Mitochondrial Physiology Network 12.09: 1-16 (2012)
O2k-Core Manual E. Oxygraph-2k Series E-F, DatLab 5.1
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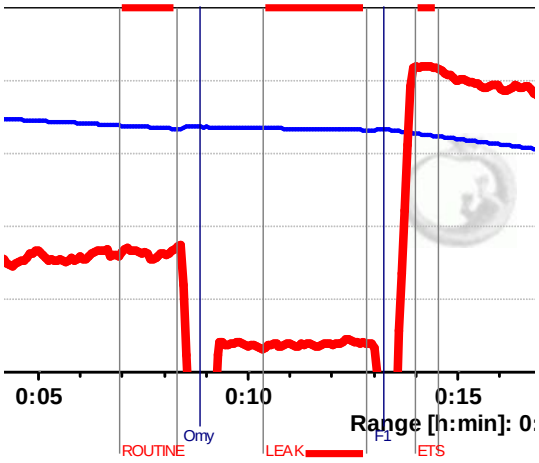
Version 5: 2012-10-24

Oxygen Flux Analysis: DatLab On-Line



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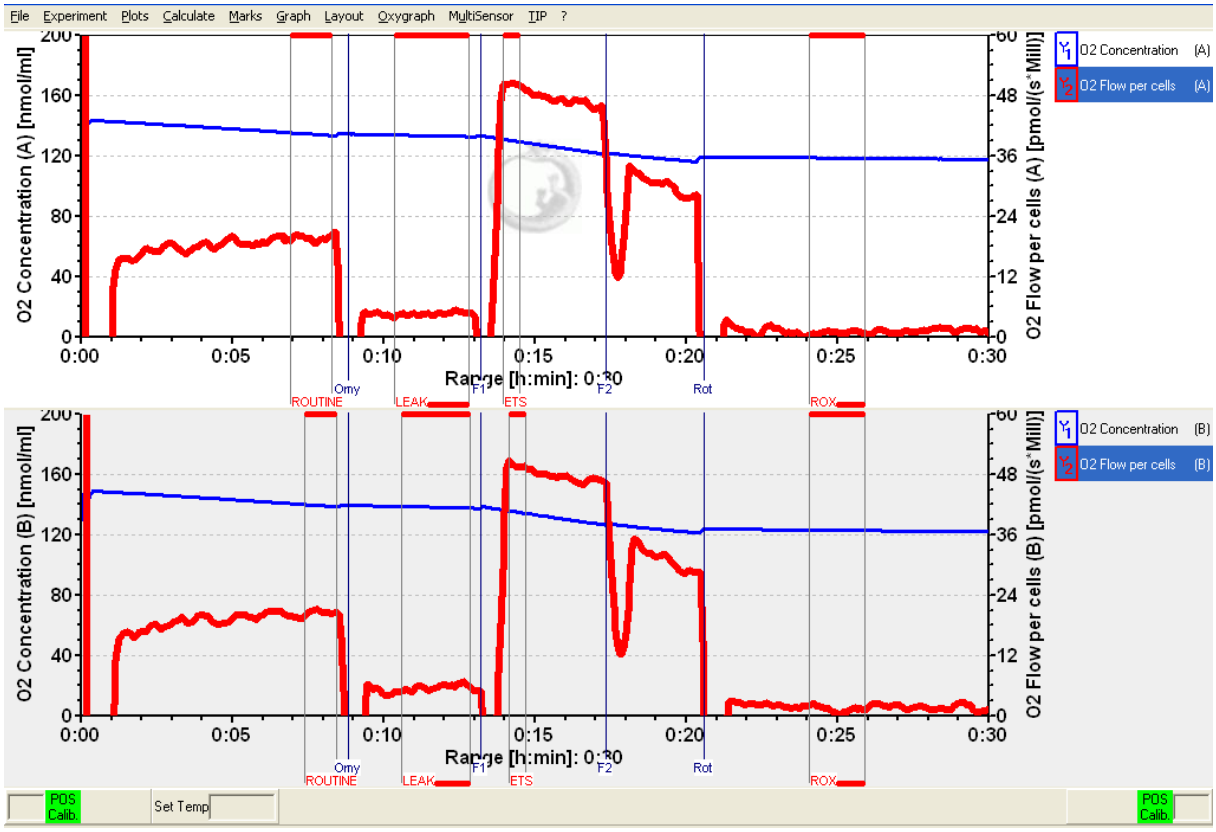
Overview: DatLab sets a novel standard in high-resolution respirometry for on-line analysis of oxygen flux measured in the OROBOROS Oxygraph-2k, and of other signals obtained in the O2k-MultiSensor System. Oxygen flux can be plotted with or without O2k-Background correction and is instantaneously expressed per mass of sample or per number of cells. Various sections on the plot of oxygen flux are marked, and corresponding average values are viewed in a table which can be simply exported into Excel or SigmaPlot. Instrumental and experimental parameters are summarized in a protocol which can be printed or saved as a pdf file. These features provide the basis for combining high-resolution with instant and user-friendly analysis.

A demonstration experiment, performed during an Oxygraph-2k Workshop on high-resolution respirometry, is used as an example for application of DatLab and DatLab-Excel templates (MiPNet08.09). All analyses can be performed on-line or off-line.

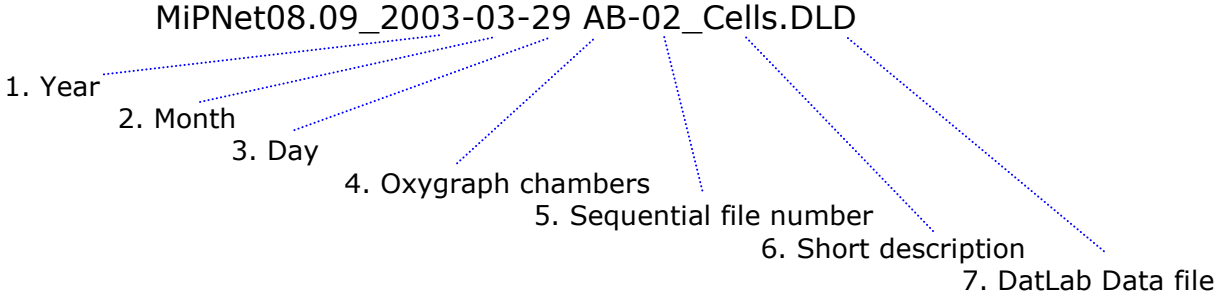
1. Oxygen Flux of a Biological Sample

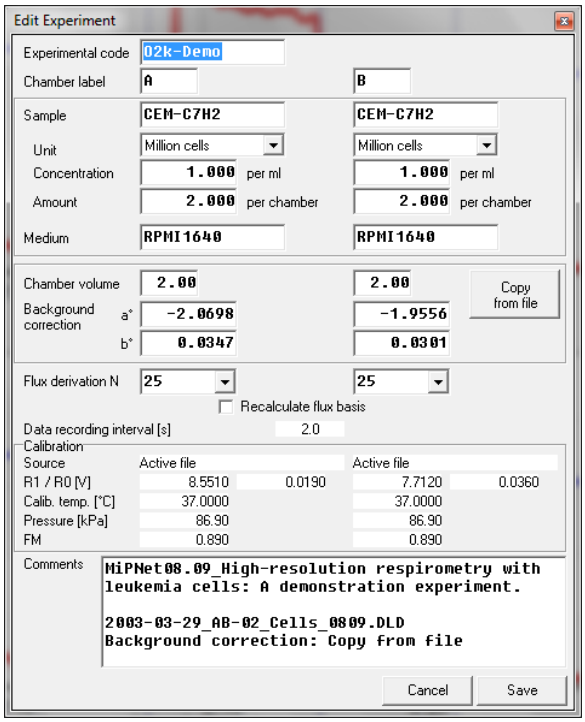
1.1. O2k-Demo file: MiPNet0809_2003-03-29 AB-02_Cells.DLD

File: MiPNet08.09_2003-03-29 AB-02_Cells.DLD
OROBOROS FileFinder: O2k-Protocols \ line MiPNet08.09 -> scroll to the right for the hyperlink.
You may save the DLD file on your PC under the subdirectory "\DatLab\DLDemo\".



MiPNet08.09_2003-03-29 AB-02_Cells.DLD O_2 concentration (blue, Y_1 axis) and O_2 flux (red, Y_2 axis) as a function of time. Data recording was started (Connect [F7]) after adding a cell suspension at a density of $1 \cdot 10^6$ cells/ml. Events are shown by vertical lines, with the <Event name> below. Marks are shown by horizontal bars between two vertical lines, with the Mark name in the lower bar.





The following steps of analysis can be performed on-line or off-line.

Edit Experiment [F3]: Select the Sample Unit Million cells ▼. Enter the cell density [10^6 cells per ml]. The amount of cells in the chamber is then shown below, depending on the chamber volume (2,00 ml).

Calibrate [F5]: See (MiPNet12.08). The calibration from the previously saved file is available as a default, but the oxygen solubility factor, F_M, is changed to 0.89 for culture medium (RPMI).

View Protocol: Press [Ctrl+F3] and Preview. The protocol has been saved as a pdf file MiPNet08.09_2003-03-29.AB-02_Cells.pdf

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high-resolution respirometry
Oxygraph-2k

DatLab 4 File protocol

File name:	2003-03-29 AB-02_Cells22A1.DLD	
Path:	C:\DatLab\DLData\DLDemo\2.2.A-Cell-Respiration\	
Experimental code:	O2k-Demo	
Chamber label:	(A)	(B)
Sample:	CEM-C7H2	CEM-C7H2
Sample concentration:	1.00 Mill. cells/ml	1.00 Mill. cells/ml
Medium:	RPMI1640	RPMI1640
Chamber volume [ml]:	2.00	2.00
Background corr. a*:	-2.0723	-1.9513
b*:	0.0347	0.0301
Calibration Source:	Active file	Active file
Sensor #:	6001	6002
R1 [V]:	8.5510	7.7120
R0 [V]:	0.0190	0.0360
Block temp. [°C]:	37.0000	37.0000
Barom. pressure [kPa]:	86.90	86.90
FM:	0.890	0.890
Set block temperature [°C]:	37.0	
Gain for oxygen sensor:	4	4
Polarization voltage [mV]:	800	800
Stirrer speed [rpm]:	750	750
Data recording interval [s]:	2.0	

Comments:
2003-03-29_AB-02_Cells22A1.DLD
Demo experiment, Oxygraph-2k Course, March 2003, Schroecken, Austria.
2.2.A1. High-resolution respirometry with leukemia cells: A demonstration experiment.
Mitochondr. Physiol. Network 8.9 (2003-2009)

Filter: Default / Chamber: both

offline Calibration O2: Active file
Calibration A R1: 8.5510 Temp: 37.0000 p(b): 86.9000

Graph layout: 05 Flux per Volume corrected▼. This layout provides a plot of volume-specific respiratory oxygen flux, which is most relevant to evaluate experimental details, for instance the flux measured in relation to the sensitivity of the instrument (1 pmol·s^{-1}·cm^{-3}; MiPNet06.05). This plot is also chosen, when measurements of sample concentration are available at a later stage only (in DatLab, press [F6] and Info for further information).

1.2. Flux per Mass or Flow per Cell

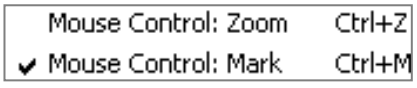
Expressions of oxygen flux (corrected for O2k-Background):

Volume-specific flux	J_{O_2}	[pmol·s^{-1}·cm^{-3}]: The experimental flux per unit of chamber volume is the basis for expressing respiration in a variety of units.
Flow	I_{O_2}	[pmol·s^{-1}·10^{-6} cells]: A system-specific quantity, in contrast to the size-specific quantities.
Mass-specific flux	J_{O_2}	[pmol·s^{-1}·mg^{-1}]
Flux Control Ratio	FCR	Normalized flux, dimensionless, relative rate.

Graph layout: 06 Specific Flux per Unit Sample▼ is used for plotting respiration per unit sample (Y_2 axis), in units defined in the [F3] Window (oxygen flow per million cells, flux per biomass or protein [mg/ml]). Respiratory flux per chamber volume is converted to an extensive quantity (flow; per cell) or a size-specific quantity (flux; per mg cell protein or mass). Flow or fluxes are always corrected for instrumental background, using the parameters entered in the window Edit Experiment [F3]. Press [F6] and Info for information.

1.3. Marks on Flux

Select Y_2 as the active plot (Example: O2 Flow per cells). Set marks for calculating average respiration at relevant metabolic states.

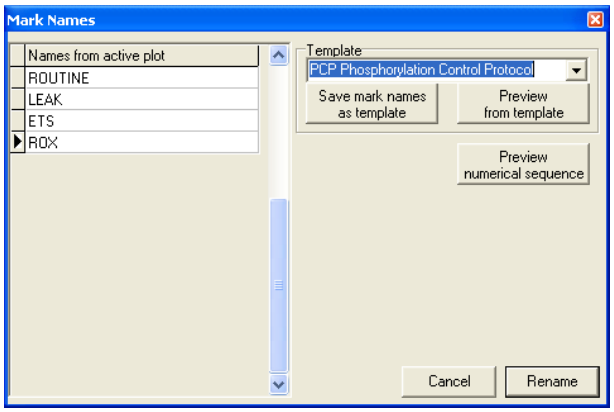


Marks: In the Graph pulldown menu, Mouse Control: Mark must be selected.

To set a mark, hold [Shift], click into the graph and drag the cursor with the left mouse button along the time axis. Sequential numbers are automatic default mark names. To delete or reduce a marked section,

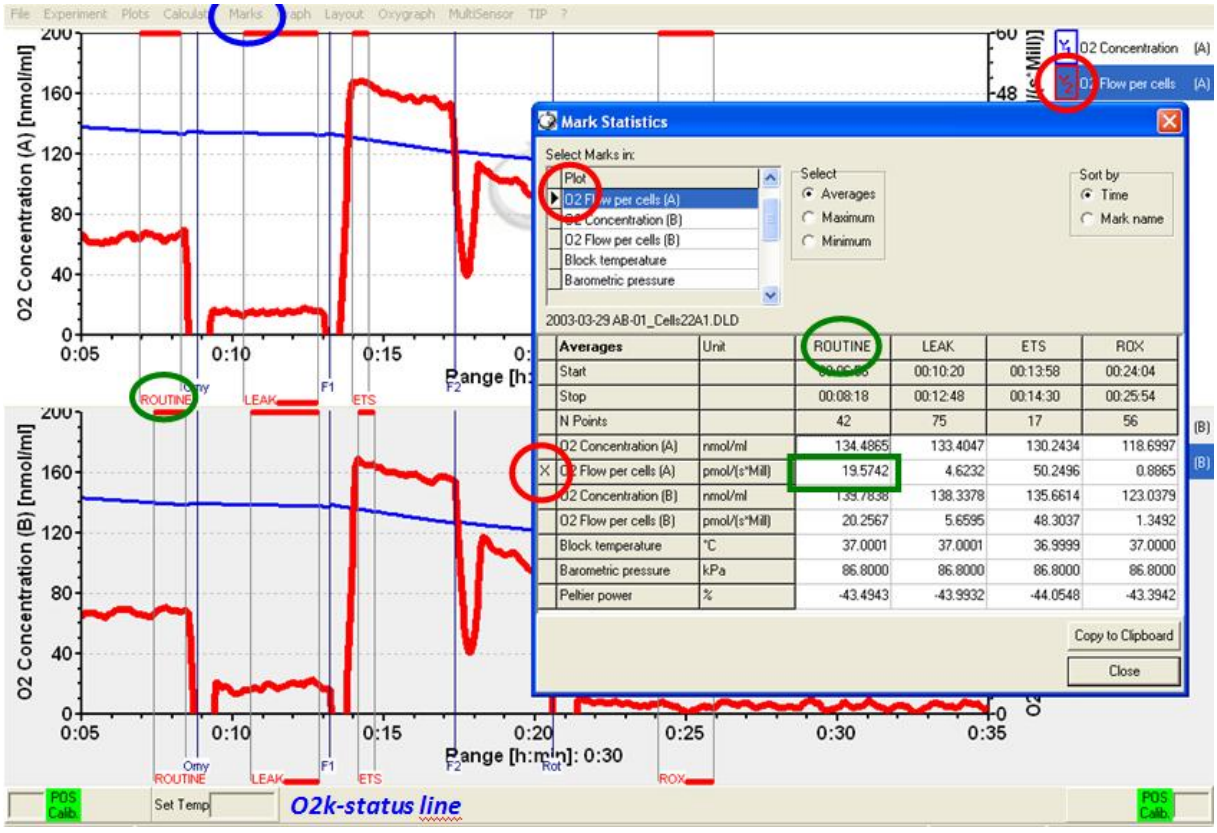
hold [Shift] and drag the cursor with the right mouse button along the time axis.

Rename a single mark by clicking onto the upper or lower bar of the mark, and edit the mark name.



Rename an entire set of marks from the Mark \ Names pull down menu. Example: 'PCP Phosphorylation Control Protocol' ▼. The first mark ROUTINE indicates routine respiration of intact cells; LEAK: LEAK state induced by addition of oligomycin (Omy). ETS: electron transport system capacity after uncoupling; ROX: residual oxygen consumption after inhibition of ETS.

Marks can be set and named on-line immediately when proceeding to the next titration. As progressively more marks are defined, more values appear in the table Mark statistics [F2].



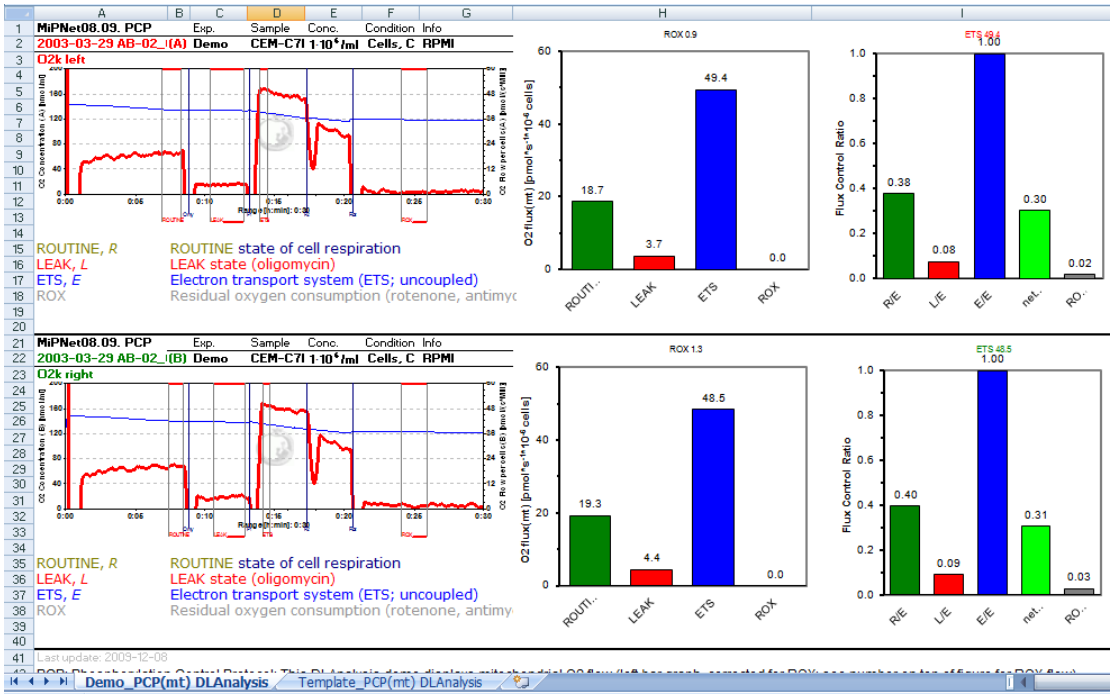
Press [F2] for viewing the table "Mark Statistics". Averages are tabulated in the bottom panel. The active plot, from which the marks are taken, is selected in the top panel and shown by an **X** in the bottom table. Averages are calculated in all plots for the marks defined on the active plot. Click on Copy to Clipboard,

and paste the data into a table of the Excel template "O2k-Analysis_Cells_0809.xls".

1.4. MiPNet0809_O2k-Analysis_Cells.xls

File: MiPNet0809_O2k-Analysis_Cells.xls
OROBOROS FileFinder: O2k-Protocols \ line MiPNet08.09 -> scroll to the right for the hyperlink.

Save this template file under the subdirectory "DatLab\DLDemo\". In "MiPNet0809_O2k-Analysis_Cells.xls", detailed instructions are provided for data transfer from DatLab to the Excel template prepared for a phosphorylation control protocol (PCP).



MiPNet0809_O2k-Analysis_Cells.xls This Excel file is the template for on-line or off-line DatLab analysis. In column J, **X** and **X** and bold lines (averages for O2 Flow per cells, in colour) indicate the plots, where the marks have been set. These values are shown in the Excel bar graphs as average respiration at defined metabolic states.

Follow the instructions step-by-step. The demo table sheet "Demo_PCP(mt)DLAnalysis(2)" may be deleted.

Initial adjustment of the template, table sheet "Template_PCP(mt) DLAnalysis": Edit the mark names according to your specific protocol. Example: "PCP Mark Reference:" (Lines 1 and 21, columns M onwards), corresponding to the sequentially marked sections of the experiment. This serves as a control if the marks have been set properly in DatLab (Lines 3 and 23, columns M onwards).

Upper and lower Excel graphs: Adjust the number of bars to be shown in the bar graphs. Click with the right mouse button on the bar graph, select data source, then click on "Rows".

Select the data source for values on the *Y*-axis, and for labels on the *X*-axis.

Edit the name of the *Y*-axis; edit the scaling and tick intervals after right mouse click on the *Y*-axis.

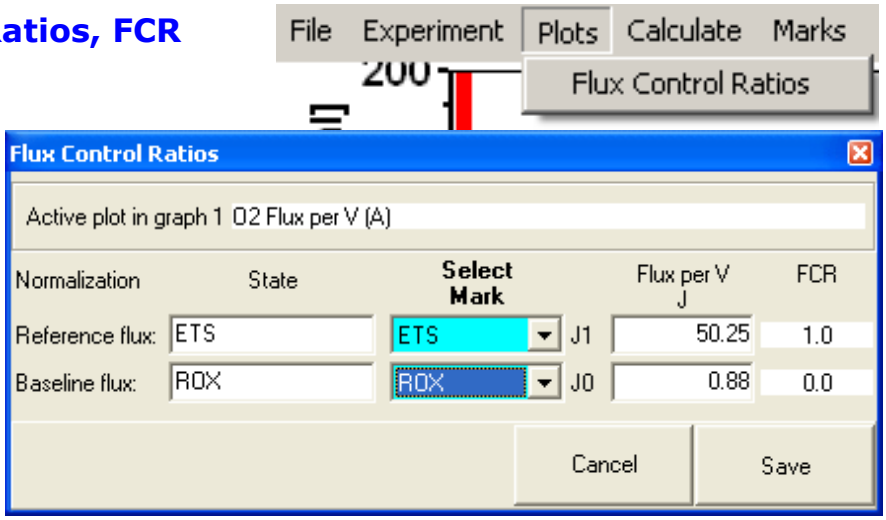
Enter experimental information as far as constant values can be used for sequential runs (Lines 2 and 22, columns C-G).

1. Copy the template table sheet "Template_PCP(mt) DLAnalysis" to obtain the table sheet "Template_PCP(mt) DLAnalysis(2)". Click with the right mouse button on the name of the table sheet in the bottom line, select "Move/copy", and click on the bottom line "Copy".
2. In the copied table sheet, edit the information for the left and right chamber.
3. (A) In the Mark statistics [F2] window of DatLab, top panel, **Select Marks in**: ▶ O2 Flow per cells(A), and Copy to Clipboard. In the Excel file, click into the red cell Left (column J, line 2) for chamber (A). Paste [Ctrl+V] to insert the copy of the Mark statistics table from the clipboard into the Excel table.
   (B) The same in green for (B). In the Mark statistics window [F2] of DatLab, select marks in ▶ O2 Flow per cells (B), Copy to Clipboard, and paste into the Excel file into the green cell Right (column J, line 22) for chamber (B).
   Scales in the Excel graph have to be adjusted according to the experimental protocol.
4. Check the number and sequence of marks imported from DatLab (lines 3 and 23) in relation to the Mark labels in your template (lines 1 and 21).
5. Insert the DatLab graphs with the traces for both chambers. (A) In DatLab, select the upper graph (left mouse click into the graph), and select "Graph\ Copy to Clipboard\WMF" [hold the Alt key, and sequentially type G P W]. In the Excel table, click on the upper red cell marked "Paste DatLab graph here", and paste [Ctrl+V].
   (B) In DatLab, select the lower graph (left mouse click into the graph), continue as for (A). In the Excel table, click on the lower green cell marked "Paste DatLab Graph here", and paste [Ctrl+V]. Select both graphs (hold shift and sequentially click left on each graph),

select "Format\Graph\Size" and set the width of the graphs to 15 cm or 6 inches.

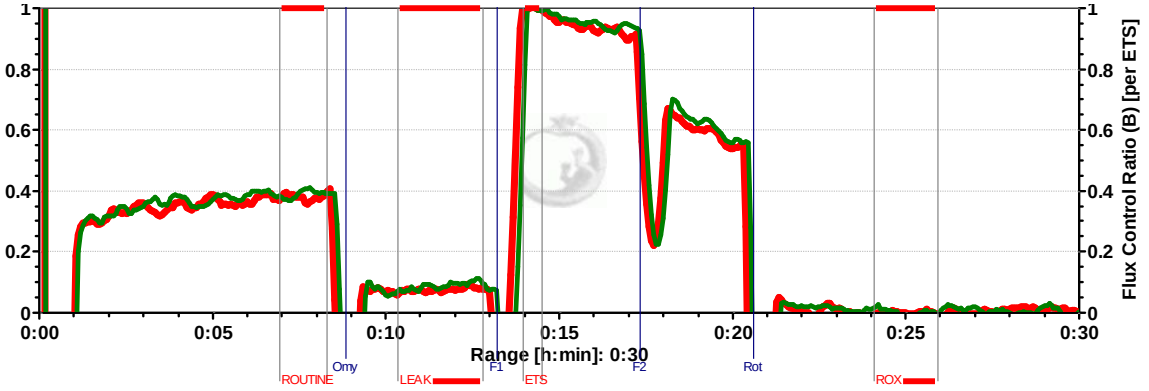
6. Optionally, enter calibration information: In DatLab, select the oxygen signal (Y_1) for chamber A and calibrate [F5], press Calibrate and Copy to Clipboard. In the Excel file (line 2), click on Paste Calibration Info, and paste [Ctrl+V]. The same for chamber B (line 22) in green. Specifically selected graphs may be entered here as well.
7. Select lines 1-40, cut [Ctrl+X], and paste the figure with data lines into a separate table sheet [Ctrl+V] where you collect all results.
8. Delete the now empty table sheet "Template_PCT(mt) DLAnalysis(2)" (right mouse click on the name of the table sheet in the bottom line; delete).

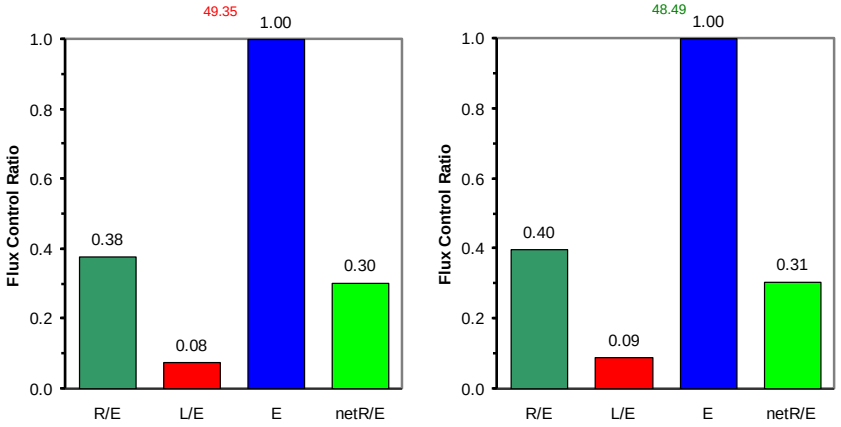
1.5. Flux Control Ratios, FCR



Internal normalization of flux may be particularly informative when relating flux to a reference state within the experimental protocol.

- In the PCT protocol, respiratory capacity of the electron transport system, ETS, in the uncoupled state is the reference flux, J_1.
- The baseline flux, J_0, determined as residual oxygen consumption (ROX) after inhibition of electron transport, is subtracted from flux.





Graph layout:

After pressing Save, the entire plot of O2 Flux is divided by the reference flux (corrected for baseline flux), to obtain normalized flux or Flux Control Ratios (FCR).

07 Gr1-Flux Gr2- O2 Conc.▼ is used in the graph above, plotting the normalized flux for both chambers in a single graph (Graph 1). The range for both *Y* axes is set to 1.0 [F6]. Oxygen concentration is plotted in Graph 2 for both chambers.

The values of the FCR are obtained graphically and numerically from Mark statistics [F2] and Copy to Clipboard, in the Excel file 'O2k-DatLabAnalysis_Cells_0809.xls' (see above).

For a discussion of Flux Control Ratios in relation to the respiratory control ratio, RCR, and uncoupling control ratio, UCR see: MiPNet10.04 and Gnaiger 2008.

Gnaiger E (2008) Polarographic oxygen sensors, the oxygraph and high-resolution respirometry to assess mitochondrial function. In: Mitochondrial Dysfunction in Drug-Induced Toxicity (Dykens JA, Will Y, eds) John Wiley: 327-352.

2. Instrumental O2k-Background Oxygen Flux

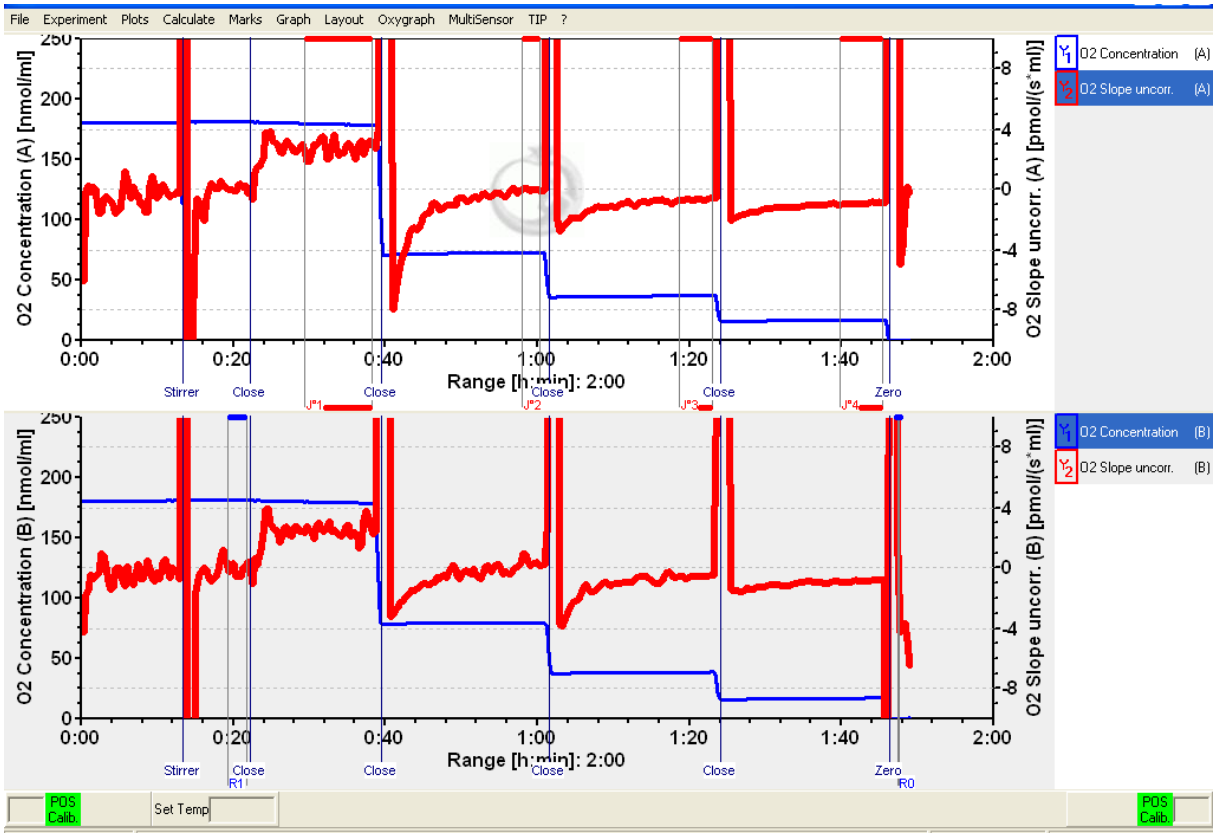
For calibration of the oxygen sensor and of the O2k-Background, only incubation medium but no biological sample is added to the Oxygraph-2k chamber at experimental conditions. In general, use the default values in the absence of specifically designed background tests.

2.1. MiPNet1406_2003-04-17 AB-01_Calib.DLD

DemoFile: MiPNet1406_2003-04-17 AB-01_Calib.DLD
OROBOROS FileFinder: O2k-Protocols \ line MiPNet14.06 -> scroll to the right for the hyperlink.

You may save the DLD file on your PC under the subdirectory "\DatLab\DLData\DLDemo\".This DatLab file can also be downloaded from www.oroboros.at.

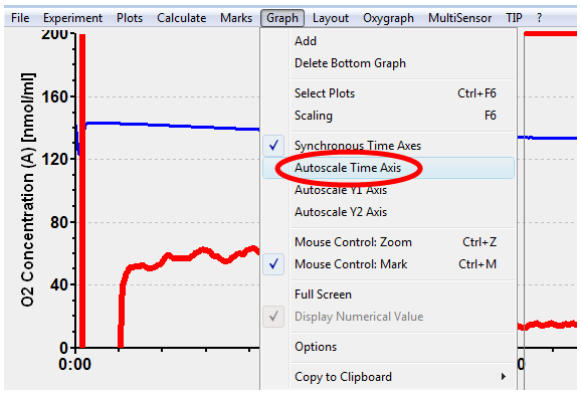
MiPNet14.06_2003-04-17 AB-01_Calib.DLD



MiPNet14.06_2003-04-17 AB-01_Calib.DLD

1. Year
2. Month
3. Day
4. Oxygraph chambers
5. Sequential file number
6. Short description
7. DatLab Data file

Graph layout: 02 Background Experiment▼. A 30 min time range is frequently used online. Time may be compressed to a range of 1, 2, .. h, or changed to "Autoscale Time Axis".



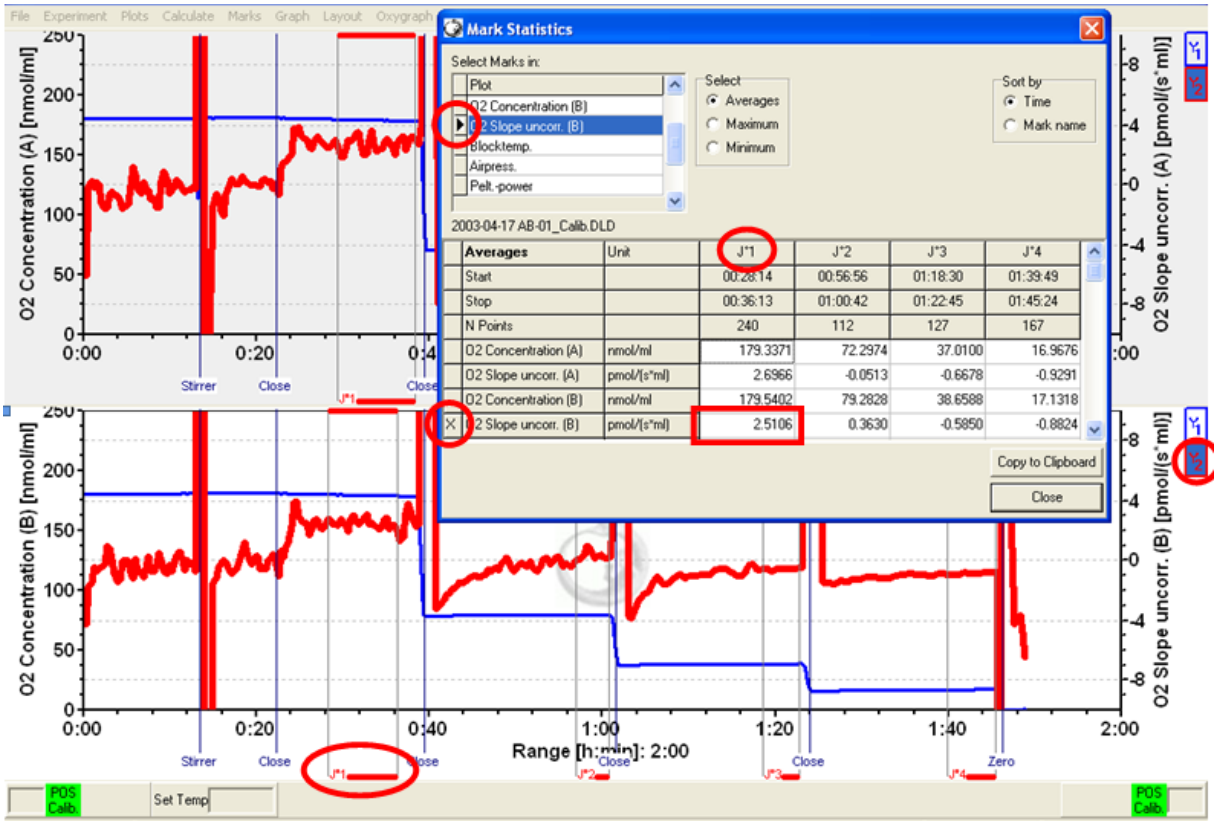
Oxygen calibration: (MiPNet12.08)

R1 An air calibration section is marked in Graph 2 (chamber B) for the average value of R1 in the calibration window [F5]. The O2k-Background test starts with air calibration using a gas phase of air above the stirred experimental medium. You may zoom into the calibration section for more detail. Equilibrium is gradually obtained between the gas and aqueous phases for air calibration of the oxygen signal. The signal is constant at equilibrium, and the slope is zero. Information on the zero oxygen signal, R_0, is obtained from a dithionite zero calibration, marked R0.

2.2. Instrumental Background

Y_2

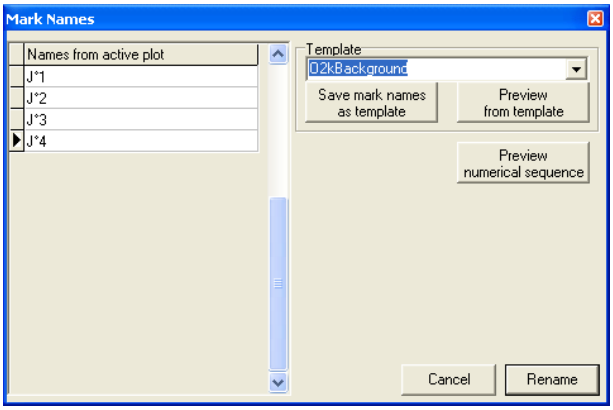
Click on Y_2 at the right side of the graph (figure legend), to select the negative slope of the oxygen signal as the active plot, which is displayed on the Y_2 axis.



J°1 After closing the chamber <Close> (2 ml), the instrumental O2k-Background oxygen flux is obtained at air saturation, marked as J°1 for the first section near air saturation (marks on Y_2 are shown for chamber A). Step-wise reduced oxygen levels were achieved by exchange of oxygen between the aqueous phase and a

gas phase flushed with nitrogen or argon, using the 50 ml gas injection syringe. The chamber was closed again at the desired oxygen levels <Close>.

O2 Slope uncorr. (A) [pmol/(s*ml)]: After closing the chamber, the oxygen consumption by the polarographic oxygen sensor is shown as a constant slope (Mark $J°_1$). 10 min are required for stabilization of the signal. Allow for sufficient time until flux has stabilized before setting a mark. Note that no mark must be set on the plot of flux for the air calibration period. At progressively lower steps of oxygen concentration, the oxygen consumption by the sensor decreases linearly, and the effect of oxygen backdiffusion is finally apparent as a positive slope or negative flux (Marks $J°_2$ to $J°_4$). Mark names are selected from the pull down menu Graph \ Names, selecting the template "O2kBackground".



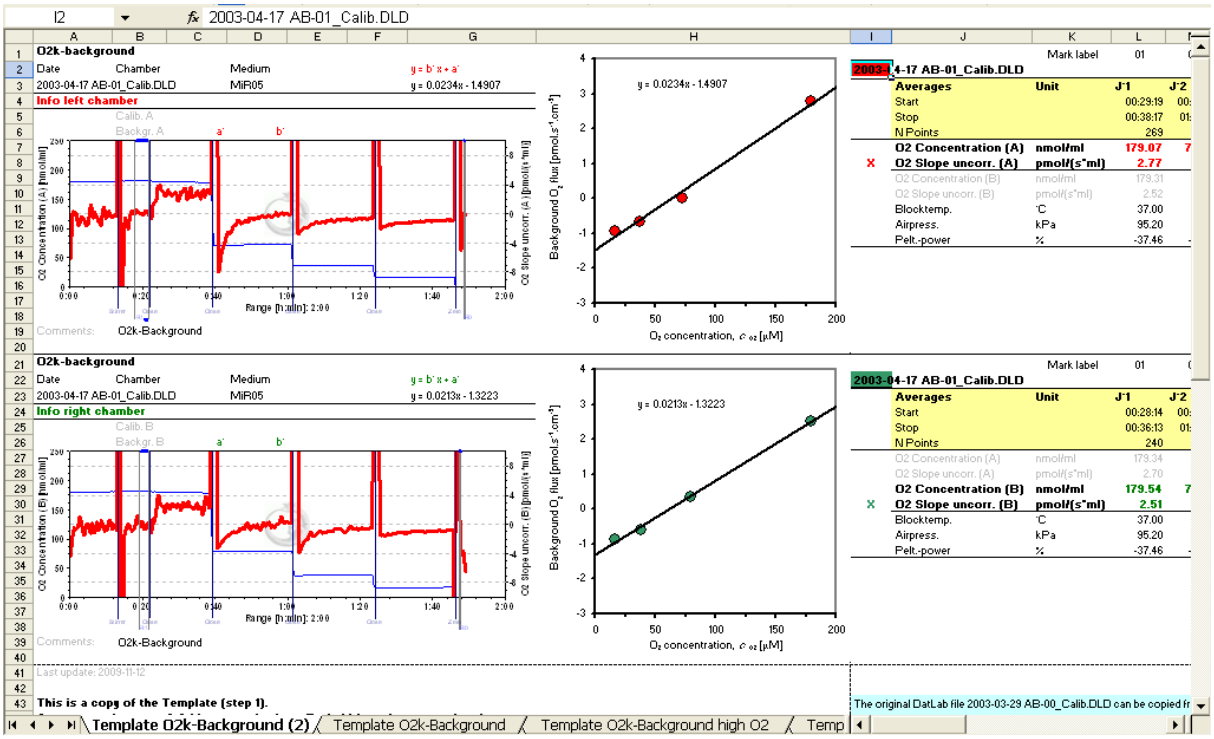
Press [F2] for viewing the table "Mark Statistics". Averages are tabulated in the bottom panel. The active plot, from which the marks are taken, is selected in the top panel and shown by an **X** in the bottom table. Averages are calculated in all plots for the marks defined in the active plot. Click on Copy to Clipboard, and paste the data into a table of the Excel template "O2k-Background.xls".

2.3. Excel File: O2k-Background.xls

File: O2k-Background.xls
OROBOROS FileFinder: O2k-Protocols \ line MiPNet14.06 -> scroll to the right for the hyperlink.

To analyze an O2k-Background test, delete the demo table sheet "Template O2k-Background(2)" in the Excel File "O2k-Background.xls", and follow the step-by-step instructions as given above in Section 1.4, or as modified below.

1. Edit the information for the left and right chamber (Medium, Volume).
2. Copy the template table sheet "Template O2k-Background" to obtain the table sheet "Template O2k-Background(2)".

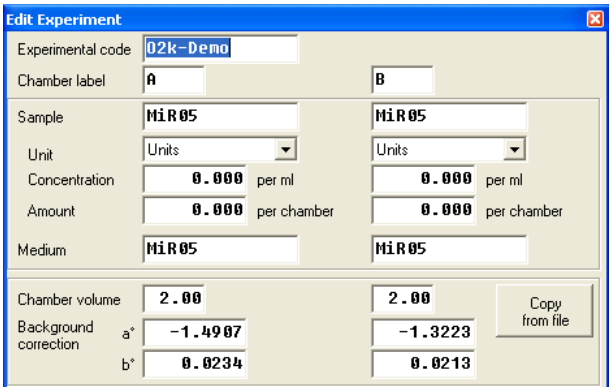


O2k-Background.xls: This Excel file is the template for analysis of O2k-Background tests. In column I, the **X** and **X** and bold lines (averages for O2 Concentration and O2 Slope in colour) indicate the plots where the marks have been set, and the values which are used in the Excel graph. The corresponding graphs show oxygen flux as a function of oxygen concentration with linear regression parameters.

3. (A) In the Mark statistics [F2] window of DatLab, select the top panel of ▶ O2 Slope uncorr.(A), and Copy to Clipboard. In the Excel file, column I, click into the red cell "Left", for chamber (A). Paste [Ctrl+V] to insert the copy of the Mark statistics table from the clipboard into the Excel table.
   (B) In the Mark statistics window [F2] of DatLab, select marks in ▶ O2 Slope uncorr.(B), Copy to Clipboard, and paste into the Excel file into the green cell "Right" (column I), for chamber (B).
   If the standard format - background oxygen flux measured at four oxygen levels - is varied, then the settings in the Excel graphs may have to be adjusted.
4. to 8. Follow step-by-step instructions in Section 1.4 (above).
   The linear instrumental background equation with slope, $b°$, and intercept, $a°$, is:

Eq.(1) $$J_{O_2}^{\circ} = b^{\circ} \cdot c_{O_2} + a^{\circ}$$

In the graph: $$y = b^{\circ} \cdot x + a^{\circ}$$



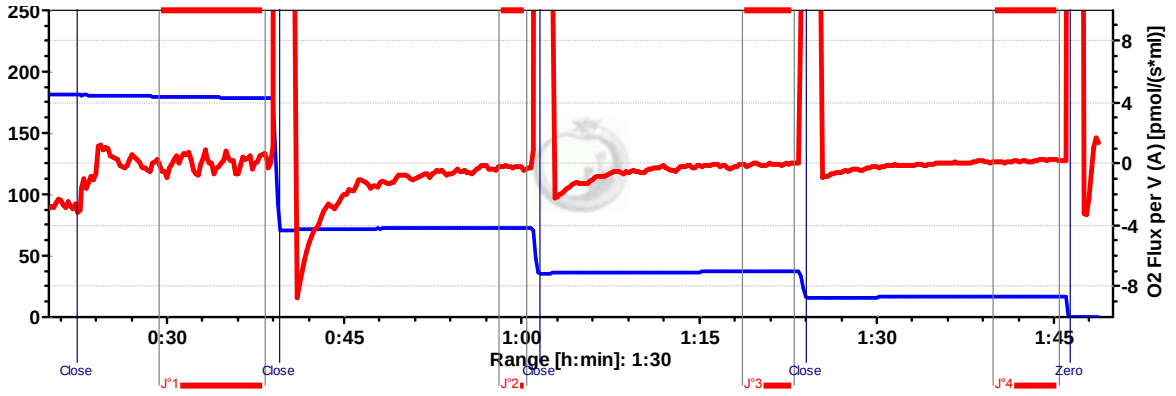
[F3]: Copy the background parameters, *a*° and *b*°, into the Edit Experiment [F3] window of DatLab, and Save. You may copy the entire equation into the Comments window, and then copy the values of *a*° and *b*° individually into the respective windows for "Background correction".

The background parameters are thus calibrated and available for the corresponding correction of O_2 flux:

Eq.(2) $$J_{O_2}(\text{corr.}) = J_{O_2}(\text{uncorr.}) - (b^{\circ} \cdot c_{O_2} + a^{\circ})$$

2.4. Background Quality Check

Graph layout: 03 Background Exp. Corrected▼. Adjust the time range according to the length of the background calibration period. The plot displayed below is the background-corrected volume-specific oxygen flux. Ideally, corrected flux of a background test should be zero at any oxygen level, when the chamber is closed (correction does not make sense when the chamber is open for air calibration). This corrected plot is useful for judging the selection of marks after sufficient equilibration times. Press [F6] and Info for information.



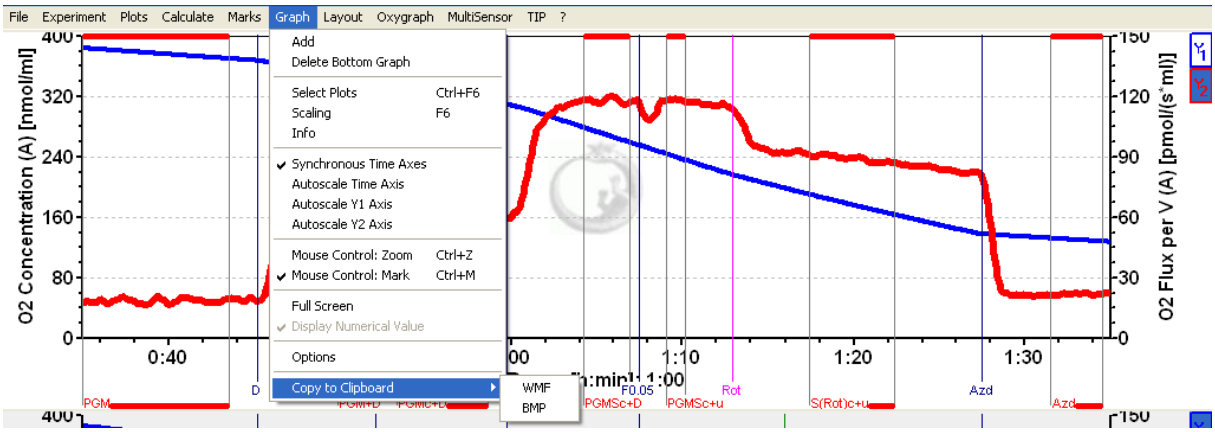
Off-line: Save the file, and print the protocol [Ctrl+F3] for future reference on oxygen calibration and instrumental background calibration parameters.

On-line: You may add a zero oxygen calibration. After completion, disconnect to save the file at this stage and continue with an experiment. Upon re-connection to the Oxygraph-2k (Close and Connect), all calibration data from this test are available as a default for the next experiment.

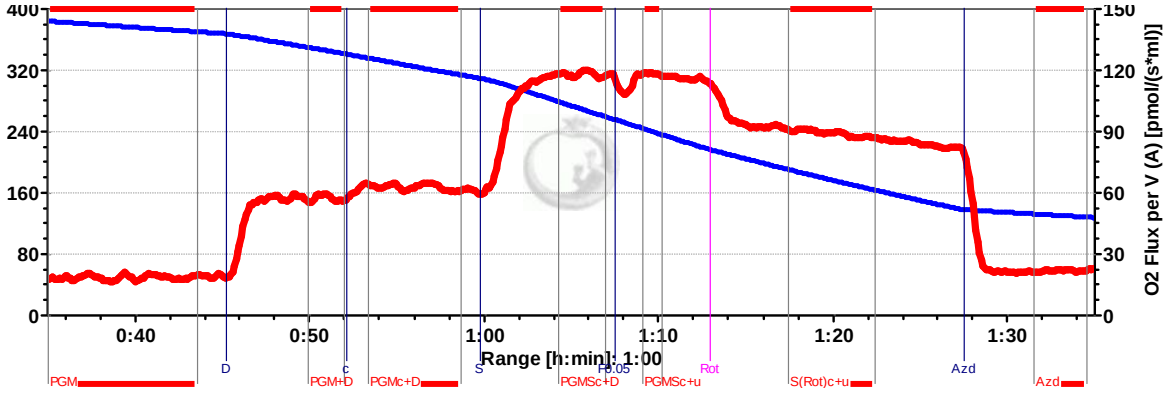
3. General Notes on Graphs

In this document, graphs were imported from DatLab:

Screenshots: In DatLab, copy the entire screen by [Ctrl+PrtSc]. Open a Word file (or PowerPoint), and paste [Ctrl+V] to obtain a figure as below.



Graph – Copy to Clipboard: In DatLab, select the active graph. In the Graph menu, click on Copy to Clipboard, and select the WMF or BMP format. Open a Word file, and paste [Ctrl+V] to obtain the figure as below.



Mark Statistics Clipboard: After copying the Mark statistics table into the Excel file, screenshots of tables with figures were copied into the Word file.

4. References

Gnaiger E (2001) Bioenergetics at low oxygen: Dependence of respiration and phosphorylation on oxygen and ADP supply. Respir Physiol 128: 277-297.

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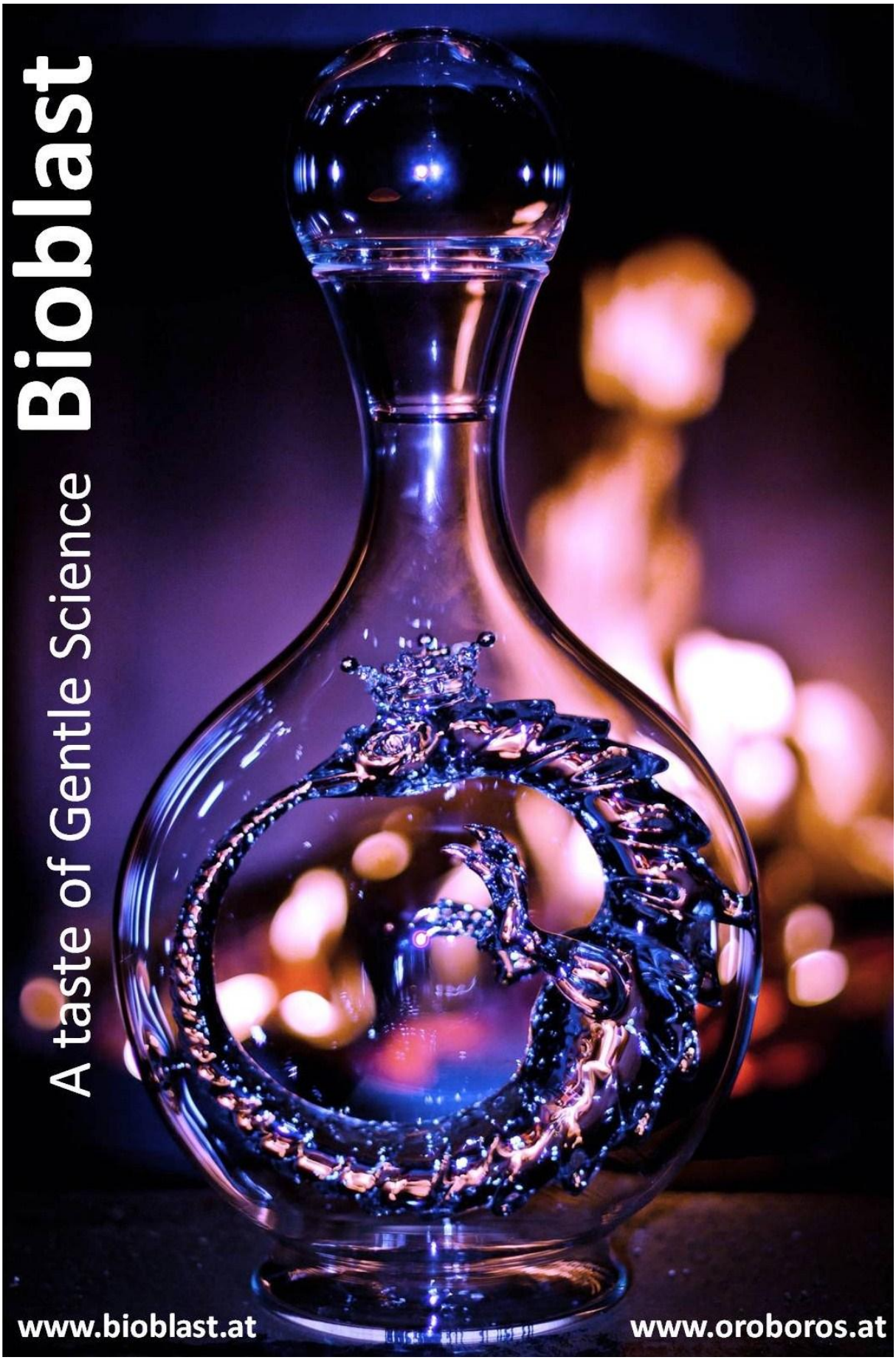
Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. Int J Biochem Cell Biol 41: 1837–1845.

Press WH, Teukolsky SA (1990) Savitzky-Golay smoothing filters. Computers in Physics Nov/Dec 1990: 869-872.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