**Assessment of individual mitochondrial respiratory chain complex activity with permeabilized LUHMES cells**

Introduction

Aim

This protocol describes the analysis of how to assess the activity of the mitochondrial respiratory chain complex I-IV activities by using selectively permeabilized LUHMES cells (human neuronal).

Purpose

Many toxic substances inhibit mitochondrial function. Also many compounds in research for pharmaceutical use inherit mitochondrial off-target effects. This assays aims to identify direct inhibition of the MRC complexes I-IV.

Limitations

Since the analysis of each MRC complex activity is based on the oxygen consumption at cIV, sometimes only indirect conclusions can be drawn. However control experiments can confirm the suggested results. Additionally, adverse effects on cytochrome c or ubiquinone are not assessed.

Method outline

Cells get selectively, i.e. only their plasma membrane, permeabilized before the experiment starts. Then, their MRC complexes are sequentially fed with specific substrates, i.e. first cI, then cII, then cIII, finally cIV. At the same time, inhibitors for upstream complexes are added with the fuel substance for the next downstream complex to enable the analysis. Finally, complex activity is analyzed simultaneously in treated samples relative to solvent control samples, based on the cIV-mediated oxygen consumption using the Seahorse device.

Method description

Chemicals and buffers

Table 1: Chemicals used in experiments

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Product | Supplier | Cat. No. | Lot. No. | Cas. No. |
| ADP | Sigma | A5285 | - | 72696-48-1 |
| Digitonin | Sigma | D141 | 0001432565 | 11024-24-1 |
| D-Mannitol | Sigma | M4125 | 007K0166 | 69-65-8 |
| DMSO | Merck | 1.09678 | K48040378 727 | 67-68-5 |
| Duroquinol | TCI | T0822 | 5QCSN | 527-18-4 |
| EGTA | Sigma | E0396 | SLBP2807V | 67-42-5 |
| Fatty-Acid free BSA | Roth | 8076.5 | 197254757 | 90604-29-8 |
| HEPES | Roth | HN77.4 | 468101696 | - |
| KH2PO4 | Ridel-deHaen | 30407 | 51530 | 7778-77-0 |
| L-Ascorbic Acid | Sigma | A-4544 | 21H01855 | 50-81-7 |
| L-Glutamine | Sigma | G3126 | 046K0009 | 56-85-9 |
| Malic acid | Sigma | M6413 | BCBJ3883V | [97-67-6](https://www.sigmaaldrich.com/catalog/search?term=97-67-6&interface=CAS%20No.&N=0&mode=partialmax&lang=de&region=DE&focus=product) |
| Malonic acid | Sigma | M1296 | BCBV3859 | [141-82-2](https://www.sigmaaldrich.com/catalog/search?term=141-82-2&interface=CAS%20No.&N=0&mode=partialmax&lang=de&region=DE&focus=product) |
| MgCl2-Hexahydrate | Roth | 2189.1 | 387253790 | 7791-18-6 |
| Potassium Hydroxide | Sigma | 30603 | SZBE1180V | 1310-58-3 |
| Pyruvic acid | Sigma | 107360 | MKCC8726 | [127-17-3](https://www.sigmaaldrich.com/catalog/search?term=127-17-3&interface=CAS%20No.&N=0&mode=partialmax&lang=de&region=DE&focus=product) |
| Succinic acid | Sigma | S9512 | 057K01281 | [110-15-6](https://www.sigmaaldrich.com/catalog/search?term=110-15-6&interface=CAS%20No.&N=0&mode=partialmax&lang=de&region=DE&focus=product) |
| Sucrose | Merck | 1076511000 | K47396351613 | 57-50-1 |
| TMPD | Sigma | T7394 | STBC1186V | [100-22-1](https://www.sigmaaldrich.com/catalog/search?term=100-22-1&interface=CAS%20No.&N=0&mode=partialmax&lang=de&region=DE&focus=product) |
| XF calibrant solution | Agilent/Seahorse | 100840-000 | 090 | - |

Table 2: reagent preparation

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Reagent | Stock | Final (on cells) | 10 x port solution | Solvent |
| ADP | 100 mM | 1 mM | 10 mM | MAS |
| Ascorbate | 200 mM | 2.0 mM | 20 mM | MAS |
| Digitonin | 25 mg/ml | 25 µg/µl | 250 µg/ml | MAS |
| Duroquinol | 500 mM | 0.250 mM | 2.5 mM | DMSO |
| Glutamate | 200 mM | 2 mM | 20 mM | MAS |
| Glutamine | 200 mM | 2 mM | 20 mM | Cell culture in H2O |
| Hydroquinone | 500 mM | 5 mM | 50 mM | Water |
| Idebenone | 250 mM | 0.25 mM | 2.5 mM | DMSO |
| Malate | 250 mM | 2.5 mM | 25 mM | MAS |
| Malonate | 250 mM | 5 mM | 50 mM | MAS |
| Pyruvate | 500 mM | 5 mM | 50 mM | MAS |
| Succinate | 250 mM | 10 mM | 100 mM | MAS |
| TMPD | 500 mM | 0.125 mM | 1.25 mM | Ethanol |

Rotenone & antimycin used a as in mitostress test (0.5 µM each)

Table 3: Formulation of 1x MAS buffer

|  |  |  |
| --- | --- | --- |
| Reagent | 1x MAS | Amount for 1.0 liter of 1x MAS |
| Sucrose | 70 mM | 23.96 g |
| Mannitol | 220 mM | 40.08 g |
| KH2PO4 | 10 mM | 1.36 g |
| MgCl2hexahydrate | 5 mM | 1.02 g (for hexahydrate; for pure MgCl2: 0.48 g)] |
| HEPES | 2 mM | 0.48 g |
| EGTA | 1 mM | 0.38 g |
| Fatty acid free BSA | 4 mg/ml | 4 g |

Reagents were dissolved in MiliQ-water and pH was adjusted to 7.2 by using KOH. After 0.22 µm filter sterilization, MAS buffer was stored at 4°C.

Preparation upfront

* Hydrate seahorse cartridge
* Have cells at 95% confluency in coated (normal PLO/fibronectin LUHMES coating, optionally with additional laminin) Seahorse plates ready, cultured at least 18 h in these plates for equilibration
* 1x MAS buffer
* Substrates and tool compounds

Experimental procedure

1. Cells were grown in T175 cell culture flasks until they reached a confluency of 95%
2. For assays, conducted one day after cell seeding in Seahorse cell culture plates, proliferating cells were seeded at a density of 60.000 cpw in 100 µl proliferation medium (PM). For assays, conducted two days after cell seeding in Seahorse cell culture plates, proliferating cells were seeded at a density of 40.000 cpw in 100 µl PM.
3. After one hour of attachment, 150 µl of PM was added to proliferating cells.
4. The Seahorse cell culture plates were incubated at 37°C in a humidified 5%-CO2 incubator until the day of assay.
5. At the day of assay, the reagents for the respective assay were prepared. Therefore 1x-MAS buffer was warmed to 37°C in the water bath.
6. The prepared stock solutions of mitochondrial effectors and oxidizable substrates had to be thawed if they were frozen.
7. MAS-buffer is used to prepare the 10x port solutions, which are loaded into the ports of the cartridges. The desired final concentrations for oxidizable substrates and mitochondrial inhibitors are listed in above tables.
8. After preparing the desired concentrated port solutions, they are loaded into the appropriate ports of the cartridge, by adding 56 µl/62 µl/69 µl/75 µl into ports A/B/C/D, respectively.
9. Until the start of the assay, the cartridge was incubated at 37°C without CO2.
10. Create an XF assay template with Mix/Wait/Measure times of 2 min/1 min/2 min, respectively. The equilibration step is excluded from the assay template. Each step is repeated two cycles.
11. After the calibration step of the XF assay template is finished, the cell culture plate is removed from the incubator.
12. The medium was replaced with pre-warmed 1x MAS-buffer (37°C) supplemented with digitonin and ADP. Therefore, the medium was carefully aspirated, and 500 µl of MAS-buffer was added to each well of the cell culture plate.
    1. MAS 12.857 ml + digitonin 13 ul (25 µg/ml on cells) of stock and ADP 130 µl of stock (1 mM on cells)
13. The utility plate was removed from the analyzer and was discarded, while the cartridge remained in the instrument.
14. Finally, the cell culture plate was inserted into the XF analyzer and the assay was started.

Data analysis

* Each experiment has to contain control treated (solvent) samples
* Oxygen levels have to be checked before deeper data analysis (mmO2, not OCR!)
* If oxygen doesn’t get depleted, the data can be analyzed.
* First, basal respiration of all wells gets normalized to the last measurement value before compound injection via port A
* Then the oxygen consumption rate of the first measurement cycle after each tool substrate/tool inhibitor mix injection undergoes comparison. Inhibition results in OCR values smaller in treated samples than in control samples, uncoupling vice versa.
* Compound effects are expressed as percent inhibition relative to control
* Over several experiments (4 weeks, 30 compounds, 4 complexes assessed), the SD of the measurement was 12.5%. Thus compound effects of >25% (=2xSD) were considered as “biologically significant”