O2k-Manual: Q-Module

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NextGen-O2k: Q-Module Manual

Timea Komlódi[1], Luiza H D Cardoso[1], Martin Gollner[2], Alexander Merth[2], Wolfgang Niedenzu[1], Markus Haider[3], Carolina Doerrier[1], Lisa Tindle-Solomon[1], Hannes Schwaninger[2], Michael Walter-Vracevic[2], Philipp Gradl[2], Anthony L Moore[4], Peter R Rich[5], Erich Gnaiger[1]

[1]Oroboros Instruments
High-Resolution Respirometry
Schoepfstrasse 18
6020 Innsbruck, Austria
Email: instruments@oroboros.at
www.oroboros.at

[2]WGT-Elektronik GmbH & Co KG
Rettenbergstraße 30a
6114 Kolsass, Austria

[3]Haider Technology Consulting
Fischnalerstraße 23
6020 Innsbruck, Austria

[4]University of Sussex
Biochemistry and Medicine
School of Life Sciences
Falmer, BN1 9QG
Brighton, United Kingdom

[5]University College London
Dept Structural and Molecular Biology
Gower Street, WC1E 6BT
London, United Kingdom

Contents

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 mitochondria and chloroplasts that transfer electrons between the respiratory and photosynthetic complexes of the ETS. The level of reduction of the mitochondrial CoQ 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 diagnosed more precisely when combining respiratory measurements with continuous monitoring of the Q-redox state.

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 ETS-reactive Q-pool in the mitochondrial inner membrane (mtIM) via a Q-pool mimetic. Since CoQ_{10} is trapped within membrane boundaries, the CoQ_{10} mimetic, **coenzyme Q_2 (CoQ_2)** is used as a probe which reacts with both the biochemical sites and the measuring electrode. It is assumed that CoQ_2 does not react directly with the CoQ_{10} in the Q-junction. However, CoQ_2 will be reduced by Complexes I and II and oxidized by Complex III at the Q_o site and reduced at the Q_i site (Rich PR, personal communication). As the redox state of the Q-mimetic equilibrates with the redox state of ETS-reactive CoQ, the measured redox state of the Q-mimetic reflects the redox state of ETS-reactive mtCoQ [3,4,5]. To avoid any side effect of the CoQ_2 on the biological system, the concentration of CoQ_2 is kept low.

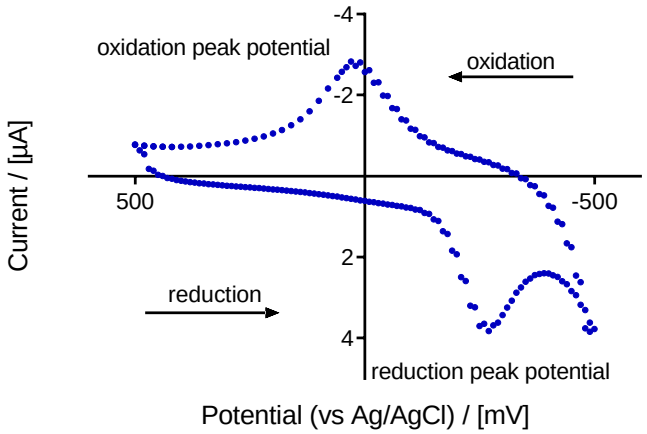
1.1. Three-electrode system

A three-electrode system is used to detect the redox changes of CoQ_2. For the Q-Module, the detecting electrode is a **glassy carbon** electrode (GCE; 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 CoQ_2 or to reduce oxidized CoQ_2 (in the Q-Module, the GC electrode is set at the oxidation peak potential). The third electrode is a **platinum electrode** (Pt) that acts as a counter electrode to complete the circuit that is rate-limited by electron transfer on the GC.

If the GCE is set to a potential oxidizing CoQ_2 (oxidation peak potential), then CoQ_2 which was reduced by the ETS undergoes oxidation on the GCE surface, resulting in a current between the GCE and Pt electrode which is measured. In this case, the concentration of the reduced CoQ2 is proportional to the current measured between GCE and Pt electrodes: the current increases in proportion to the concentration of reduced CoQ_2. Conversely, if the GCE is set to the CoQ_2 reduction potential, the oxidized CoQ_2 undergoes reduction on the GCE surface and current flows into 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 to (*1*) determine the oxidation and reduction peak potentials of CoQ_2 in the specific experimental conditions used, (*2*) check the quality of the sensor, and (*3*) test the interference of chemicals used with the Q-sensor. 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 electrodes varies linearly over time in cyclical phases, while the current is detected between GC and Pt. The current is plotted as a function of the applied electrical potential in the cyclic voltammogram trace, where the characteristic peaks refer to the maximum rate of CoQ_2 oxidation (oxidation peak potential) and the maximum rate of reduction (reduction peak potential).



Cyclic voltammogram of coenzyme Q_2 (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

The Q-Module consists of the Q-Sensor (composed by the Q-Stopper with embedded electrodes plus a reference electrode), the electronics in the O2k – ideal for both cyclic voltammetry and measuring the Q redox ratio – and the DatLab software.

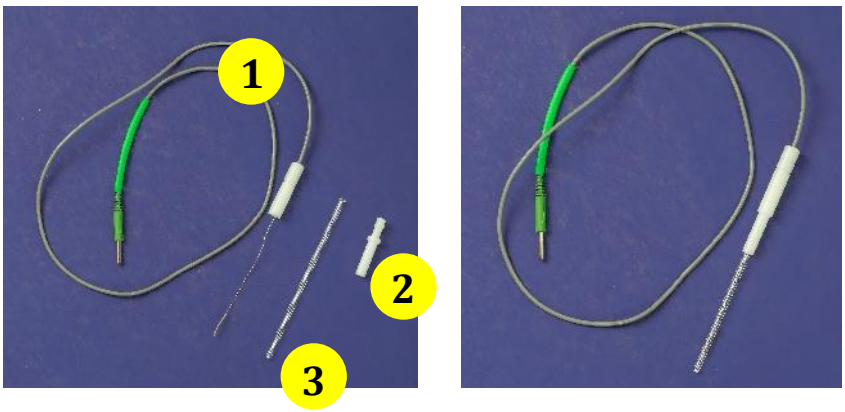
OroboQ -Service box contains:

- 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 2 needles
- 2× Polishing Powder 0.3 µm
- 2× Polishing Powder 0.05 µm
- 4× Polishing Cloth
- 3× Q-Filter Papers (10/Pkg)
- 2× box of 8 spare O-ring\ Viton® \ 12.5 × 1 mm for PVDF and PEEK stoppers
- MiPNet24.12NextGen-O2k: Q-Module
- MiPNet24.16 DatLab8.0:CV-Module

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

Before the reference electrode (Ag/AgCl electrode) can be put into operation, the glass reference barrel must be filled with the electrolyte solution supplied for the reference electrode (3 M KCl saturated with AgCl). To do this, the electrode will need to be taken apart.

Parts of the reference electrode:
(1) RE-Cable Connection, upper part of electrode housing, with cable and silver wire
(2) RE-Electrode Holder, lower part of electrode housing
(3) RE-Glass barrel



At the tip of the glass barrel there is a diaphragm made of a porous ceramic material (frit) produced with Vycor®. For further details on the maintenance of the Vycor frit, please see section 10.

Assembly:

1. 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).
2. 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 (0.2 inch) from top. Remove bubbles with the filling tube.
3. 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.

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 Vycor frit 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.

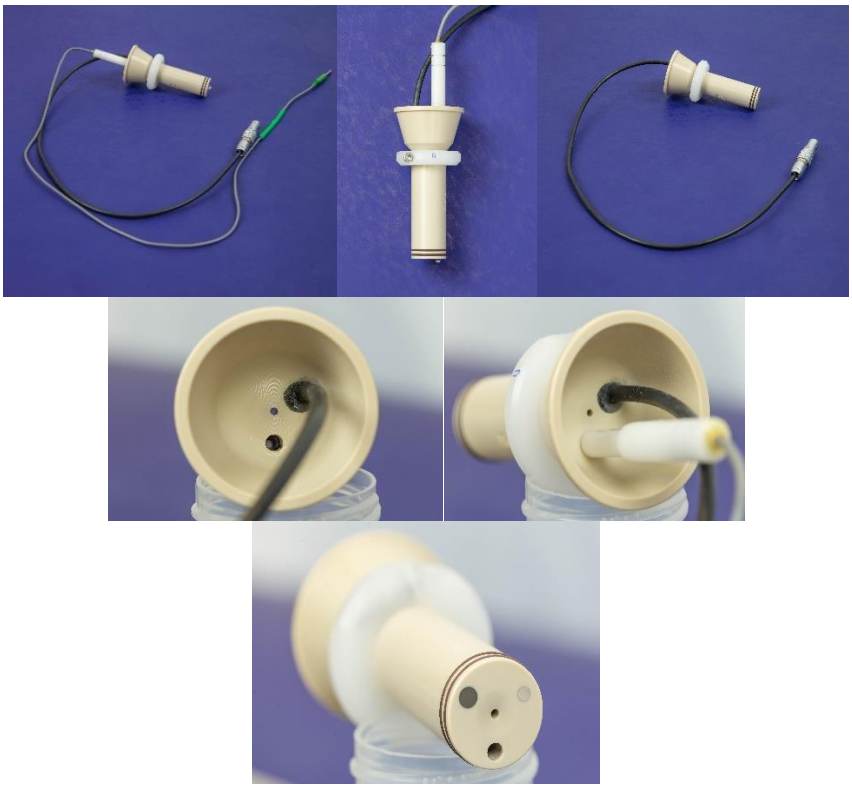
Storage: Always clean the electrode before storage.

- Short term: Place the tip of the electrode in a test tube or beaker containing 3 M KCl solution and protected from light. Falcon-type 15-mL vials are well suited, cover with aluminum foil to protect from light. 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 it from light.

2.2. Q-Sensor: cleaning and polishing

The Q-Stopper is designed to introduce the reference electrode through the stopper into the O2k-chamber. The end of the Q-Stopper is concave with one gas-escape/titration capillary in the middle and three electrode inlets. Two electrodes, the GCE 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 into the stopper.



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.

Polishing of glassy carbon and platinum electrodes:

The GCE and Pt electrode 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 (video).

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

1. Place the Petri dishes with the Polishing Cloth on a flat surface.
2. Using the tip of a spatula, add Polishing Powder 0.3 µm onto one of the polishing clothes. Add a few drops of distilled water.
3. Hold the sensor in a vertical position and polish the GCE and Pt electrode in the thin paste in a figure-eight motion 10-15 times.
4. 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.
5. Repeat steps 2 and 3 using Polishing powder 0.05 µm and the other Polishing Cloth (use always the same polishing cloth with the same polishing powder). Repeat step 4 (washing).
6. Finally, wash the polishing clothes with ultra-pure water and let them dry before storage.

Cleaning of the Q-Sensor before use:

1. After polishing the GCE and Pt electrodes (see above), rinse the reference electrode also with ultra-pure water and insert it into the Q-Stopper before an experiment (video).

Cleaning of the Q-Sensor after use of CV:

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

Cleaning of the Q-Sensor after a biological experiment:

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

Storage of the Q-Stopper:

Q-Stopper (GC and Pt are built in the regular Q-Stopper): Always clean the Q-Stopper before storage. For long storage, place it in a sealed box (OroboQ-Service box).

- 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 it from air.

2.3. Assembly of the Q-Sensor

1. Polish and wash the Q-Stopper before each experimental use (section 2.2).
2. Fill the glass barrel of the reference electrode with 3 M KCl solution saturated with AgCl and assemble the reference electrode (MiPNet15.03 and video).
3. 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.
4. Add respiration medium into the O2k-chamber, insert fully the Q-Sensor (Q-Stopper with the inserted reference electrode) into the O2k-chamber.
5. Connect the cable of the Q-Sensor to the Q port and the cable of the reference electrode to the Q-Ref port.
6. Connect DatLab to the instrument and start your experiment.
7. Run CV to determine the oxidation peak potential for GCE (see section 3) if it is needed.

3. Quality control: Cyclic voltammetry

1. Polish the GCE and Pt electrode (section 2.2) before each experimental use.
2. Clean the Q-Stopper after polishing with ultra-pure water.
3. Clean the O2k-chambers before experimental use (MiPNet19.03_O2k-cleaning and ISS).
4. 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).
5. Open DatLab 8.0-CV-Module to perform cyclic voltammetry (MiPNet24.16 DatLab 8.0 CV Manual).
6. First, run a background CV without adding CoQ_2. No peaks should appear on the cyclic voltammogram.
7. After finishing that background CV, add 30 µM CoQ_2 and start again CV.
8. Save your CV file and start cleaning the O2k-chambers, Q-Stoppers, and reference electrodes (section 2.2).
9. Clean the O2k-chamber by rinsing five times with ultra-pure, then soak for 10 min in $EtOH_{abs}$ and rinse five times with ultra-pure water again.
10. Before the biological experimental run, polish the GCE and Pt electrode again.

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 for calibrating the O2k-chamber volume, comparable to volume-calibration with standard stoppers (MiPNet19.18A O2k-start and video).

1. 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 for the injection capillary and ~0.04 mL for the inlet of the reference electrode. Therefore, the volume to calibrate a chamber volume of 2 mL with the Q-Sensor is 2.1 mL.
2. 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 the shaft with a paper towel (do not use a paper towel directly on the Vycor frit of the reference electrode). Insert the electrode into the Q-Stopper.
3. Place the stopper on top of the chamber with the 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.
4. Fix the position of the volume calibration ring by tightening the screw to finalize the volume calibration.

4.2. Instrumental background flux

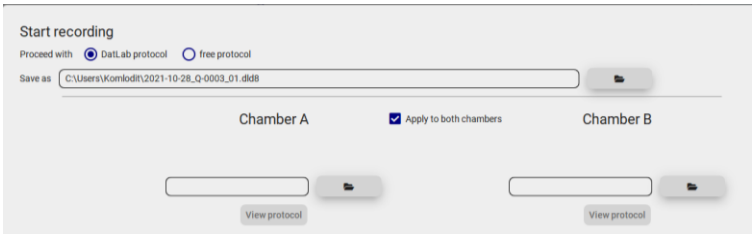
Instrumental oxygen background parameters are used to correct real-time oxygen flux (MiPNet14.06 Instrumental O2 background). The instrumental background tests must be carried out with the Q-Sensor and reference electrode in place. The values obtained with the standard stoppers for respirometry cannot 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 8

The Q-Module is operated via DatLab 8 (DL).

1. Plug in the two cables of the Q-Sensor into the Q and Q-Ref labelled ports on the O2k-Main Unit.
2. Switch on the O2k and open DL. Enter the username and click Login. Start the connection process by clicking on Connect and start measurement. The `Start recording´ window pops up with two options to proceed:

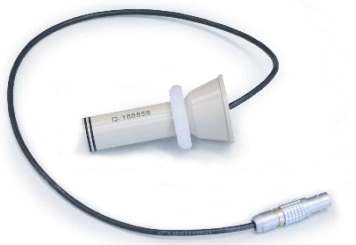
   a. DatLab protocol: If this box is checked, and a DLP for Q measurement is selected, it will be automatically set to record O_2 and Q. Select the protocol clicking on the folder button below "Chamber A" and "Chamber B" and select a protocol for each chamber or the same for both.



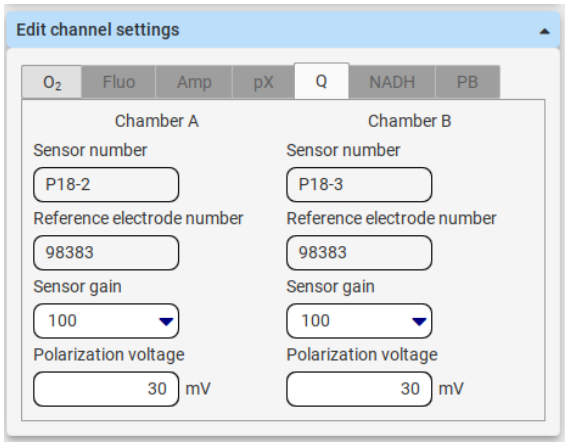
b. Free protocol: If this box is checked, a list of channels will appear below "Chamber A" and "Chamber B". Select the box for Q. Adjust the other parameters (temperature, data recording interval, illumination in the chamber, stirrer speed) if needed.

3. Click on the folder button after "Save as" to select a different folder to save the file, or to change the file name. If this is not done, the files will be saved under the "Users" folder.
4. Click on Start recording.

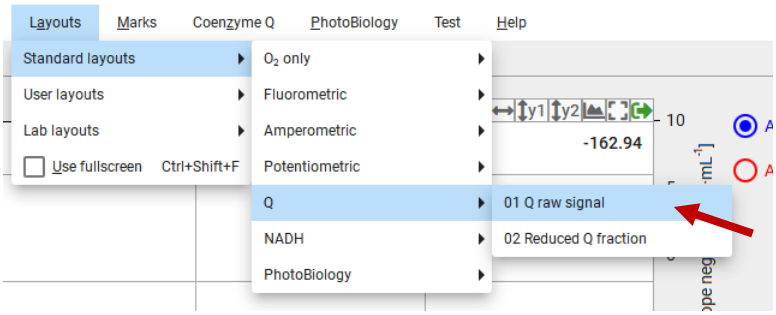
In the right-side menu under the `Edit channel settings´ menu, open the Q tab to edit the settings. The Q-Stopper `sensor number´ is automatically recognized by the instrument and appears here. The serial number of the reference electrode can be found on its cable and must be manually entered.



If cyclic voltammetry (CV) was run prior to the experiment, DL asks whether the Polarization voltage should be set to the oxidation peak potential obtained during CV, in mV. The Sensor gain must be set before starting the measurement as 100 if no DL protocol was used. If CV was not run, set these values manually, e.g., Polarization voltage as 30 mV and Sensor gain as 100 before starting the measurement; press enter after typing in the values. It is strongly recommended to run CV and use its values for the experimental run.
The current detected, raw Q-signal, is multiplied by the gain, *i.e.* at gain 100 the raw Q-signal is expressed as [µA/100].



5. Select Layouts\Standard layouts\Q\01 Q raw signal in the menu to see the O2 channel and Q channel simultaneously.



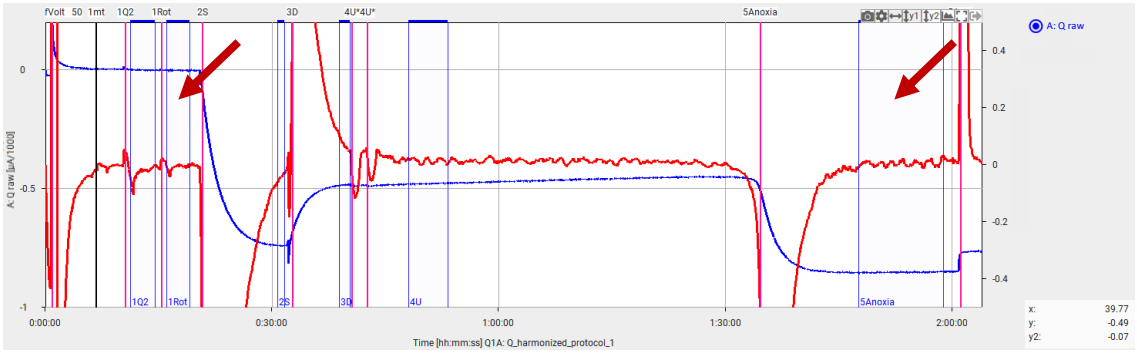
For more instructions on the use of DatLab 8, see:
» MiPNet26.13_NextGen-O2k_manual

6. Data analysis

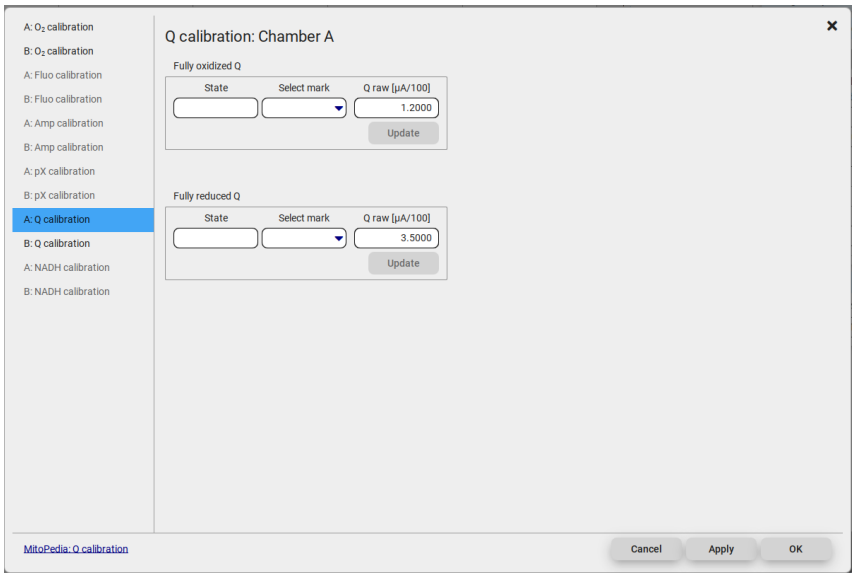
6.1. Q-signal analysis

The calculations of the reduced Q fraction can be performed with DatLab 8 and are provided in this MiPNet, under topic 6.3, complying with Oroboros transparency policy.

1. In DatLab 8, set the marks to the raw Q-signal (Y1 axis) when the signal is stable. Y2 axis, Q slope, is not meaningful for data analysis, do not set the marks to this trace.
2. The marks on the fully reduced and fully oxidized Q state as defined in the protocol are used to calibrate the signal (in the example below, marked with red arrows).

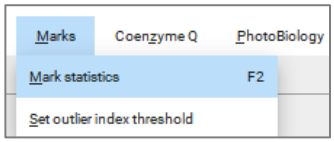


To calibrate, click Calibration in the upper menu and then Open, or press F5. Select A: or B: Q calibration. Click on the arrows ▼ to select the marks for fully oxidized Q and fully reduced Q; click Update after selecting each mark. When both marks are selected adequately, click Apply.

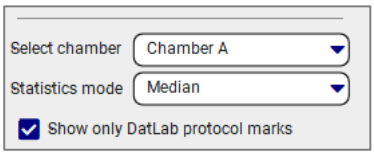


3. After Q calibration change the layout by clicking on Layout/Standard layouts/Q/02 Reduced Q fraction to see the reduced Q fractions.

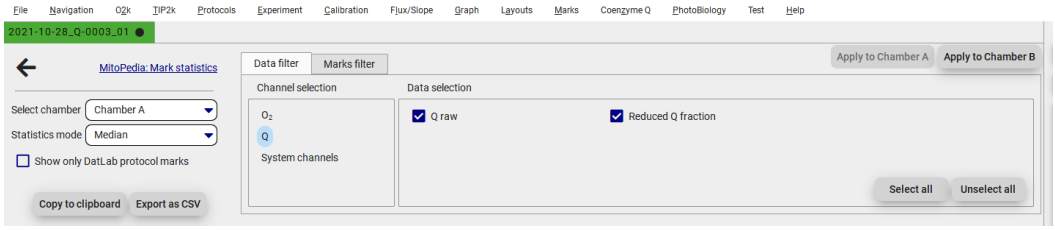
4. Click on Marks in the upper menu, then Mark statistics, or press F2.



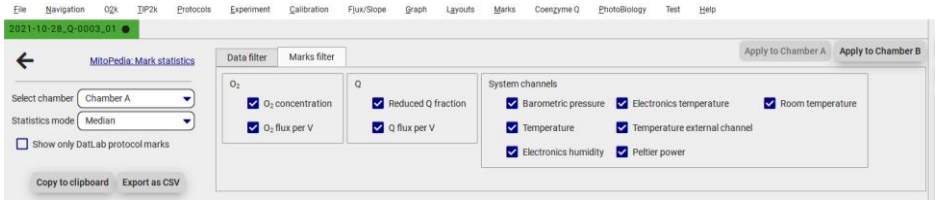
5. Select the chamber A or B in the menu on the left side. Median is selected in the Statistics mode for every mark by default (as recommended), however, it is possible to change this parameter.
If a DatLab protocol was used, leave the option "Show only DatLab protocol marks" selected, otherwise, unselect this option to see the marks.



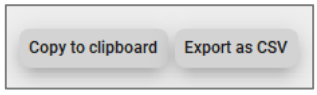
6. In the Mark statistics window, in the Data filter tab, select Q under Channel selection, and under Data selection, select the normalizations that are needed for your data analysis. The option Apply to chamber A/B allows the user to apply the same settings to the other chamber.



7. In the Marks filter tab, select the marks at Q-signal, **Q**. The option Apply to chamber A/B allows the same settings to be applied to the other chamber.



8. With the options Copy to clipboard and Export as CSV, it is possible to, respectively, copy the data and paste in a spreadsheet program, or save as a .csv format. In the latter case, it is recommended to open the file with a program such as LibreOffice and save as .xls before opening the file in the program Excel.

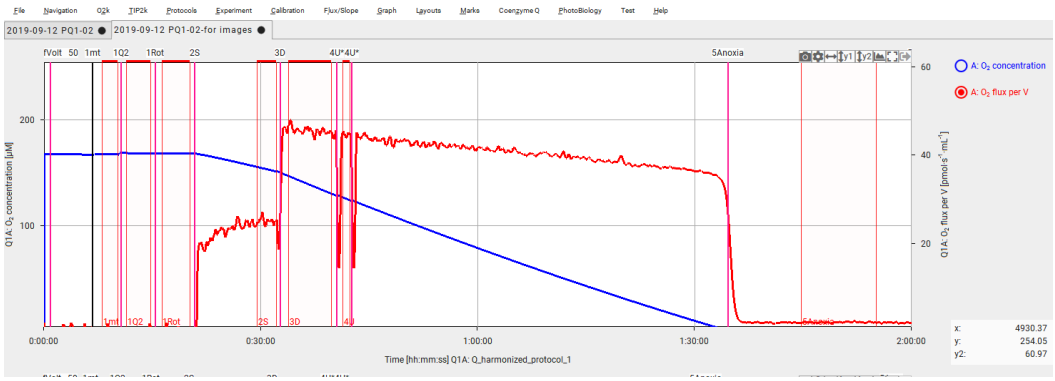


6.2. Oxygen flux analysis

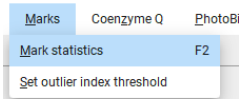
The calculations of the O_2 fluxes can be performed with DatLab 8 and are provided under the following link complying with Oroboros transparency policy:

» https://wiki.oroboros.at/index.php/Flux_/_Slope#O2

1. Set the marks to the O_2 flux (Y2 axis). Always select the marks when the flux is stable. Y1 axis, O_2 concentration, is not used for data analysis, do not set the marks to this trace.



2. Click on Marks in the upper menu, then Mark statistics, or press F2.



3. In the Mark statistics window, select O2 under Channel selection, and under Data selection, select the normalizations that are needed for your data analysis. Follow the same steps as for Q analysis.

6.3. Calculation

The reduced Q fraction (x_{Qred}) is expressed as the mole fraction of reduced Q in each coupling and pathway control state in a SUIT protocol [5]. To calculate x_{Qred}, the raw Q-signal (I_{raw}) is normalized for the fully oxidized CoQ_2 signal (I_{ox}) and fully reduced CoQ_2 signal (I_{red}). x_{Qred} is a fraction of I_{red}.

Reduced Q fraction is calculated as follows:

$$x_{Qred} = (I_{raw}-I_{ox})/(I_{red}-I_{ox})$$

The fully oxidized CoQ_2 signal is measured in the presence of mitochondria, and the CoQ_2 but in the absence of ADP and respiratory fuel substrates. Mitochondrial preparations may contain endogenous substrates which can slightly reduce CoQ_2. Therefore, the CI inhibitor rotenone might be needed to inhibit respiration with endogenous substrates. For some preparations, the use of rotenone is not necessary, to be determined experimentally.

To quantify the amount of reduced or oxidized Q is beyond the possibilities of our instrument. Mass spectrometry or HPLC (High Performance Liquid Chromatography) are required to determine the absolute amount of the oxidized and reduced CoQ.

7. Demo experiment

7.1. 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 (MiPNet 22.10 MiR05-Kit) was used.
- Coenzyme Q_2 (CoQ_2): MW: 318.41 mg (Sigma Aldrich: C8081), dissolved in $EtOH_{abs}$. Stock solutions of 10 mM and 1 mM were prepared, respectively, for cyclic voltammetry and for analysis of the raw Q signal with isolated mitochondria. For further details:
  » https://www.bioblast.at/index.php/Coenzyme_Q2.
- Cyclic voltammetry: 30 µM CoQ_2 (6 µL of 10 mM solution in each O2k-chamber), MiR05. For set up see Section 3 and for DatLab8.0 CV instructions see MiPNet24.16_DatLab8.0_CV Manual.

7.2. Experimental setup

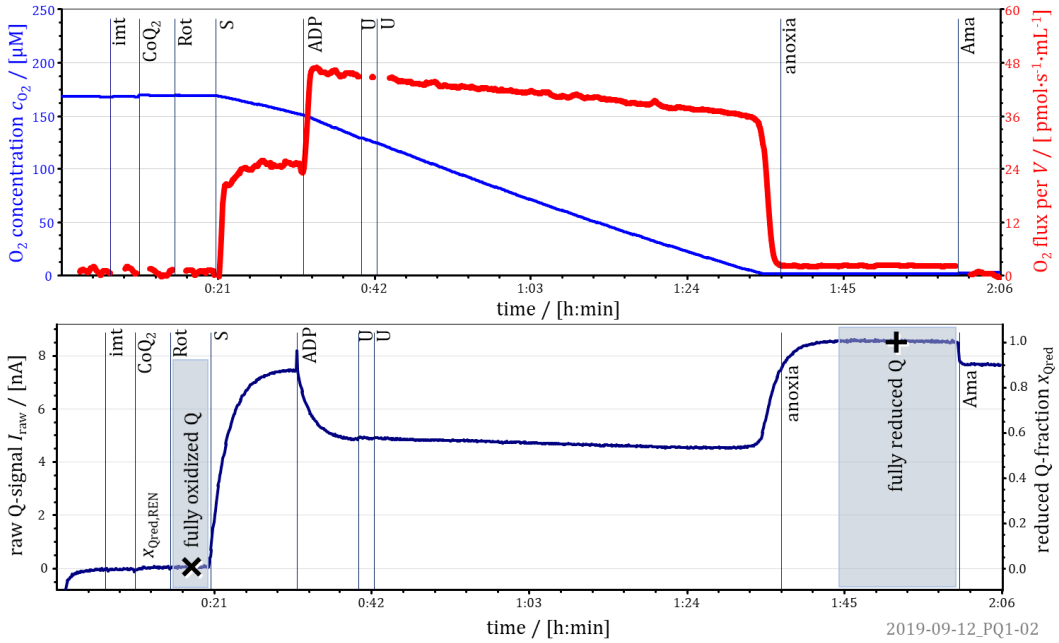
- Polarization voltage: dependent on the oxidation peak potential of CoQ_2 with the given Q-Sensor in the given O2k-chamber (Section 3 for CV).
- Gain: 100
- DL-Protocol: SUIT-006 Q mt D071

Abbreviation list and concentrations used:
Mt mitochondria
CoQ_2 coenzyme Q_2, 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, 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 CoQ_2 (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 CoQ_2. 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 CoQ_2 and therefore, it would overestimate the fully oxidized CoQ_2. Next, a saturating concentration of ADP was added to initiate oxidative phosphorylation (OXPHOS) which was reflected in the oxidation of CoQ_2. 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 CoQ_2 and taken as 1 for the calculations of the reduced Q fractions. (As a control of the fully reduced CoQ_2, 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 CoQ_2). The fully oxidized CoQ_2 can be observed in the presence of mitochondria, CoQ_2 and Rot but absence of respiratory substrate and ADP and taken as 0 to calculate the reduced Q fractions.

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.



O_2 flux and Q redox. Simultaneous measurement of O_2 flux and raw Q-signal using mitochondria isolated from mouse heart. The experiments were carried out in MiR05, 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: 2019-09-12 PQ1-02.DLD. Figure from Komlodi et al, 2021.

8. Troubleshooting

8.1. Artificial signals

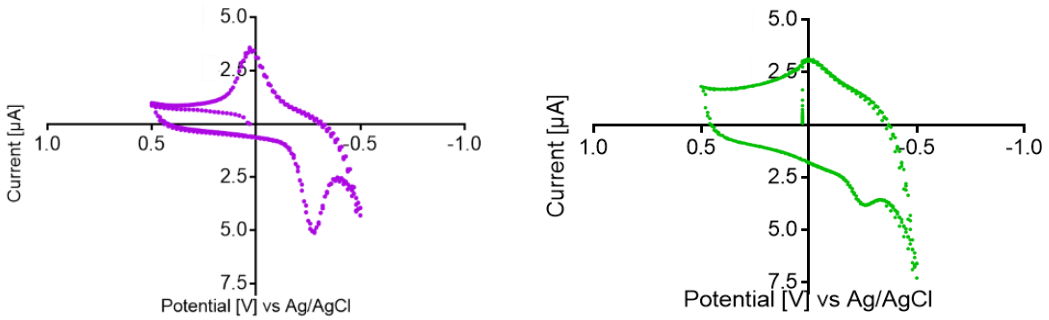
There are chemicals which influence the raw Q-signal; therefore, they cannot be used with the Q-Module. The following chemicals interfere with the Q-signal: ascorbate, TMPD (Tetramethyl-p-phenylenediamine dihydrochloride), azide, dithionite, cytochrome *c*, KCN (potassium cyanide) and NADH. A chemical 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.

8.2. 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:

1. Polishing GC and Pt electrodes with alumina powder (0.5 µm and 0.03 µm).
2. Cleaning the Q-Sensor, reference electrode and O2k-chamber with ultra-pure water, 70 % ethanol and $EtOH_{abs}$ (see, 2.2).
3. Filling up the glass barrel of the reference electrode with new 3 M KCl solution.
4. Checking the quality of Vycor frit of the glass barrel.



Typical and unacceptable voltammograms: Cyclic voltammogram in the presence of coenzyme Q_2 (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, polarization window: between -500 mV and +500 mV, scanning speed:100 mV/s, gain: 1; 30 µM CoQ_2 was used for each test. The left figure shows a typical cyclic voltammogram while the right figure shows an unacceptable one.

8.3. Drifting of Q-signal with and without CoQ_2 and biological sample

If the CV trace or oxidation peak potential is not acceptable, it might lead to an unstable or noisy raw Q-signal in the absence or presence of CoQ_2, which leads to artefacts when calculating the reduced Q fraction or in the worst-case scenario, the reduced Q fraction 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.

8.4. Damaged surface of glassy carbon

Under normal circumstances, the surface of GCE 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.

8.5. Respiration

The simultaneous measurement of respiration with redox state of mtCoQ is one of the biggest advantages of the NextGen-O2k. The inhibitory effect of CoQ_2 mimetic on respiration should also be tested compared to a chamber where no CoQ_2 was titrated (https://bioblast.at/index.php/Carrier_control_titrations).

8.6. 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.

9. Supplementary

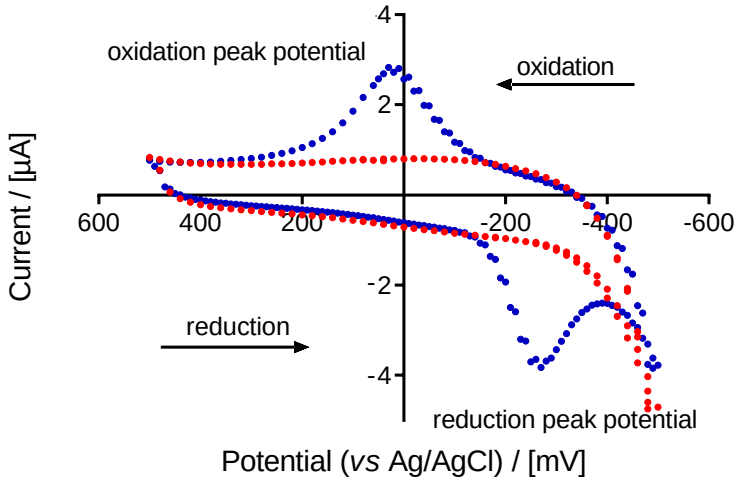
9.1. Measurement parameters in cyclic voltammetry

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 mitochondrial respiration medium MiR05; 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 CV.
- Initial polarization voltage is the potential where the scanning starts. It must be close to the peak potential to avoid a coating of GCE which would lead to side reactions [2]. In the case of CoQ_2, +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 GCE [2]. Any type of modification of GCE will inhibit the electron transfer on the surface of the electrode. In the case of CoQ_2 -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 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 (CoQ_2). If the scanning rate is very slow, there is a risk that CoQ_2 is transported to and from the electrode surface via migration and convection rather 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 GCE as a result of the changing electrode potential and results in high baseline current that obscure features in a CV [2]. To avoid these side reactions and provide free diffusion for CoQ_2, 100 mV/s was applied as a scanning speed.
- Gain: Amplification of the raw Q-signal which is expressed in µA. In the O2k the gain, $F_{Q,G}$, can be selected in DatLab 8 within the `O2k control´ menu, with values 1, 10, 100 or 1000 to amplify the raw current. At a gain of 100, the raw Q-signal is expressed in µA/100 which is equivalent to 10 nA.
- 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 GCE (Peter R Rich, personal communication).
- Final concentration of CoQ_2: The lowest possible concentration of CoQ_2 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. Concentrations lower than 30 µM of CoQ_2 did not give us detectable peaks in

the CV at gain of 1 V/µA, while using higher than ~ 90 µM of CoQ_2 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 ETS-reactive CoQ 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 CoQ_2 or any other chemicals. If any peaks appear in the current, further polishing of GCE or cleaning of the O2k-chamber and Q-Stopper and reference electrode are required.



Coenzyme Q_2: Cyclic voltammogram in the absence and presence of CoQ_2 (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 CoQ_2, while red dots show the control or background CV, without CoQ_2.

10. 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.

1. Take two reference electrodes dipped in a glass beaker filled with 3 M KCl solution.
2. Connect these two electrodes to the same voltammeter and measure the electric potential between them in the KCl solution.
3. 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.

11. References

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12. Author Contributions

Komlódi T and Gnaiger E were responsible for the project development. Komlódi T, and Cardoso LHD performed related experiments and tested the software. Gnaiger E, Gradl P, Komlódi T, Gollner M, Merth A, Schwaninger H and Walter-Vracevic M were responsible for instrumental development. Haider M and Niedenzu W developed the software. Komlódi T, Cardoso LHD, 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. Iglesias-Gonzalez J and Hunger M as former members of Oroboros Instruments contributed to this MiPNet.

13. Acknowledgements

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