O2k-Procedures:
Magnesium Green analysis

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Measurement of mitochondrial ATP production using Magnesium Green: DL-Protocols and data analysis with DatLab 7.4

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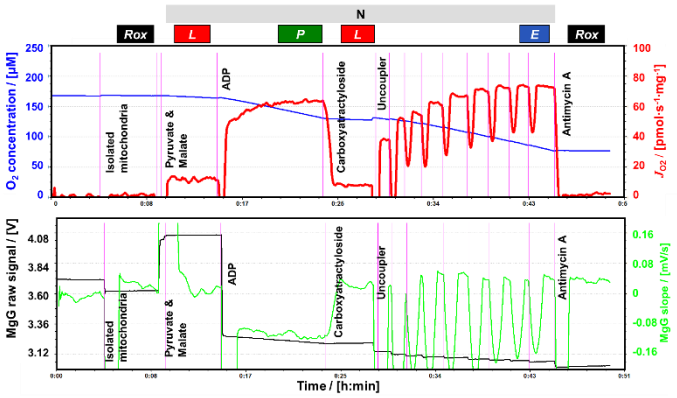


Contents

1. General information

Magnesium Green™ (MgG) is a fluorescent dye that absorbs and consequently emits light when bound to Mg^{2+} at excitation/emission maxima of ~506/531 nm (Fluorescent Magnesium Indicators, 2005). Both ADP and ATP bind Mg^{2+}, thus decreasing the MgG signal. Since ADP and ATP have different affinities to Mg^{2+}, the dye can be used to analyse the mitochondrial exchange of ADP with ATP, which can be taken as a measure of ATP production (Chinopoulos et al, 2009, Chinopoulos et al, 2014).

Further details: https://wiki.oroboros.at/index.php/Magnesium_Green

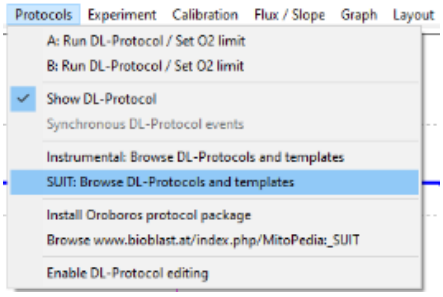
Substrate-uncoupler-inhibitor-titration (SUIT) protocols are designed to study respiratory control in a sequence of coupling and pathway control states induced by multiple titrations within a single experimental assay. DatLab 7.4 has been specifically designed to guide the user through the SUIT protocols (DL-Protocols in DatLab). Coupling control and ATP production are measured with the SUIT-006 MgG protocol in different

mitochondrial preparations (isolated mitochondria, tissue homogenate, permeabilized cells which are permeabilized prior to addition to the O2k-chamber). Excel templates are provided for data analysis of O_2 flux and ATP production in mitochondrial preparations using MgG.

2. DatLab 7.4 Protocols (DLPs) for Magnesium Green

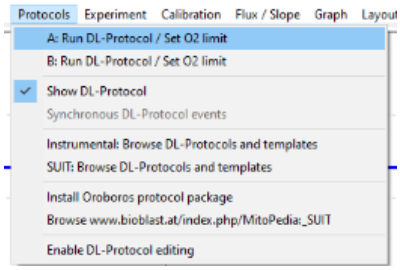
View the SUIT-006_MgG_mt_D055 demo experiment in DatLab for an overview of this protocol:

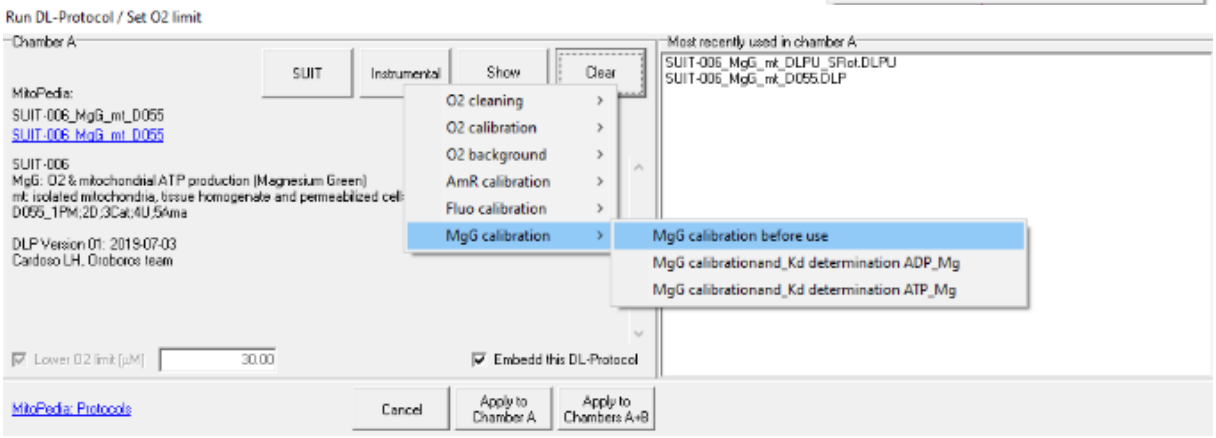
1. Select Protocols and click SUIT: Browse DL-Protocols and templates.
2. Open the SUIT-006 folder, then open the SUIT-006_MgG/ SUIT-006_MgG_mt_D055 folder. Select the DLP SUIT-006_MgG_mt_D055.DLP.



Run an experiment with MgG using the DLPs:

1. Select Protocols and click A: or B: Run DL-Protocol/Set O2 limit.
2. Click Instrumental, select MgG calibration, and MgG calibration before use. Select Apply to chamber A/B or Apply to chambers A+B.





3. Without using this run for calibration, start a new file.
4. Select Protocols and click A: or B: Run DL-Protocol/Set O2 limit.
5. Click SUIT and open sequentially the following folders: SUIT-006, SUIT-006_MgG, SUIT-006_MgG_mt_D055. Select SUIT-006_MgG_mt_D055.DLP.
6. Select Apply to chamber A/B or Apply to chambers A+B.

Further details: https://wiki.oroboros.at/index.php/SUIT-006_MgG_mt_D055

Instrumental DLPs are used for determination of the apparent K_d' of ADP to Mg^{2+} and ATP to Mg^{2+}. These files include the calibration of the MgG signal.

View the demo experiment of the Instrumental DLPs:

1. Select Protocols and click Instrumental: Browse DL-Protocols and templates.

2. Open the MgG calibration folder. Inside of this folder, the DLPs MgG_Calibration_and_Kd_determination_ADP_Mg.DLP and MgG_Calibration_and_Kd_determination_ATP_Mg.DLP can be found.

Run the calibration/K_d' determination:

1. Select Protocols and click A: or B: Run DL-Protocol/Set O2 limit.
2. Click Instrumental and select MgG calibration.
3. Select MgG_Calibration_and_Kd_determination_ADP_Mg.DLP or MgG_Calibration_and_Kd_determination_ATP_Mg.DLP.
4. Click Apply to chamber A/B or Apply to chambers A+B.

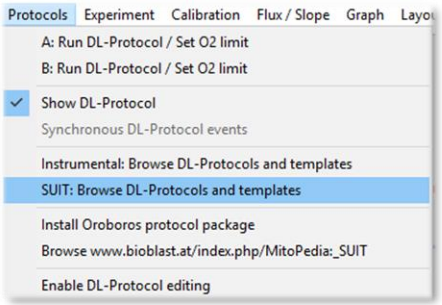
2.1. Stepwise approach for MgG experiments with DLPs

1. Clean the O2k-chambers before use according to the SOP MiPNet19.03 O2k-cleaning and ISS (Di Marcello et al, 2019): Select O2k-cleaning_BeforeUse.DLP in the *Instrumental\O2 cleaning* folder.
2. Add the medium to the O2k-chambers for the experiment.
3. Run the O2 calibration according to MiPNet06.03 POS-calibration-SOP (Gnaiger, 2020): select O2_calibration_air.DLP in the *Instrumental\O2 calibration* folder.
4. Run the MgG calibration and K_d' determination, using the DLPs: MgG_Calibration_and_Kd_determination_**ADP**_Mg.DLP and MgG_Calibration_and_Kd_determination_**ATP**_Mg.DLP (in the *Instrumental\MgG calibration* folder).

   If you wish to test the optimal concentrations of the chelators EDTA and EGTA, the right moment to do this is during this experimental run. Perform it right after the addition of the sample and substrates (e.g. pyruvate and malate), but before the addition of $MgCl_2$. First, titrate EDTA until there are no significative changes of the signal, and then EGTA in the same way. In the next experimental runs, apply these final concentrations of EDTA or EGTA obtained in this run.
5. Follow with cleaning of the O2k-chambers after the use, according to the SOP MiPNet19.03 O2k-cleaning and ISS (Di Marcello et al, 2019): run O2k-cleaning_AfterUse.DLP (folder *Instrumental\O2 cleaning*).
6. Repeat step **1**.
7. Add MgG and $MgCl_2$ to the O2k-chamber: run MgG_before_use.DLP (folder *Instrumental\MgG calibration*).

   1 mM $MgCl_2$ is added, but for some sample types it is recommended to use more, e.g. 1.5 mM $MgCl_2$ was used for HEK293 cells permeabilized with digitonin (Chinopoulos et al, 2014).
8. Start the experiment: run SUIT-006_MgG_mt_D055.DLP (in the *SUIT\SUIT-006\SUIT-006_MgG\SUIT-006_MgG_mt_D05* folder 5).
9. Repeat step 4.

Note: Step **3** (and therefore steps **4** and **5**) do not need to be performed every day: it is possible to do multiple experimental runs following the steps **1**, **2**, **6, 7** and **8** directly. It is also possible to run the SUIT-006 experiment first, wash the chambers, and then run the MgG calibration/K_d' determination (steps **1**, **2**, **6**, **7**, **4**, then **1**, **3** and repeat step **4**).



MgG does not appear to inhibit mitochondrial respiration (Cardoso et al 2021), however, it is advisable to test with each sample and experimental conditions, by running the same protocol with and without MgG.

3. Data Analysis

3.1. Calibration and K_d' determination

1. In DatLab 7.4, select Protocols and click Instrumental:Browse DL-Protocols and templates.
2. Open the MgG calibration folder that contains Excel templates:
   a. Template_MgG_Calibration_and_Kd_determination_ADP_and_ATP_to_Mg.xlsx;
   b. Template_MgG_Calibration_and_Kd_determination_ADP_and_ATP_to_Mg_demo.xlsx – a demo version of the template.
3. Create a copy of the template for data analysis and rename it. You can rename the template by opening it and choosing the option 'Save as´ in the top menu.
4. After running the DLPs (MgG_Calibration_and_Kd_determination_**ADP**_Mg .DLP and MgG_Calibration_and_Kd_determination_**ATP**_Mg.DLP), open the DatLab files.
5. Select [Y1: Amp] as an active plot for setting the marks and placing the marks on the [Mg^{2+}] steady states according to the protocol, after every $MgCl_2$, ADP or ATP titrations.

Fluorescence signal calibration

1. Open `Template_MgG_Calibration_and_Kd_determination_ADP_and_ATP_to _Mg.xlsx´.
2. Copy the marks: In the open DLD file go to Marks, DL-Protocol marks and select Slope uncorrected + all info. There, in Plot for Marks, select Amperometric,Amp [V] (or MgG raw [V]). In Channel, ensure that both Oxygen, O2 and Amperometric, Amp are selected. Keep the default selection Median and Sort by Time.
3. Click Copy to clipboard.
4. In the Excel tab `Copy marks here´, paste (Ctrl+V) into the yellow cell B2 for the protocol `MgG_Calibration_and_Kd_determination_ADP_Mg´ or in B16 for `MgG_Calibration_and_Kd_determination_ATP_Mg´.
5. The values will be automatically transposed to the tab `Calibration - free Mg2+´, in the yellow cells in columns G and O. The calibration equation and constants (a, b, c) appear on the right side of this tab, and the calculated free Mg^{2+} concentrations [mM] are shown in the green cells in columns H and P.
6. The values from the marks `10Mg1.0´ to `29D4.75´ (MgG_Calibration_and_Kd_determination_ADP_Mg) or `10Mg1.0´ to `21T2.2´ (MgG_Calibration_and_Kd_determination_ATP_Mg) are used for the K_d' calculation.

Determining the K_d' of ADP and ATP to Mg^{2+}

To use `Template_MgG_Calibration_and_Kd_determination_ADP_and_ATP_to _Mg.xlsx´, it is necessary to have installed the free Excel add-in `Solver´. Follow the instructions below to perform the installation. Once this step is done, there is no need to do it again for the same computer/Excel.

1. In Excel 365, choose the `more options´ arrow (arrow pointing down in the upper bar);
2. Choose `more commands´;
3. Choose `add-ins´ in the left bar;
4. Manage: Excel add-ins, Go;
5. Choose `Solver Add-in´, OK.

After performing the fluorescence signal calibration, calculate the K_d':

1. The Mg^{2+} concentrations for the ADP and ATP titration marks appear automatically in the tabs `Kd determination - ADP to Mg2+´ and `Kd determination - ATP to Mg2+´, in columns G (yellow).
2. After clicking the cell for the K_d' (N5), click on `Data´ in the upper bar of the ribbon menu.
3. Choose Analyse/Solver (or question mark and arrow symbol) on the right side of the Excel ribbon menu.
4. The window `Solver Parameters´ opens with the set parameters. Check if the parameters are correct and click `Solve´.
   a. `Set Objective´: J25;
   b. `By changing variable cells´: N4, N5;
   c. `Subject to the Constraints´: N5>=0.
5. The window `Solver Results´ opens. Choose `Keep Solver Solution´ and click OK.
6. The K_d' appears in the cell N5, as calculated by the least squares method.

Follow these instructions for ADP and ATP titrations separately and repeat these steps every time you need to calculate the K_d' from new experimental data and/or parameters (e.g. with different respiration media, substrates, or samples).

3.2. Biological Experiment

Oxygen flux analysis

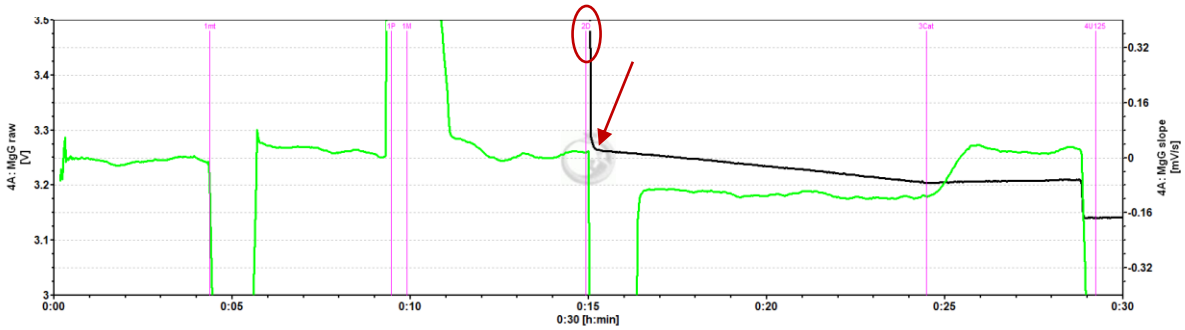
O_2 flux analysis is performed with the template `O2 analysis template DL7.4.xlsx´. More information can be found here: MiPNet24.06 Oxygen flux analysis with DatLab7.4 (Cardoso, 2019).

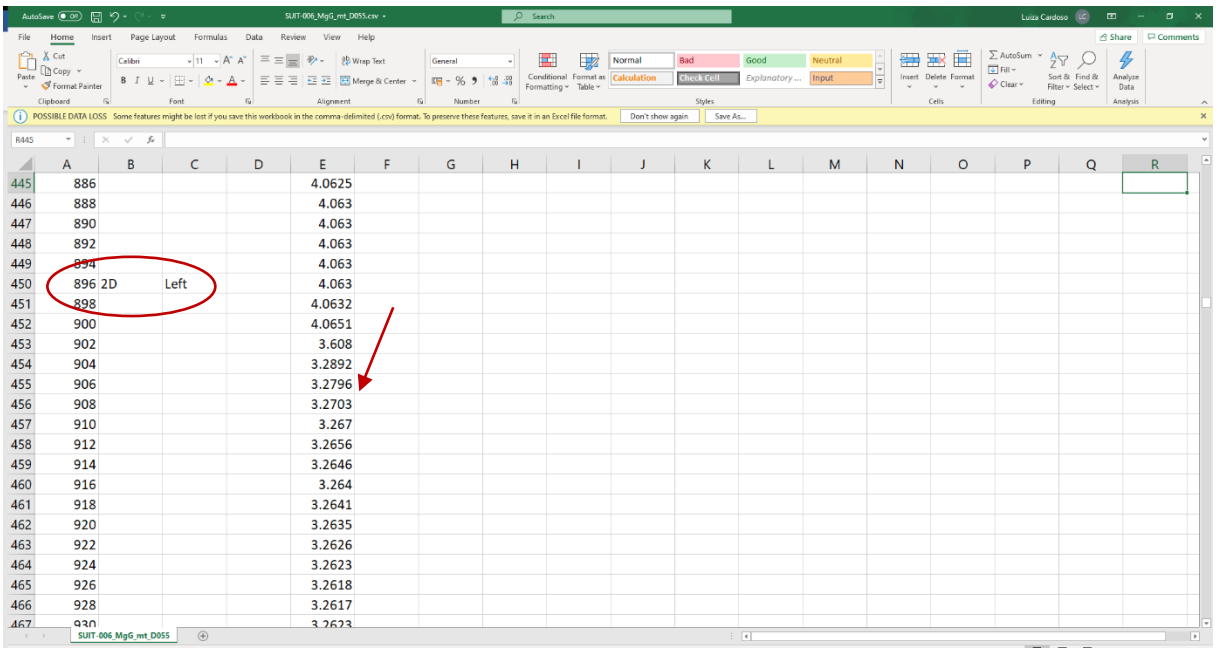
ATP production analysis

1. In DatLab 7.4, select Protocols from the menu and click SUIT:Browse DL-Protocols and templates.
2. Select *SUIT-006\SUIT-006_MgG\SUIT-006_MgG_mt_D055* folder. The following Excel files can be found:
   a. Template - MgG ATP production analysis.xlsx;
   b. Template - MgG ATP production analysis_demo.xlsx - a demo version of the template.
3. Create a copy of `Template - MgG ATP production analysis.xlsx´ for each O2k chamber for data analysis, and rename.
4. For calibration, the same marks are used as in `MgG_Calibration_and_Kd _determination_ADP_Mg.DLD´ or `MgG_Calibration_and_Kd_determination _ATP_Mg.DLD´ (not in the SUIT-006.DLD file). Ensure that you use data from the same O2k-chamber/Fluo-Sensor used. Copy to clipboard in mark statistics.
5. Copy the clipboard into cell B2 (yellow) of the tab `Calibration - copy marks here´. The Amp raw values [V] are automatically copied to the tab `Calibration

- free Mg2+´ (column G, yellow). The calibration is automatically performed, the equation and constants appear on the right side of this tab, and the calculated free Mg^{2+} concentrations [mM] are shown in column H (green cells).

6. Install the Solver add-in from Excel: Follow the instructions in section 'Determining the K_d' of ADP and ATP to Mg^{2+}' 3.1.2 above, if needed.
7. Open the Excel tab `ATP production´ and add the experimental parameters in the orange box (columns B and C, rows 20 to 24):
   a. K_d' values of ADP to Mg^{2+} (cell C20) and ATP to Mg^{2+} (cell C21). These values must be previously determined in the same respiration media, in the presence of sample and substrates that are used in the experiment (see instructions above).
   b. Edit the `[Mg2+] added (mM)´, `[ADP] added (mM)´ and `[ATP] added (mM)´ (cells C22, C23 and C24), if necessary.
8. In the .DLD file of the experiment (SUIT protocol), choose: File, then Export, Data to text file (*.csv). In the window that opens, select the plot for Amp raw [V] (or renamed as MgG raw [V]) for the desired chamber; in Select time range choose full range; time unit: choose [s], then click export to save the .csv file.
9. Open the .csv file. The events are exported, look for the 2D event. After the ADP addition, a fast drop in the MgG fluorescence can be seen (due to the binding of Mg^{2+} to added ADP). After this, a slower drop in the MgG signal is seen, this is related to the exchange of ADP for ATP, this later phase is to be copied. It is important to copy the values only after the fast drop of the signal. The Amp raw [V] values normally are between 2 and 5 V.





	A	B	C	D	E
445	886				4.0625
446	888				4.063
447	890				4.063
448	892				4.063
449	894				4.063
450	896	2D	Left		4.063
451	898				4.0632
452	900				4.0651
453	902				3.608
454	904				3.2892
455	906				3.2796
456	908				3.2703
457	910				3.267
458	912				3.2656
459	914				3.2646
460	916				3.264
461	918				3.2641
462	920				3.2635
463	922				3.2626
464	924				3.2623
465	926				3.2618
466	928				3.2617
467	930				3.2623

10. Import the experimental values from your .DLD file and copy into the tab `ATP production´.
    Caution: The .csv file might not look like shown above. If it does not, check the general settings of your operating system and Excel and select 'English' as default language setting. You can also select the tab 'File' in Excel, select 'Options' and 'Advanced'. Make sure that the decimal separator is set to '.' there.
11. In the `ATP production´ tab , paste the values for time [s] and Amp raw [V] in the grey (J) and yellow (K) columns.
12. Calculate the correction factor with the Solver add-in. This step must be done to set the initial ATP concentration as 0 (zero).
13. Select the cell for the correction factor in the green box (C28) of `ATP production´ tab.
14. In Excel 365, choose the `Data´ option in the upper bar. Choose Analyse/Solver (or question mark with arrow symbol) on the right;
15. A window will open with the parameters set. Check if the parameters are correct and click `Solve´:
    a. `Set objective´: M4
    b. `To´: `Value of´: 0
    c. `By changing variable cells´: C28
16. In the next window that opens, choose `Keep solver solution´ and click `OK´.
17. The ATP concentrations will be corrected such that the initial concentration, right after ADP addition, is close to zero.
18. **Results:** ATP concentrations [mM] are given in column M. With these values, it is possible to calculate ATP flux per time, and P»/O_2 ratios [ATP flux/O_2 flux] taking into consideration that equivalent units are used (e.g. amol·s^{-1}·mL^{-1} for both fluxes).

The calculations used for calibration, determination of K_d' values, and ATP production are based on Chinopoulos et al (2014).

4. Further Information

Here you find additional information on how to do experiments using MgG:
» MitoPedia: Magnesium Green

In-depth explanation of flux measurements with DatLab:
» MitoPedia: Flux / Slope

5. References

Fluorescent Magnesium Indicators, Molecular Probes, Invitrogen Detection Technologies, Revised: 05–May–2005, available at: »https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp01290.pdf

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6. Acknowledgements

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