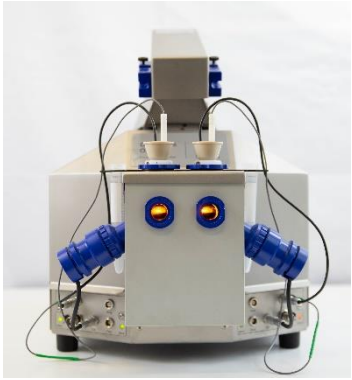
NextGen-O2k: Q-Module Manual



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Content

1. Introduction

The Q-Module has been developed as an integral part of the NextGen-O2k to detect the redox changes of the Q-pool in the mitochondrial electron transfer system (ETS) and in chloroplasts. Ubiquinone, also known as coenzyme Q (CoQ or Q) and plastoquinones are essential mobile components of the mitochondria and chloroplasts that transfer electrons between the respiratory and photosynthetic complexes of the ETS. The level of reduction of the Q-pool is dependent on the relative activities of the enzymes that reduce and oxidize it in the ETS. Therefore, deficiencies in the mitochondrial ETS, originating from the malfunction of respiratory enzymes (e.g. Complex I), can be easily detected by measuring the changes of the Q redox state with respect to respiratory activity.

The original idea is based on a patent developed by Rich PR [1] and utilizes a three-electrode system to indirectly determine the redox state of the Q-pool in the mitochondrial inner membrane (mtIM) via a Q-pool mimetic. Since CoQ10 is trapped within membrane boundaries, the CoQ10 mimetic, **Coenzyme Q2 (CoQ2)** is used as a probe which reacts with both the biochemical sites and the detecting electrode. It is assumed that CoQ2 does not react directly with the CoQ10 in the Q-junction. However, CoQ2 will be reduced by Complexes I and II and oxidized by Complex III (Rich PR, personal communication). If the ratio of the rates of oxidation and reduction of the CoQ2 equals the ratio of rates of oxidation and reduction of the natural CoQ10, then the redox state of CoQ2 will reflect the redox state of the Q-pool [3,4]. In order to avoid any disturbing effect of the CoQ2 on the biological system, the concentration of CoQ2 is kept low.

1.1. Three-electrode system

In order to detect the redox changes in the CoQ2, a three-electrode system is used. For the Q-Module, the detecting electrode is a **glassy carbon** (GC) electrode (working electrode) which is set to a given potential versus a **silver/silver chloride** (Ag/AgCl) reference electrode. The applied potential on the surface of the GC should be sufficient to either oxidize reduced CoQ2 or to reduce oxidized CoQ2 (in the Q-Module, the GC electrode is set at the oxidation peak potential). The third electrode is a **platinum electrode** (Pt; counter electrode) that acts as a counter electrode to complete the circuit that is rate-limited by electron transfer on the GC.
When the GC is poised at the oxidation peak potential, reduced CoQ2 undergoes oxidation at the surface of the GC. The current that flows between GC and Pt is proportional to the concentration of reduced CoQ2. Therefore, with the GC set at the oxidation peak potential, the current will increase as the amount of reduced CoQ2 increases. The electric flow (current, I_{el} [A]) is converted into a voltage (electric potential, V_{el} [V]) by the equipment and amplified ($U=I \cdot R$; U: voltage; I: current; R: resistance).

Of note, GC can also be set at the reduction peak potential of the CoQ2. Under this condition the oxidized CoQ2 is reduced on the surface of GC and a current will flow in the opposite direction.

1.2. Cyclic voltammetry: quality control

Cyclic voltammetry (CV) is a type of electrochemical measurement which is applied in the Q-Module as a quality control step to determine the redox potential of CoQ2 in the specific experimental conditions used. In voltammetry, information about the analyte is obtained by measuring the current as the electric potential is varied. In CV, the electric potential between the GC and the Ag/AgCl electrode ramps/changes linearly versus time in cyclical phases, while the current is detected between GC and Pt. The detected current is plotted versus the applied voltage to obtain the typical cyclic voltammogram trace (Figure 1). The presence of CoQ2 that is oxidized/reduced will result in current between GC and Pt, which is seen as characteristic peaks in the voltammogram at a defined potential.

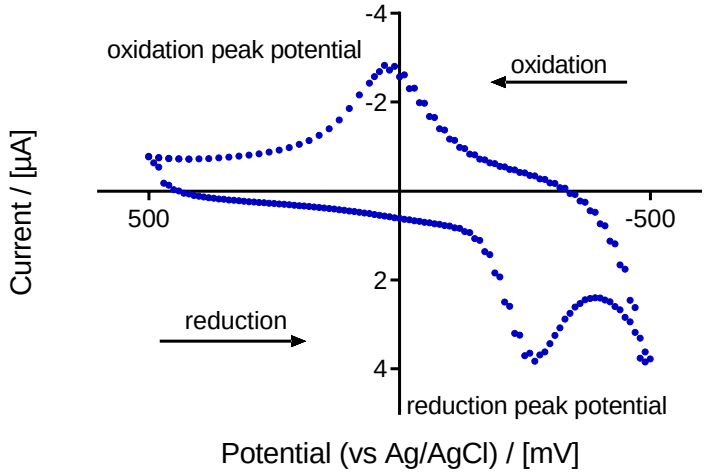


Figure 1. Cyclic voltammogram of Coenzyme Q2 (Sigma Aldrich, C8081;2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone, Ubiquinone-2; MW 318.2 g/mol) measured with the Q-Sensor. Measurements were carried out in non-stirred MiR05-Kit medium, at 37 °C using the NextGen-O2k. Initial potential: +30 mV, polarization window: between -500 mV and +500 mV, scanning speed: 100 mV/s, gain: 1; 30 µM Q2 was used for each test. The oxidation peak potential shows the maximum rate of ubiquinol (reduced form) oxidation and the reduction peak potential is the point of maximum rate of quinone reduction.

2. Setup of the Q-Module

Q-Module consists of the Q-Stopper with embedded electrodes, the electronics in the O2k – ideal for both cyclic voltammetry and measuring the Q redox ratio, and the DatLab software. The Q-Stopper with the reference electrode is called the Q-Sensor, which is plugged in the NextGen-O2k.

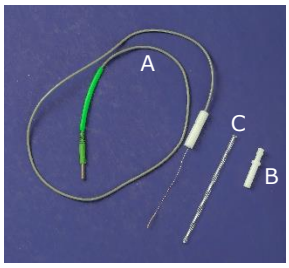
Q2k -Service box contains:

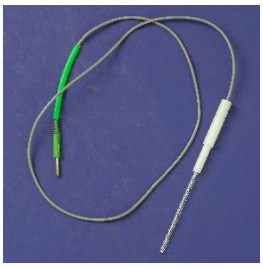
- 4 spare Viton O-rings (12 × 1 mm), with volume calibration ring
- 2× Reference-Electrode\2.4 mm: 2.4 mm diameter glass barrel
- 4× Q-Stopper\beige PEEK\conical shaft\side+2.6 mm+1.3 mm central port: with glassy carbon and platinum electrode embedded
- 10× Replacement-Barrel for Reference-Electrode\2.4 mm diameter glass
- Electrolyte\Reference-Electrode
- ISE-Filling Syringe with needle
- 2× OroboQ-Polishing Powder 1 (0.3 µm)
- 2× OroboQ-Polishing Powder 2 (0.05 µm)
- 2× OroboPOS-Polishing Cloth
- 1× TIP2k-Filter Papers for Q (10/Pkg)
- MiPNet24.12NextGen-O2k: Q-Module
- MiPNet24.16 DatLab8.0:CV-Module

2.1. Ag/AgCl electrode – reference electrode (RE)

The reference electrode\2.4 mm is an Ag/AgCl electrode with an internal filling solution of 3 M KCl saturated with AgCl.
Before the electrode can be put into operation, the glass reference barrel must be filled with the electrolyte supplied for the reference electrode. To do this, the electrode will need to be taken apart.
Parts of the reference electrode:

A RE-Cable Connection, upper part of electrode housing, with cable and silver wire
B RE-Electrode Holder, lower part of electrode housing
C RE-Glass barrel





2.1.1. Assembly

a) Unscrew the white plastic cap of the reference electrode removing the upper part of the cap with the attached silver wire. Pull the glass barrel out of the lower part of the cap (video).

b) The electrolyte solution is added to the glass tube using the provided electrolyte bottle and polyethylene tube: Insert filling tube into the electrolyte bottle. Push until tube locks into place. Insert tube into reference barrel and squeeze bottle. Fill reference barrel up to

approximately 0.5 cm (approx. 0.2 inch) from top. Remove bubbles with the filling tube.

c) After filling the glass barrel with the reference electrolyte, the silver wire is inserted back into the glass tube and the electrode cap is re-assembled.

2.1.2. Cleaning the electrode

To wash the reference electrode between runs, rinsing is recommended in the specific sequence: water, 70% ethanol, pure ethanol, and water. This procedure should be sufficient to prevent carry-over even of hydrophobic inhibitors since the reference electrode is made of non-hydrophobic materials. Immersion into 99.9% ethanol ($EtOH_{abs}$) should be avoided to prevent blocking of the ceramic diaphragm in an assembled electrode. When using the electrode in solutions containing higher concentrations of protein, the electrode can be soaked in a dedicated enzyme cleaning solution or a chromic/sulfuric acid glass cleaning solution after each use for 10-15 seconds to remove the protein from the glass and the reference junction. This prolongs the lifetime of the electrode.

2.1.3. Storage

Always clean the electrode before storage.

Short term: Place the tip of the electrode in a test tube or beaker containing reference electrolyte (3 M KCl) and protected from light. Falcon-type 15 mL vials are well suited. If necessary, refill electrolyte before use.

Long-term (>4 weeks): Remove the glass barrel containing the electrolyte and store the entire glass barrel in a closed test tube filled with the reference electrolyte. Rinse the silver wire and electrode cap to remove the salt solution and dry using an absorbent towel. Store in the accessory box or any closed container to keep dust off the electrode and protect from light.

2.2. Q-Sensor with built-in glassy carbon and platinum electrodes

The Q-Stopper has been designed close to the shape of a regular O2k-Stopper to introduce the reference electrode through the stopper into the O2k-chamber. The standard O2k-Stopper has a concave shape on the end inserted into the chamber, with a single capillary (gas-escape/titration capillary) in the centre of the stopper. The end of the Q-Stopper is also concave with one gas-escape/titration capillary in the middle and three electrode inlets. Two electrodes, the GC- and the Pt-electrode are already tightly built-in into the Q-Stopper and cannot be removed. The Ag/AgCl reference electrode is inserted through an inlet in the stopper.

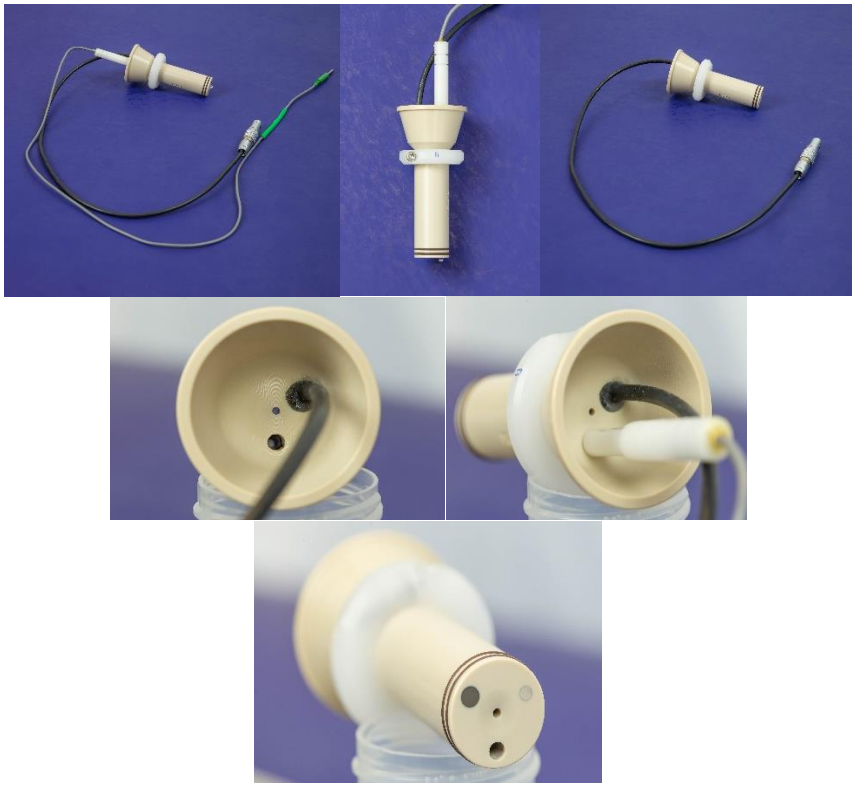


Figure 2. Q-Sensor assembly. First row from left to the right: Q-Sensor with the reference electrode, Q-Stopper without the reference electrode. Second row: Q-Stopper viewed from above without and with the reference electrode. Third row: Q-Stopper viewed from bottom. Glassy carbon electrode (black) and platinum electrode (shiny, silver) are built-in part of the stopper. One gas-escape/titration capillary and an inlet for the reference electrode can be seen.

2.2.1. Polishing of glassy carbon and platinum electrodes

The GC and Pt electrodes are tightly built-in into the Q-Stopper which is delivered in a sealed box. Before its use, the two electrodes need to be polished and cleaned.

The GC must be treated with extreme care. Do not touch with fingers, nor expose to detergents or greasy liquids (video). Avoid using sonication for cleaning the Q-Stopper.

a) Place the Petri dishes with the OroboPOS-Polishing Cloth on a flat surface.
b) Using the tip of a spatula, add OroboQ-Polishing Powder 1 (aluminum oxide, 0.3 µm) onto one of the polishing clothes. Add a few drops of distilled water.
c) Hold the sensor in a vertical position and polish the GC and Pt electrodes in the thin paste in a figure-eight motion 10-15 times.
d) Wash the polishing powder carefully off the end of the sensor 3 times with distilled water and wipe the surface of the Q-Stopper with a soft paper tissue between each washing step (video).

e) Repeat the step b) and c) using OroboQ-Polishing powder 2 (aluminium oxide, 0.05 µm) and the other OroboPOS-Polishing Cloth (use always the same polishing cloth with the same polishing powder). Repeat the step d) (washing).
f) Finally, wash the polishing clothes with distilled water and let it dry before storage.

2.2.2. Cleaning of the Q-Sensor before use

a) Polish GC and Pt electrodes before each experimental use (see section 2.2.1).
b) Clean the Q-Stopper after polishing with distilled water to remove polishing powder (section 2.1.2).
c) Rinse the reference electrode also with distilled water and insert it into the Q-Stopper before an experiment (video).

2.2.3. Cleaning of the Q-Sensor after use of CV

a) Remove the reference electrode from the Q-Stopper and rinse it in the following sequence: distilled water, pure ethanol ($EtOH_{abs}$), distilled water. Wipe the glass part of the reference electrode with a tissue at each washing step and store it in a Falcon tube filled with 3 M KCl solution (video).
b) Repeat the washing steps with the Q-Stopper: three times distilled water, three times $EtOH_{abs}$, three times distilled water. Thoroughly wipe the surface of the GC- and Pt-electrode with a soft tissue between each washing step.
c) In between experimental runs store the Q-Sensor in a sealed box or in an empty Falcon tube.

2.2.4. Cleaning of the Q-Sensor after a biological experiment

a) Remove the reference electrode from the Q-Stopper and rinse it in the following sequence: distilled water, 70% EtOH, $EtOH_{abs}$, distilled water. Wipe the glass part of the reference electrode with a tissue at each washing step and store it in a Falcon tube filled with 3 M KCl solution (video).
b) Repeat the washing steps with the Q-Stopper: three times distilled water, three times 70% EtOH, three times $EtOH_{abs}$, three times distilled water. Thoroughly wipe the surface of the GC- and Pt-electrode with a soft tissue between washing steps.
c) In between experimental runs, store the Q-Sensor in a sealed box or in an empty Falcon tube.

2.2.5. Storage of the Q-Stopper

Q-Stopper (GC and Pt are built in the regular Q-Stopper): Always clean the Q-Stopper before storage.
Short-term: In between experiments, store the Q-Stopper in an empty Falcon tube.
Long-term: Between experimental days and for long term, store the Q-Stopper in a sealed box to keep dust out and protect from air.

2.3. Assembly of the Q-Sensor

a) Polish and wash the Q-Stopper before each experimental use (section 2.2.1).
b) Fill up the glass barrel of the reference electrode with 3 M KCl solution and assemble the reference electrode (MiPNet15.03 and video).
c) Rinse the reference electrode with water and insert the glass barrel of the reference electrode into the inlet of the Q-Stopper (advisable to rinse the inlet with water) all the way, taking care not to break the glass.
d) Add respiration medium into the O2k-chamber, insert fully the Q-Sensor (Q-Stopper with the inserted reference electrode) into the O2k-chamber.
e) Connect the cable of the Q-Sensor to its `Q´ plug and the cable of the reference electrode in the `Q-Ref´ plug.

f) Connect DatLab to the instrument and start your experiment.
g) Run CV to determine the oxidation peak potential for GC (see section 3) if it is needed.

3. Quality control: Cyclic voltammetry

a) Polish the GC and Pt electrodes (section 2.2.1) before each experimental use.
b) Clean the Q-Stopper after polishing with distilled water.
c) Clean the O2k-hambers before experimental use (MiPNet19.03 O2k-cleaning and ISS).

d) Add the respiration medium into the O2k-chambers (same as is used for biological experiments) and insert the Q-Stopper with the reference electrode (video).
e) Open DatLab 8.0-CV-Module to perform cyclic voltammetry (MiPNet24.16 DatLab 8.0 CV Manual).
f) First, run a background CV without adding CoQ2. No peaks should appear on the cyclic voltammogram.
g) After finishing that background CV, add 30 µM CoQ2 and start again CV.
h) Save your CV, take note of your oxidation peak potential, and start cleaning the O2k-Chambers, Q-Stoppers and reference electrodes (section 2.2.3).
i) Clean the O2k-Chamber in the sequence of distilled water (rinse five times), $EtOH_{abs}$ soak for 10 min and rinse five times with distilled water again.
j) Before the biological experimental run, polish again the GC and Pt electrodes.

4. Operating instructions

4.1. Volume calibration with the Q-Stopper

When using a Q-Sensor, the Q-Stopper and reference electrode must be in place when calibrating the O2k-chamber volume, comparable to volume-calibration with standard stoppers (MiPNet19.18A O2k-start and video).

a) Add to the dry O2k-Chamber, containing the stirrer bar, a water volume accounting for the final chamber volume (2 mL) plus the additional dead volume in the capillary and spaces between electrodes and inlets. For the Q-Sensor (Q-Stopper and reference electrode), this additional volume is approximately 0.07 mL. Therefore, the volume to calibrate a chamber volume of 2 mL with the Q-Sensor is 2.07 mL.

b) Prepare the Q-Stopper (loosen the calibration ring, dry the stopper), making sure that the titration capillary and the electrode inlet are dry. Remove the reference electrode from the storage solution. Dry their shafts with a paper towel (do not use a paper towel directly on the diaphragm/Vycor frit of the reference electrode). Insert the electrodes into the Q-Stopper.

c) Place the stopper on top of the chamber with a loosened volume-calibration ring slid down to the chamber holder. Insert the Q-Sensor (Q-Stopper plus reference electrode) slowly into the chamber, carefully observing first the diminishing gas phase in the chamber. Stop the insertion as soon as the first drop of liquid appears on the top of the stopper. This may be visible first on top of the gas-ejection

capillary (comparable to the standard stoppers), but it may also occur at the edge of the reference electrode.

d) Fix the position of the volume calibration ring by tightening the screw to finalize the volume calibration.

4.2. Instrumental background flux

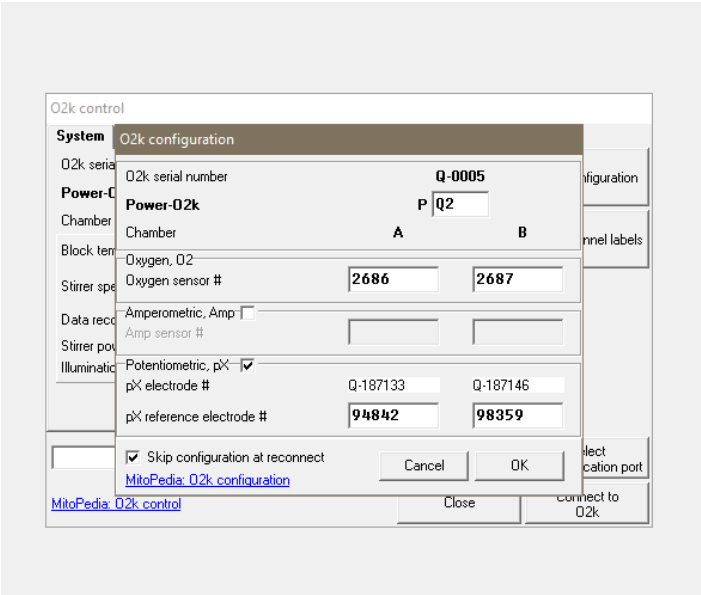
Instrumental oxygen background parameters are used to correct real-time oxygen flux (MiPNet14.06 Instrumental O2 background). The instrumental background tests must be carried out with the Q-Sensor and reference electrode in place. The values obtained with the standard stoppers for respirometry cannot be used for Q-Sensor experiments. It is important that, while the instrumental oxygen background test with dithionite injections is running, the Q-Module must be switched off (see Section 5).

5. DatLab 7.4

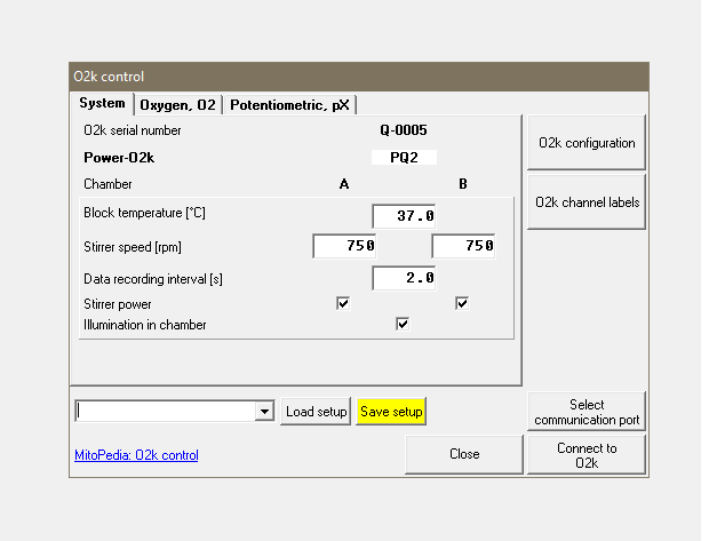
The Q-Module is operated via DatLab7.4 (DL).

a) Plug in the two cables of the Q-Sensor into the `Q´ and `Q-Ref´ labelled plugs on the O2k-Main Unit.

b) Switch on the O2k and open DL. Enter the username and start the connection process by clicking Connect to O2k. The `O2k configuration´ window pops up. Check the box for Potentiometric, pX, measurements, and type the serial number of pX reference electrode into the according field. The number of the Q-Stopper is automatically recognized by the instrument. The serial number of the reference electrode can be found on its cable.



c) Confirm the input with OK, which brings up the `O2k control´ window. Check the default measurement parameters in the System tab.



d) In the Potentiometric, pX tab set the Offset-voltage according to the oxidation peak obtained during the CV and select 100 as gain for Q-sensor.

e) Click on Connect to O2k. Choose a folder to save your DL measurement file (.dld) and continue by clicking on Save to establish the connection between DL and your O2k.

f) Select your DL-Protocol in the Run DL-Protocol/Set O2 limit window, which pops up automatically (MiPNet22.16 DL-Protocols) .

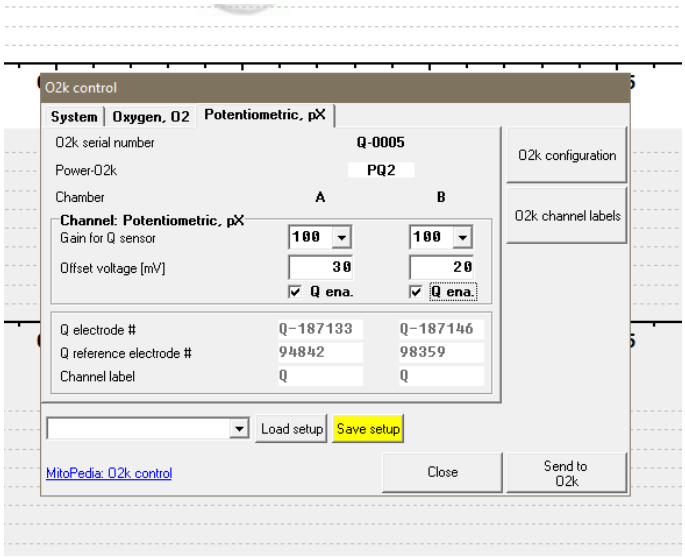
g) Select Layout\O2&pX\Standard layouts\ 01 Potentiometric in the menu to see the O2 channel and pX channel for Q simultaneously.

h) Change the scaling of the pX raw channel (Y1 axis): Select Graph\Scaling. Select Graph 2 and change the scaling of the Y1 axis: minimum: 0; range: +1. Press OK to apply the new scaling.

i) Select Oroboros O2k tab in the menu, select O2k control window (F7) and choose O2k channel labels. Rename the Potentiometric, pX channel as Q.

j) Open again the O2k control window and select Potentiometric tab.

k) In the Potentiometric tab enable Q measurements by checking the Q ena. box.



l) Press Send to O2k to send the commands to the instruments.

6. Demo experiment

6.1. Materials

- Isolated mitochondria (mt): Heart mitochondria were isolated from mouse. Final concentration: 0.14-0.15 mg/mL.
- Respiration medium: Standard mitochondrial respiration medium, MiR05-Kit (see reference: MiPNet 22.10 MiR05-Kit) was used.
- Coenzyme Q2 (CoQ2): MW: 318.41 mg (Sigma Aldrich: C8081), dissolved in 100% ethanol. Stock solutions of 10 mM and 1 mM were prepared, respectively for cyclic voltammetry and for analysis of the Q redox ratios with isolated mitochondria. For further details please see: https://www.bioblast.at/index.php/Coenzyme_Q2.
- Cyclic voltammetry: 30 µM CoQ2 (6 µL of 10 mM solution in each O2k-chamber) , MiR05-Kit; for setting see in Section 8.1., for experiment see section 3., and for DatLab8.0 CV software see MiPNet24.16 DatLab8.0 CV Manual.

6.2. Experimental setup

- Polarization voltage: dependent on the oxidation peak potential of CoQ2 with the given Q-Sensor in the given O2k-chamber (section 3. for CV).
- Gain: 100
- DL-Protocol: SUIT-006 Q mt D071

Abbreviation list and concentrations used:
mt: mitochondria
Q2: coenzyme Q2, 1 µM
Rot: rotenone; 0.5 µM
S: succinate; 10 mM
D: ADP; 2.5 mM
U: uncoupler, carbonyl cyanide m-chlorophenyl hydrazine (CCCP), 0.5 µM steps
Ama: antimycin A; 2.5 µM

The O2k-chambers, containing 2 mL respiration medium MiR05-Kit, were closed and the Q-Sensor was set to the experimental configurations (see the instructions above). After enabling the Q-Sensor, the baseline of the Q signal was recorded. The experiment was started by the addition of 30 µL of sample and 2 µL Q2 (1 mM stock solution, to achieve 1 µM final concentration).
It was followed by rotenone and succinate addition to initiate LEAK respiration, which was reflected also in the reduction of the Q-pool. Rot addition was needed 1) to avoid oxaloacetate formation which would inhibit succinate dehydrogenase, and 2) to inhibit the endogenous substrate oxidation, which might slightly reduce the Q-pool and therefore, it would overestimate the fully oxidized Q state. Next, a saturating concentration of ADP was added to initiate oxidative phosphorylation (OXPHOS) which was reflected in the oxidation of the Q-pool. Uncoupler was titrated to detect electron transfer (ET) capacity. In mouse heart mitochondria usually, U does not further increase O_2 flux, therefore, no changes were observed in the Q signal. Mitochondria consumed all oxygen in the O2k-chamber leading to anoxia, which correlates with fully reduced Q-pool and taken as 1 for the calculations of Q redox ratios. (As a control of the fully reduced Q state we also added a Complex III inhibitor Ama, but it causes artefact in the Q signal, which questions its application as an inducer of the fully reduced Q state.) The fully oxidized Q-pool can be observed in the presence of mitochondria, CoQ2 and Rot and taken as 0 to calculate the Q redox ratios.

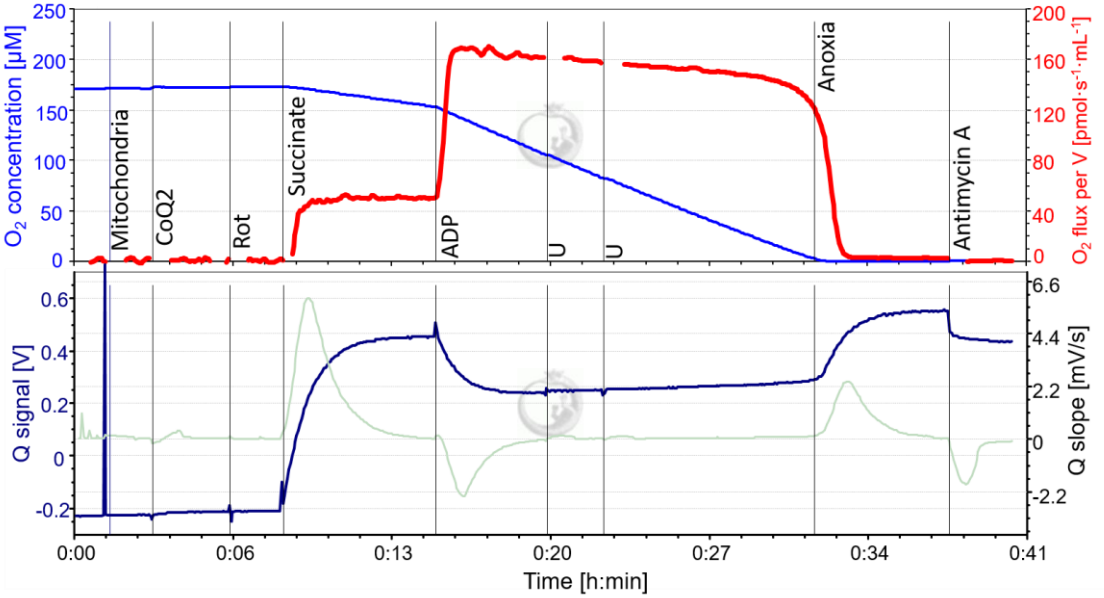


Figure 3. Simultaneous measurement of O_2 flux and Q redox ratio using mitochondria isolated from mouse heart. The experiments were carried out in MiR05-Kit, at 37 °C. The glassy carbon of the Q-Sensor was poised at +30 mV. In the upper figure, blue trace shows the O_2 concentration [Y1 axis; µM], red trace represents the volume-specific O_2 flux [Y2-axis; [pmol·s^{-1}·mL^{-1}], in the lower figure, dark blue trace shows the raw Q signal [Y1 axis; V], grey trace demonstrates the Q slope [Y2 axis; mV/s]. DLD file: 2020-06-23 PN4-03.DLD

To facilitate the O_2 flux in the chamber, leading to anoxia, it is recommended to use a high concentration of mitochondria (more than 0.05 mg/mL of protein). Alternatively, the O_2 concentration in the chamber could be decreased, before the sample addition, by injecting N_2 in the gas phase of the chamber in the opened position. For reoxygenations during experiments with the Q-Module, it is recommended to use catalase (MiR06, MipNet14.13) in the respiration medium and titrate H_2O_2 whenever reoxygenation is necessary, avoiding chamber opening.

6.3. Analysis

6.3.1. Oxygen flux analysis

The calculations of the O_2 fluxes are provided under the following link complying with Oroboros transparency policy.
https://wiki.oroboros.at/index.php/Flux_/_Slope#O2

In the MiPNet 24.06 (section 3.1) you can find information about the marks setting to the O_2 flux.

a) In the SUIT-006 Q mt D071 Excel analysis template: click on the yellow cell B4 and paste only the O_2 fluxes from DatLab [Ctrl+V].

b) The calculated values for the specific O_2 flux, specific O_2 flux (bc), *FCR* and *FCR* (bc) on each step of the protocol can be found in the rows 22 to 25, starting at column K.

6.3.2. Q signal analysis

a) In the DatLab 7.4, set the marks separately to the O_2 flux and the raw Q signal (Y1 axis). (Y2 axis, Q slope, is not meaningful, do not set the marks to this trace).
   1. Go to Marks and select Slope uncorrected + all info. In the new window select pX raw [V] in Plot for Marks.
   2. Channel: Potentiometric, pX. Leave only this channel selected.
   3. Select: Median.
   4. Sort by: Time(default).
   5. Then, click on Copy to clipboard to copy the selected values.

b) In the Excel template: Click on the yellow cell B28 and paste [Ctrl+V] raw Q data from DatLab.

c) The calculated values for Q redox ratio on each step of the protocol can be found in the row 46.

6.4. Calculation

The Q redox ratio (Q_X/Q_{total}; Q_X/Q_t) is a ratio calculated between the given Q signal (Q_X) in the presence of different substrates/inhibitors/uncouplers and the fully reduced Q (Q_r) detected under anoxia (see mark below in figure 4), both corrected for the fully oxidized Q (Q_{ox}).
The fully oxidized Q state (Q_{ox}) is measured in the presence of mitochondria, rotenone and the CoQ2, which is subtracted from the raw Q signal for every step before the calculation of the ratios. For some preparations, the use of rotenone is not necessary, to be determined experimentally.

1) $Q_X=Q_{raw}-Q_{ox}$
2) $Q_t= Q_r-Q_{ox}$

Then, the Q_X/Q_t can be calculated from Q_X and Q_r both corrected for Q_{ox}.
To quantify the amount of reduced or oxidized Q is beyond the possibilities of our instrument. Mass spectrophotometer or HPLC (High Performance Liquid Chromatography) are required to determine the amount of the Q_r and Q_{ox} in our system.

7. Troubleshooting

7.1. Artificial signals

There are chemicals which influence the raw Q signal; therefore, they cannot be used with the Q-Module. The following chemicals interfere with the Q signal: ascorbate, TMPD (Tetramethyl-p-phenylenediamine dihydrochloride), azide, dithionite, cytochrome *c*, KCN (potassium cyanide) and NADH. A chemical background test should be run when using new chemicals without any biological sample present in the solution to test the interference of chemicals and the Q signal.
Cyclohexylammonium salts of some chemicals, e.g. glycerol-3-phosphate, can also interfere with the Q signal.

7.2. Unsuitable cyclic voltammogram

Performing CV before experiments is always a good quality control step to see how the Q-Sensor works. Not only the oxidation and reduction peak potentials are important parameters in CV, but also the shape of CV gives information about the quality of electrodes. The CV background shape should not present peaks nor be too wide in the y axis. If the shape of CV is not acceptable, the following procedures can be done to solve this problem:

a. Polishing GC and Pt electrodes with alumina powder (0.5 µm and 0.03 µm).
b. Cleaning the Q-Sensor, reference electrode and O2k-Chamber with distilled water, 70% ethanol and pure ethanol (see, 2.2.4).
c. Filling up the glass barrel of the reference electrode with new 3 M KCl solution.
d. Checking the quality of Vycor frit of the glass barrel.

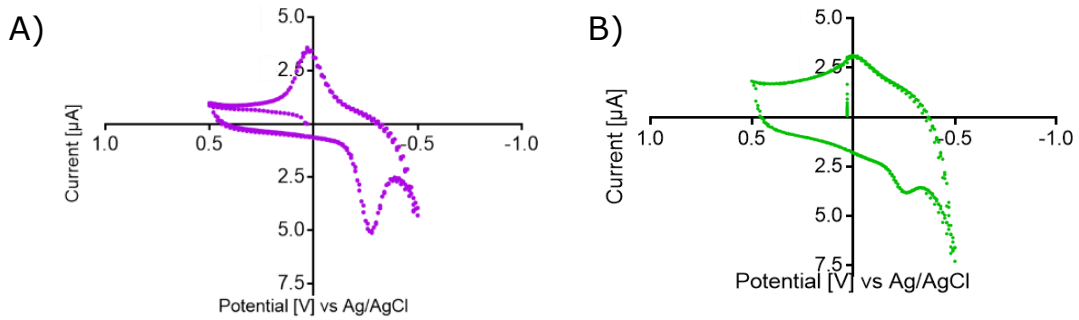


Figure 4. Cyclic voltammogram in the presence of Coenzyme Q2 (Sigma Aldrich, C8081;2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone, Ubiquinone-2; MW 318.2 g/mol) measured with same Q-Sensor on different experimental days. Both measurements were carried out in non-stirred MiR05-Kit, at 37 °C using NextGen-O2k. Initial potential: +30 mV, polarisation window: between -500 mV and +500 mV, scanning speed:100 mV/s, gain: 1; 30 µM CoQ2 was used for each test. Figure **A** shows a typical cyclic voltammogram while figure **B** shows an unacceptable one.

7.3. Drifting of Q signal with and without CoQ2 and biological sample

If the CV trace or oxidation peak potential is not acceptable, it will lead to an unstable or noisy raw Q signal in the absence or presence of CoQ2, which leads to artefacts when calculating the Q redox ratio or in the worst-case scenario, the Q redox ratio cannot be calculated. If a drift occurs in the Q signal, usually, changes are observed already in the shape of the CV trace or in the peak potentials. Therefore, the procedures mentioned in section 8.2 can solve the problem.

7.4. Damaged surface of glassy carbon

Under normal circumstances, the surface of glassy carbon should be black, smooth, and shiny. Any visible scratches on its surface might lead to disturbances in both the CV and the Q signal. If the CV shape is not acceptable and the raw Q signal is also drifting after further polishing, and the procedures written in section 8.2 cannot solve the problem, a new Q-Sensor might be ordered. Of note, any electrical problems with the instrument should be excluded by testing another Q-Sensor in the same O2k-chamber.

7.5. Respiration

The simultaneous measurement of respiration with Q redox ratio is one of the biggest advantages of the NextGen-O2k. The inhibitory effect of CoQ2 mimetic on respiration should also be tested compared to a chamber where no CoQ2 was titrated (https://bioblast.at/index.php/Carrier_control_titrations).

7.6. Breakage of the reference electrode

The reference electrode is inserted manually into the inlet of the Q-Stopper. The insertion of the reference electrode into the thin hole of the stopper might lead to the breakage of the glass. In the accessory box there are glass barrels for replacement. Application of a new glass barrel requires a new volume calibration and instrumental background because the diameter of the glass barrels can differ slightly.

8. Supplementary

8.1. Measurement parameters in cyclic voltammetry

The following parameters are important when running a CV [2].

- Solution/medium: cyclic voltammogram must be run in the same medium as used for the experiments with biological sample. In our case, we used our respiration medium, MiPNet 22.10 MiR05-Kit.

- Data recording interval: to record a cyclic voltammogram, the data must be recorded every 0.2 s. If the data recording interval is 2 s, there are not enough data points available for a CV.

- Initial polarization voltage is the potential where the scanning starts. It must be close to the peak potential to avoid a coating of GC which would lead to side reactions [2]. In the case of CoQ2, +30 mV was used as an initial potential that is close to the peak potential where the maximum rate of quinol oxidation happens. CV was also tested at +60 mV, which did not influence the peak potential values.

- Polarization window: During scanning, the narrowest possible range of potentials should be applied. Unnecessarily low and high potentials cannot be applied because it might lead to chemical modification or coating of GC [2]. Any type of modification of GC will inhibit the electron transfer on the surface of the electrode. In the case of CoQ2 -500 mV and +500 mV were chosen as a potential window.

- Number of CV cycles: Theoretically, one cycle (potential changes from -500 mV to +500 mV and then back to -500 mV) should be enough to observe the oxidation and reduction peak potential values, but in order to check any other side-reactions over the experimental time, more cycles are required. A total of 5 cycles are performed with the DatLab CV software.

- Scan speed: The scanning speed is ideal if it supports free diffusion of analyte (CoQ2). If the scanning rate is very slow, there is a risk that CoQ2 is transported to and from the electrode surface via other processes than diffusion [2]. If the scanning speed is too fast, it leads to double layer charging current, which comes from the rearrangement of solution molecules at the surface of GC as a result of the changing electrode potential and results in high baseline current that obscure features in a CV [2]. In order to avoid these side reactions and provide free diffusion for CoQ2, 100 mV/s was applied as a scanning speed.

- Gain: Amplification of the signal. In the O2k the gain, $F_{O2,G}$,can be selected in DatLab 7.4 within the `O2k control´ menu, with values 1, 10, 100 or 1000 V/A, where 1 V/µA is the basal gain at a gain setting of 1. The raw signal after amplification, is related to the original current, I_{el}= raw signal · $F_{O2,G}$.

- Non-stirred solution: Stirring of the solution can influence the reactions during CV. If you have only quinone (oxidized CoQ), only a

wave of quinone reduction is visible, the wave of quinol (reduced CoQ) oxidation cannot be seen, because the quinol is stirred off from the surface of GC (Peter R Rich, personal communication).

- Final concentration of CoQ2: The lowest possible concentration of CoQ2 should be used for CV which gives us well-defined peaks in the current in the CV to determine the peak potential values for oxidation and reduction. Lower than 30 µM of CoQ2 did not give us detectable peaks in the CV at gain of 1 V/µA, while using higher than ~ 90 µM of CoQ2 we can reach the limit of detection.

- Temperature: Cyclic voltammetry should be performed at the same temperature as the detection of the redox state of the Q-pool with biological sample, because the temperature slightly affects the peak potential values.

- Quality control: It is advisable to run CV without the analyte in the same solution to record the background CV, where no peak potentials should be observed, if the O2k-Chamber and Q-Sensor are not contaminated by CoQ2 or any other chemicals. If any peaks appear in the current, further polishing of GC or cleaning of the O2k-Chamber and Q-Stopper and reference electrode are required.

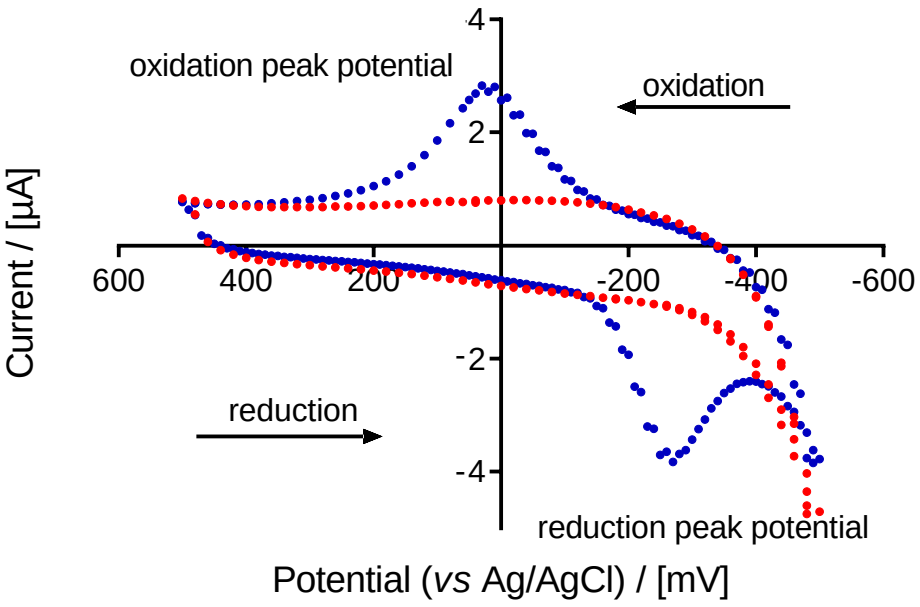


Figure 5. Cyclic voltammogram in the absence and presence of Coenzyme Q2 (Sigma Aldrich, C8081;2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone, Ubiquinone-2; MW 318.2 g/mol). Measurements were carried out in non-stirred MiR05-Kit, at 37 °C by using the NextGen-O2k. Initial potential: +30 mV, polarization window: between -500 mV and +500 mV, scanning speed:100 mV/s, gain: 1; 30 µM Q2 was used for each test. Blue dots represent the cyclic voltammogram of Coenzyme Q2, while red dots show the control or background CV, without Coenzyme Q2.

8.2. Quality control

The porous ceramic part (Vycor frit) at the end of the glass barrel of the reference electrode should always be in good condition and prevented from drying out, which causes crystallization of the electrolyte salt in the pores and makes it unusable. Even if it is stored in a 3 M KCl solution, from time to time the quality of the Vycor frit should be tested.

a) Take two reference electrodes dipped in a glass beaker filled with 3 M KCl solution.
b) Connect these two electrodes to the same voltammeter and measure the electric potential between them in the KCl solution.
c) The quality of the Vycor frit is acceptable if the potential between the electrodes is close to 0 mV. In practice, the electric potential is never 0 mV; it approaches 0 mV. Glass barrels with a potential higher than 0.5 mV should not be used.

9. References

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Author contributions

Komlodi T and Gnaiger E were responsible for the project development. Komlodi T, Hunger M and Cardoso LHD performed related experiments. Gnaiger E, Gradl P, Komlodi T, Gollner M, Merth A, Schwaninger H and Walter-Vracevic M were responsible for instrumental development. Komlodi T, Cardoso LHD, Iglesias-Gonzalez J and Tindle-Solomon L prepared the MiPNet. Moore AL as a scientific consultant contributed with valuable suggestions and provided the original prototype of the Q-electrode. We would also like to thank to Rich PR for his contributions.