O2k – Procedures
H_2O_2 flux analysis DL7.4

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Hydrogen peroxide flux analysis using Amplex UltraRed assay in MiR05-Kit with DatLab 7.4

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1. General Information

Substrate-uncoupler-inhibitor-titration (SUIT) protocols are designed to study respiratory control in a sequence of coupling and pathway control states induced by multiple titrations within a single experimental assay. DatLab 7.4 has been specifically designed to guide the user through SUIT protocols (DL-Protocols in DatLab). Excel templates are provided for data analysis of O_2 flux and hydrogen peroxide (H_2O_2) flux using Amplex UltraRed assay in MiR05-Kit for isolated mitochondria, tissue homogenate (except of liver homogenate), permeabilized cells and living cells. Each DL-Protocol is defined with a unique D-number (D###), for a detailed list see:

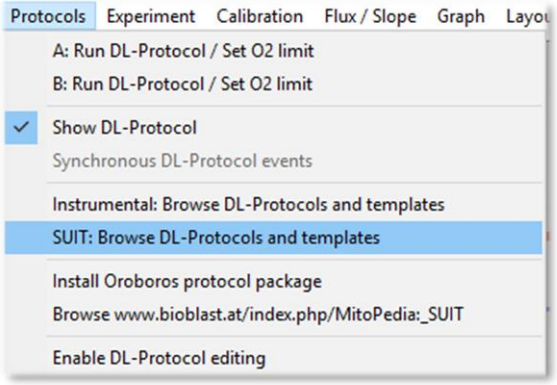
»https://www.bioblast.at/index.php/SUIT_protocol_library#List_of_SUIT_protocols_with_D-numbers

Use the SUITbrowser to find the best SUIT protocol for your research questions:

»https://suitbrowser.oroboros.at/

2. Data analysis -getting started

Upon completion of real time respirometry measurement in DatLab 7.4 or anytime while performing the DatLab analysis, open our Excel template to analyze your data in a time-efficient way.



1. In DatLab 7.4, select the menu Protocols and click on SUIT:Browse DL-Protocols and templates.
2. Select your SUIT protocol, open SUIT-###_AmR folder. Inside this folder, you will find another folder for the specific DL-Protocol (named SUIT-###_AmR_mt/ce/ce-pce_D###). In each folder, two Excel files can be found:
   a. A blank template (named SUIT-###_AmR_mt/ce/ce-pce_D###.xlsx)
   b. A demo version of the template (named SUIT-###_AmR_mt/ce/ce-pce_D###_demo.xlsx), which provides an example of the file already with data.
3. Create a copy of the SUIT-###_AmR_mt/ce/ce-pce_D### analysis template for your data analysis and rename it. You can rename the template by opening it and choosing the option `Save as´ in the archive top menu.

3. Data analysis

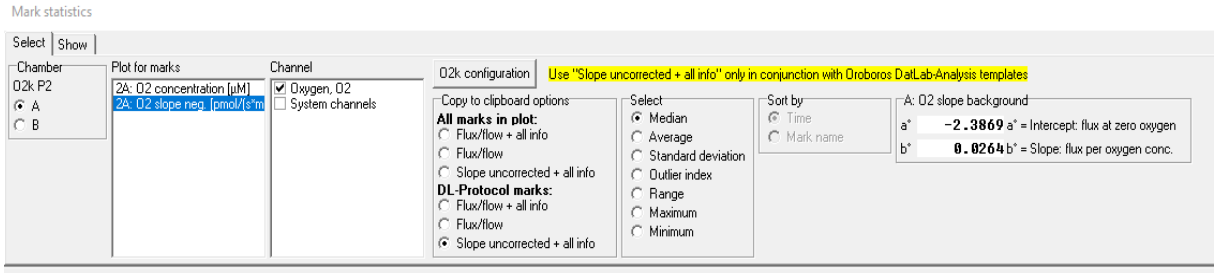
In the excel template you can select the setting by ticking the boxes `Titration volume correction´ and `Known sample concentration´. More information can be found here: MiPNet24.06 Oxygen flux analysis with DatLab7.4 (See section 2).

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

1. In DatLab 7.4, after setting the marks separately to the O_2 flux and H_2O_2 flux, go to Marks (F2), and select Slope uncorrected+all info. In the new window select:
   a. Your chamber of interest.
   b. Plot for Marks: O2 slope neg. [pmol/s*mL].
   c. Channel: Oxygen, O2. Leave only this channel selected.
   d. Select: Median.
   e. Sort by: Time (default).
   f. Then, click on Copy to clipboard to copy the selected values.



2. In the Excel template: Click on the yellow cell L4 and paste these selected values (only O_2) from DatLab [Ctrl+V].
3. The calculated values for the *FCR*, *FCR* (bc), specific O_2 flux and specific O_2 flux (bc) on each step of the protocol can be found in the rows 19 to 22, starting at column V.
4. Paste the DatLab graphs showing the traces for the chamber after autoscaling the time axis (´Graph\Autoscale time axis´):
   a. In DatLab: Select the graph (left mouse click into the respirometry graph of interest) → select Graph\Copy to Clipboard\WMF.
   b. In the Excel template: Click on the yellow cell A6: `Paste DatLab graph here, reduce to width 22 cm (8 inches)´ → press [Ctrl+V] to paste.
   c. Select the graph (right click on the graph) → select Size and properties and set the width of the graph to 22 cm (8 inches).

3.2. Hydrogen peroxide flux analysis

The calculations of the H_2O_2 fluxes are provided under the following link complying with Oroboros transparency policy:
»https://bioblast.at/index.php/Flux_/_Slope#How_to_Analyse_with_DatLab_7.4_2

1. For mark setting, see MiPNet20.14 AmplexRed H2O2-production.
   In DatLab 7.4, set the marks separately to the O2 flux and H2O2 flux (green line):
   a. Go to Layout menu and click into O2&Amp and select Standard Layouts/01 Amp Amperometric_Raw signal.
   b. Go to Marks and select Slope uncorrected + all info. In the new window select H2O2 slope [mV/s] in Plot for Marks.
   c. Channel: Amperometric,Amp. Leave only this channel selected.
   d. Select: Median.
   e. Sort by: Time (default).
   f. Then, click on Copy to clipboard to copy the selected values.
2. In the Excel template: Click on the yellow cell L41 and paste [Ctrl+V] Amp slope from the DatLab.
3. Copy the sensitivity values (Amp calib.) from the DatLab: go to Calibration\Amperometric, Amp to open Amp calibration window (be sure that you select the one for your trace of interest, i.e., A or B).
   a. Select marks (1)0.0 and (1)0.1 and copy the sensitivity value [Ctrl+C] form the box and paste [Ctrl+V] in the excel template in cell V36.
   b. Continue for each pair of H_2O_2 calibration and paste in the yellow boxes (at the right of cell V36) to the corresponding titration steps. In the empty yellow boxes use the previous sensitivity values.

c. The value for sensitivity "before sample" is taken from the previous calibration file recorded in the absence of any sample. Open this calibration file and in the Amp calibration window select (0)0.0, (0)0.1 and (0)0.2 to calculate the sensitivity value. Then, copy the value in the excel template into cell U36. This box cannot be left empty, otherwise the H_2O_2 values cannot be calculated. An example of a calibration file can be found here: in DatLab, open in the Protocols menu: Instrumental: Browse DL-Protocols and templates and open the provided demo file in the AmR calibration folder.

4. Copy the Amp graph as it is explained in the 3.1.4 and paste on the yellow cell A30.
5. Specific H_2O_2 flux can be found in row 30 and specific flux H_2O_2 (bc) in row 31, both starting at column V.

4. Calculation of the background fluorescence slope

The Excel template is ideal for analysis of O_2 and H_2O_2 fluxes measured in MiR05-Kit supplemented with DTPA, as it corrects for MiR05-Kit's specific background fluorescence slope. The background fluorescence slope of the AmR assay varies depending on the MiR05-Kit lot. The Excel (.xlsx) files available on the webpage have different sheets, each with the correction for the background fluorescence slope specific to the lot number. You need to use the Excel template sheet which corresponds with the lot number of the MiR05-Kit you use:

»https://wiki.oroboros.at/index.php/Amplex_UltraRed#H2O2_flux_analysis_and_mark_setting

If you do not add DTPA or use homemade MiR05 instead of the MiR05-Kit, we recommend calculating the background fluorescence slope. The calculation of the background fluorescence slope is also advisable when a new batch of Amplex UltraRed is used or a newly prepared homemade MiR05 is applied. The SUIT protocol (AmR background fluorescence slope.DLP) and the excel analysis template (AmR background fluorescence slope.xlsx, for a demo version, see: AmR background fluorescence slope demo.xlsx) with detailed instructions are available on the following page:

»https://wiki.oroboros.at/index.php/Amplex_UltraRed#Calculation_of_background_fluorescence_slope_of_MiR05

5. Further reading

» MitoPedia: Measurement of hydrogen peroxide
» Flux / Slope O2
» Flux / Slope H2O2

6. References

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7. Acknowledgements

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