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Ex vivo Assessment of Mitochondrial Function in Human Peripheral Blood Mononuclear Cells Using XF Analyzer

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[Abstract] Cellular health and function, as we know today, depend on a large extent on mitochondrial function. The essential function of mitochondria is the energy production, more precisely ATP production, via oxidative phosphorylation. Mitochondrial energy production parameters therefore represent important biomarkers. Studies on human cells have mainly been performed on *in vitro* cell cultures. However, peripheral blood mononuclear cells (PBMCs) are particularly suitable for such examinations. That's why this protocol describes a method to measure key parameters of mitochondrial function in freshly isolated PBMCs with the latest technology, the XF Analyzer. For this *ex vivo* approach PBMCs are first isolated out of human anticoagulated blood. Next, they are attached to the surface of special microplates pre-coated with Poly-D-Lysine. During the subsequent measurement of oxygen consumption rate (OCR) as well as extracellular acidification rate (ECAR) the stress reagents oligomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone and antimycin A are injected. Several mitochondrial parameters can be calculated from the results obtained. The application of this protocol allows the analysis of various influences, such as pharmaceuticals or environmental factors, on human cells.

Keywords: Human peripheral blood mononuclear cells (PBMCs), Mitochondria, XF Analyzer, XF Cell Mito Stress Test, Oxygen consumption rate, Mitochondrial respiration, *Ex vivo*

[Background] Mitochondria play a critical role in maintaining normal cellular function. It is now common knowledge that they not only produce ATP via oxidative phosphorylation but, for example, are also involved in the metabolism of amino acids, lipids and nucleotides, diverse signaling and redox processes as well as quality control and degradation processes including mitophagy and apoptosis (Pfanner *et al.*, 2019). However, mitochondria represent the major site of ATP synthesis in normal cells (Akbari *et al.*, 2019). For this purpose, an electrochemical proton gradient is generated across the mitochondrial inner membrane through the multi-subunit enzyme complexes I–IV. This proton gradient is used by the ATP synthase, also known as complex V, to turn ADP into ATP (Chaban *et al.*, 2014).

The process of oxidative phosphorylation is associated with the reduction of oxygen to water. Accordingly, the oxygen consumption rate of cells can be used for assessing mitochondrial function (Smolina *et al.*, 2017). This principle is the basis of Seahorse XF Analyzers (Agilent Technologies). They provide the possibility to measure not only oxygen consumption rate (OCR), but also the rate of extracellular acidification (ECAR), which is a key indicator of glycolysis. The realtime measurements are

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carried out in multi-well plates which are provided with solid-state sensors consisting of two fluorophores. One is quenched by oxygen (O_2) and the other one is sensitive to pH-value changes. The fluorophores are excited via light-emitting fiber optic bundles, which subsequently detect the fluorescence changes as a result of oxygen consumption or extracellular acidification (Plitzko and Loesgen, 2018). Furthermore, XF Analyzers enable up to four different injections per well during the measurement. All the properties mentioned constitute a significant advantage over the conventionally used clark-type oxygen electrodes for determining oxygen consumption.

Peripheral blood mononuclear cells (PBMCs) as sample in a XF Analyzer implies that the cells have to be attached to the surface of the microplates. Most commonly, this immobilization is done by means of protein solutions such as Cell-Tak™ (Jones *et al.*, 2015; Traba *et al.*, 2016; Lee *et al.*, 2019) or Poly-D-Lysine (Hartman *et al.*, 2014; Nicholas *et al.*, 2017; Thaventhiran *et al.*, 2019). We examined both Cell-Tak™ and Poly-D-Lysine and considered Poly-D-Lysine as most suitable coating method. Since we conducted the Agilent Seahorse XF Cell Mito Stress Test, optimal concentrations of the injected compounds oligomycin, FCCP, antimycin A and rotenone had to be tested as well. A typical curve of a Mito Stress Test is shown in Figure 1. Since oligomycin is an inhibitor of ATP synthase, OCR decreases after its injection. In contrast, OCR increases sharply after FCCP injection, which is an uncoupler of oxidative phosphorylation. The last injection of antimycin A and rotenone, again leads to a decline of OCR, as these two compounds inhibit complex III respectively I of the electron transport chain. The resulting curve is used to calculate various parameters of mitochondrial function (see Figure 1).

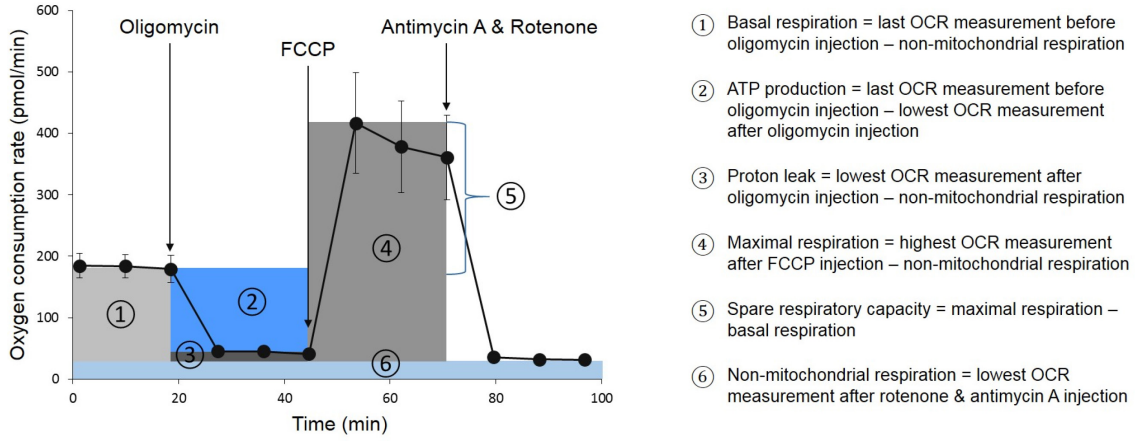

Figure 1. Assessment of mitochondrial respiration parameters by means of Agilent Seahorse XF Cell Mito Stress Test. On the left side a typical course of an OCR measurement with injections of oligomycin after the third, FCCP after the sixth and antimycin A together with rotenone after the ninth measuring point is shown. From this curve, various parameters can be calculated, which are marked in different colors. The calculations of the parameters are shown on the right side.

The protocol described in detail hereafter has been applied to examine the influence of 12 days of *in vivo* caloric reduction in humans. A significant increase of mitochondrial respiratory parameters could

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be detected in PBMCs of a subgroup of the test persons (Schöller-Mann *et al.*, 2020). Besides such *ex vivo* studies the protocol can be used to screen any soluble substance, including pharmaceuticals, dietary supplements or contaminants, due to their *in vitro* effects on PBMCs.

Materials and Reagents

1. 15 ml, 50 ml screw cap tubes (SARSTEDT, catalog numbers: 62.554.502; 62.547.254)
2. 1.5 ml, 2 ml reaction tubes (SARSTEDT, catalog numbers: 72.706; 72.695.500)
3. 20 µl, 200 µl, 1,000 µl pipette tips (SARSTEDT, catalog numbers: 70.1116; 70.760.002; 70.762)
4. 5 ml, 10 ml, 25 ml serological pipettes (SARSTEDT, catalog numbers: 86.1253.001; 86.1254.001; 86.1685.001)
5. 50 ml Leucosep™ tubes (Greiner Bio-One GmbH, catalog number: 227290)
6. Seahorse XF24 FluxPak containing XF24 sensor cartridges, XF24 cell culture microplates and XF Calibrant Solution (Agilent Technologies, catalog number: 100850-001)
7. Human venous blood, EDTA-anticoagulated – S-Monovette® 7.5 ml K3E (SARSTEDT, catalog number: 01.1605.001)
8. Ficoll-Paque™ PLUS (GE Healthcare, catalog number: 17-1440-03)
9. Poly-D-Lysine solution, 1.0 mg/ml (Merck Millipore, catalog number: A-003-E, storage temp. -20 °C)
10. Trypan Blue solution 0.4% (Sigma-Aldrich, catalog number: 93595)
11. Dulbecco's Modified Eagle's Medium (DMEM), high glucose (Sigma-Aldrich, catalog number: D7777, storage temp. 4 °C)
12. Dimethyl sulfoxide (DMSO) (Carl Roth, catalog number: A994)
13. Oligomycin from *Streptomyces diastatochromogenes* (Sigma-Aldrich, catalog number: O4876, storage temp. -20 °C; mixture of isomers A, B, and C), 2.5 mM in DMSO
14. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich, catalog number: C2920, storage temp. 4 °C), 2.5 mM in DMSO
15. Rotenone (Sigma-Aldrich, catalog number: R8875), 2.5 mM in DMSO
16. Antimycin A from *Streptomyces* sp. (Sigma-Aldrich, catalog number: A8674, storage temp. -20 °C), 2.5 mM in DMSO
17. NaCl (Carl Roth, catalog number: 3957)
18. NaOH (Carl Roth, catalog number: 6771)
19. KCl (Carl Roth, catalog number: 6781)
20. $Na_2HPO_4 \cdot 12H_2O$ (Carl Roth, catalog number: N350)
21. KH_2PO_4 (Carl Roth, catalog number: 3904)
22. 1× PBS (see Recipes)
23. 0.9% NaCl solution (see Recipes)
24. 10 M NaOH solution (see Recipes)
25. Poly-D-Lysine working solution (50 µg/ml) (see Recipes)

26. 2.5 mM oligomycin solution (see Recipes)
27. 2.5 mM FCCP solution (see Recipes)
28. 0.1 M rotenone solution (see Recipes)
29. 2.5 mM rotenone solution (see Recipes)
30. 2.5 mM antimycin A solution (see Recipes)
31. Assay medium (see Recipes)

Equipment

1. Pipettes: Eppendorf Research® Plus 10 µl, 20 µl, 200 µl, 1,000 µl (Eppendorf, catalog numbers: 3123000020; 3123000039; 3123000055; 3123000063)
2. Pipetting aid: PipetBoy acu 2 (Integra Biosciences, catalog number: 155 000)
3. Neubauer counting chamber improved (Carl Roth, catalog number: PC72.1)
4. Inverted microscope: Primovert (Carl Zeiss, catalog number: 415510-1100-000)
5. Water bath (GFL, catalog number: 1003)
6. Incubator without CO_2 (GFL, catalog number: 4010)
7. Swing out rotor centrifuge: 5804 R (Eppendorf, catalog numbers: 5805000010; 5804709004)
8. Water purification system: ELGA® PURELAB flex 3 (Veolia, catalog number: PF3XXXXM1)
9. pH meter: FE20 FiveEasy™ (Mettler Toledo, cataog number: 30266626)
10. Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, catalog number: 100737-100)

Software

1. Wave Controller Software (Agilent Technologies, version 1.8.1.1)
2. Optional: Wave Desktop Software (Agilent Technologies)

Procedure

The experimental procedure for the detection of mitochondrial function in human PBMCs using a XF Analyzer is shown schematically in Figure 2. This is followed by a detailed description of the individual work steps.

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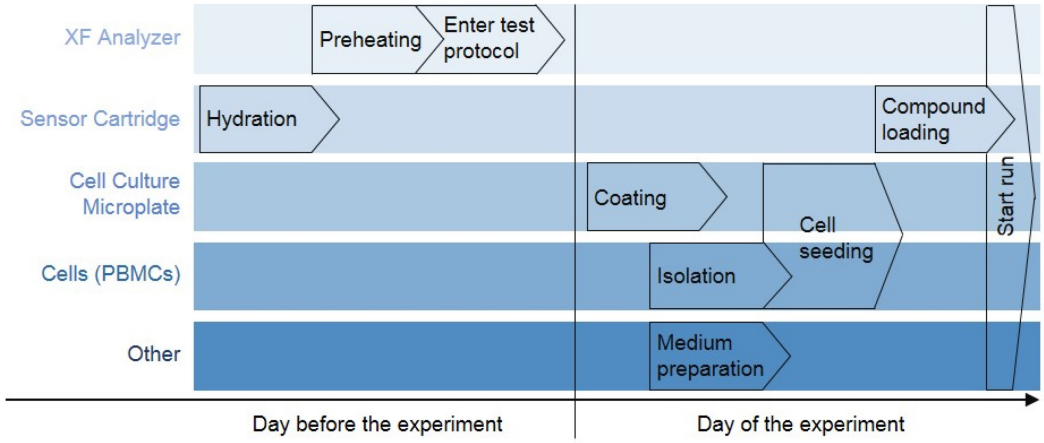

Figure 2. Workflow diagram of mitochondrial function measurement in human PBMCs using a XF Analyzer

A. Day before the experiment
   1. Hydration of XF24 sensor cartridge (consisting of the cartridge lid, the sensor cartridge itself and the utility plate; see Figure 3B and 3C):
      a. Add 1 ml XF Calibrant Solution into each well of the utility plate.
      b. Remove air bubbles that might arise at the sensors with a pipette tip.
      c. Incubate the assembled plate in a non-CO_2 37 °C incubator overnight.
   2. Switch on the XF24 Analyzer (see Figure 3A) and let it warm up to 37 °C overnight.

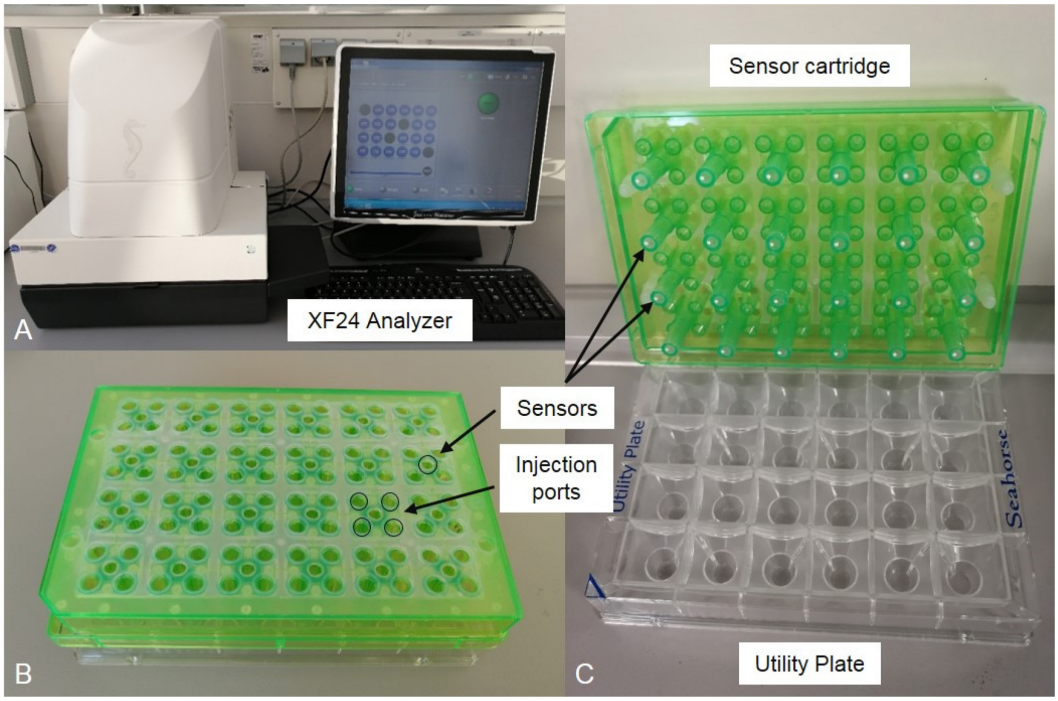

Figure 3. XF24 Analyzer (A) and XF24 sensor cartridge (B and C). The sensor cartridge, including markings of the sensors, is shown from above (B) as well as from below (C). In addition, the injection ports are marked (B) and the utility plate is depicted (C).

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3. Programming of measurement protocol on the XF Analyzer:
   a. Equilibration: 30 min
   b. Measurement period 1:
      i. Mixing: 4 min
      ii. Waiting: 2 min
      iii. Measuring: 3 min
      → 3 repetitions
   c. Injection of port A (50 µl of 7.5 µM oligomycin; final concentration 0.75 µM)
   d. Measurement period 2:
      i. Mixing: 4 min
      ii. Waiting: 2 min
      iii. Measuring: 3 min
      → 3 repetitions
   e. Injection of port B (55 µl of 10 µM FCCP; final concentration 1 µM)
   f. Measurement period 3:
      i. Mixing: 4 min
      ii. Waiting: 2 min
      iii. Measuring: 3 min
      → 3 repetitions
   g. Injection of port C (60 µl of 16.7 µM rotenone/antimycin A; final concentration 1.67 µM)
   h. Measurement period 4:
      i. Mixing: 4 min
      ii. Waiting: 2 min
      iii. Measuring: 3 min
      → 3 repetitions

B. Day of the experiment
   1. Coating of XF24 cell culture microplate:
      a. Add 30 µl of Poly-D-Lysine working solution (50 µg/ml) into each well and incubate the plate for 1 h at RT.
      b. Discard the supernatant, wash each well with 300 µl ddH_2O and let the plate air dry under sterile conditions (approximately 30 min).
   2. Isolation of PBMCs (continue with this step during microplate coating; see Figure 4):
      a. Add 15 ml of Ficoll-Paque™ PLUS (RT) into a 50 ml Leucosep™ tube and centrifugate at 1,000 × *g* for 30 s at RT.
      b. Dilute an appropriate volume of anticoagulated human blood at a ratio of 1:2 with 0.9% NaCl solution (at least 7.5 ml and at most 15 ml).
      c. Transfer the resulting volume (at least 15 ml and at most 30 ml) into the prepared Leucosep™ tube and centrifuge at 1,000 × *g* for 10 min at RT in a swing out rotor with

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brakes switched off.

d. Harvest the PBMC fraction which appears as white layer between the plasma and the Ficoll above the porous barrier of the Leucosep™ tube [for detailed information see manufacturer's instructions or refer to Matt and Bergemann, (2019)]. To do this use a 1,000 µl Eppendorf pipette and collect the PBMCs in a 50 ml screw cap tube.
e. Wash the cells once with 10 ml PBS and centrifuge at 250 × *g* for 10 min.
f. Repeat the washing step twice with 5 ml PBS each.
g. Determine the number of cells using a Neubauer counting chamber improved.

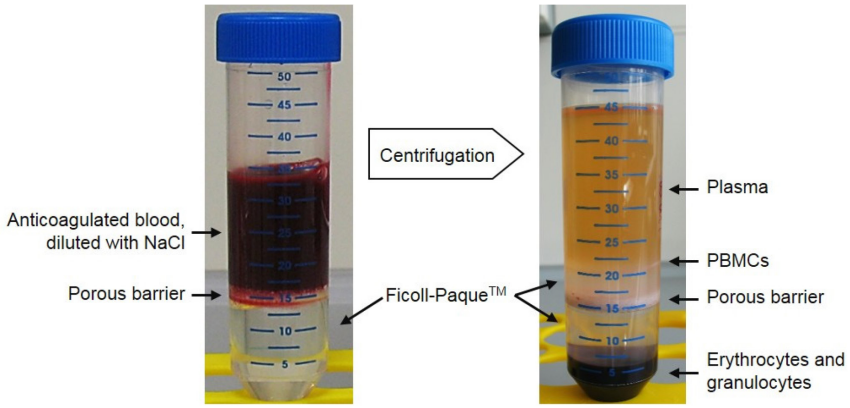

Figure 4. Isolation of PBMCs with a Leucosep™ tube. On the left side, the tube is filled with Ficoll-Paque™ below and anticoagulated and diluted blood above the porous barrier. On the right side, after density gradient centrifugation, the tube shows the following layers from top to bottom: plasma – PBMCs – Ficoll-Paque™ – porous barrier – Ficoll-Paque™ – erythrocytes and granulocytes.

3. Prepare the assay medium and preheat at 37 °C before use.
4. Seeding PBMCs onto pre-coated XF24 cell culture microplate:
   Note: 4 to 5 wells per condition are recommended. The wells A1, B4, C3 and D6 are normally used as background wells (without cells). An exemplary plate layout as well as an image of PBMCs seeded on Poly-D-Lysine is shown in Figure 5.
   a. Take 5 × 10^5 of the isolated PBMCs per well and centrifuge at 250 × *g* for 5 min.
   b. Resuspend the cell pellet in 100 µl assay medium per well.
   c. Add 100 µl of cell suspension per well.
   d. Fill the background wells with 450 µl assay medium.
   e. Centrifuge the plate at 250 × *g* for 1 min in a swing out rotor with brakes switched off.
   f. Add 350 µl assay medium to the wells with cells (final volume in each well: 450 µl).
   g. Incubate the plate at 37 °C without CO_2 until measurement (on average about 1 h).

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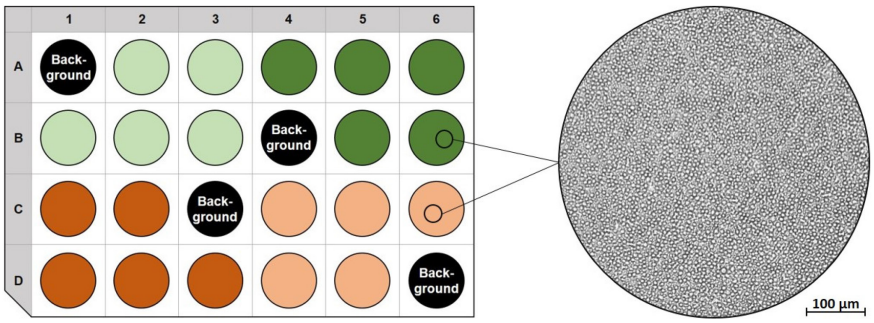

Figure 5. Exemplary plate layout of an XF24 cell culture microplate (left side) and a bright field microscopy image of PBMCs seeded on a Poly-D-Lysine coated XF24 cell culture microplate (right side). Left side: The wells A1, B4, C3 and D6 are used as background wells (without cells). One color (light green, dark green, dark orange, light orange) represents one condition each. There are 5 wells per condition. Right side: The image shows PBMCs in 100× magnification.

5. Loading of XF24 sensor cartridge with compounds:
   a. Prepare compounds as follows:
      i. Dilute 4.86 µl of 2.5 mM oligomycin solution in 1,615 µl assay medium (results in 7.5 µM).
      ii. Dilute 6.48 µl of 2.5 mM FCCP solution in 1,614 µl assay medium (results in 10 µM).
      iii. Dilute 10.82 µl of 2.5 mM rotenone solution and 10.82 µl of 2.5 mM antimycin A solution in 1,598 µl assay medium (results in 16.7 µM each).
   b. Load the injection ports of the XF24 sensor cartridge as follows:
      Note: Avoid the formation of air bubbles in the injection ports and handle the XF24 sensor cartridge with care after loading in order to prevent dripping down of the compounds.
      i. Fill all ports A with 50 µl of 7.5 µM oligomycin.
      ii. Fill all ports B with 55 µl of 10 µM FCCP.
      iii. Fill all ports C with 60 µl of 16.7 µM rotenone/antimycin A.
6. Start the run in the XF Analyzer (follow the instructions of the software):
   a. Place the XF24 sensor cartridge (without lid) in the XF Analyzer and start the calibration.
   b. After completion of the calibration, replace the utility plate by the XF24 cell culture microplate and start measurement.
7. Normalization of measurement results to the number of cells:
   a. Resuspend PBMCs in the final volume of each well (615 µl) by repeatedly pipetting up and down.
   b. Determine the number of cells per well using a Neubauer counting chamber improved.

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Data analysis

1. Divide the counted number of cells per well by 10,000 and enter the results in the Wave software under 'Normalize' in order to normalize the data (see Figure 6).

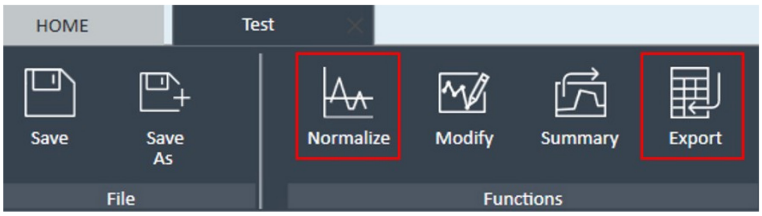

Figure 6. Screenshot of the Wave software with markings for normalization and data export

2. Define outliers based on comparing the course of the OCR of single wells of one condition and exclude them from analysis.
   Note: At least three replicates should be used for analysis.
3. Export the results to the Seahorse XF Cell Mito Stress Test Report Generator, which is linked to the Wave software (see Figure 6). This software tool automatically calculates and reports the parameters, which are analyzed by the Mito Stress Test.
4. Take the calculated values from the Report Generator and analyze them with an appropriate statistical software.

Representative Data

A typical result of an oxygen consumption rate measurement using a XF Analyzer and the stress reagents oligomycin, FCCP, antimycin A and rotenone (Mito Stress Test) is shown in Figure 7.

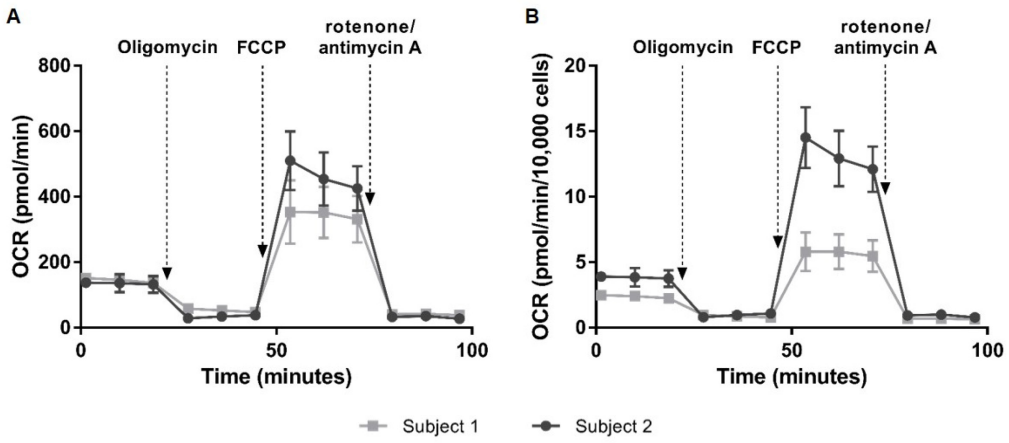

Figure 7. Oxygen consumption rate (OCR) of a Mito Stress Test of PBMCs measured with a XF Analyzer. The results of two different subjects are depicted not normalized (A) and normalized (B).

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Notes

1. Since human blood samples are often available only once per condition, the measurement of a standard PBMC sample, which is stored deep-frozen, is recommended. This can be used for an additional normalization step.
2. Port D of the XF24 sensor cartridge is not used in the described experiment and therefore all ports D can be left blank. Of the ports A, B and C all 24 wells have to be filled in order to ensure an equal injection of the compounds.

Recipes

1. 1× PBS
   8 g NaCl
   0.20 g KCl
   2.88 g $Na_2HPO_4 \cdot 12H_2O$
   1.24 g KH_2PO_4
   ddH_2O to 1 L, pH 7.4
2. 0.9% NaCl solution
   9 g NaCl
   ddH_2O to 1 L
3. 10 M NaOH solution
   40 g NaOH
   ddH_2O to 100 ml
4. Poly-D-Lysine working solution (50 µg/ml)
   50 µl Poly-D-Lysine solution, 1.0 mg/ml
   950 µl ddH_2O
5. 2.5 mM oligomycin solution
   5 mg oligomycin
   2.528 ml DMSO
6. 2.5 mM FCCP solution
   10 mg FCCP
   15.737 ml DMSO
7. 0.1 M rotenone solution
   1 g rotenone
   25.353 ml DMSO
8. 2.5 mM rotenone solution
   25 µl of 0.1 M rotenone solution
   975 µl DMSO
9. 2.5 mM antimycin A solution

25 mg antimycin A
18.529 ml DMSO

10. Assay medium
    0.675 g Dulbecco's Modified Eagle's Medium, high glucose
    ddH_2O to 50 ml, pH 7.4

Acknowledgments

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Competing interests

The authors declare no competing interests.

Ethics

The experiments were conducted in accordance with the declaration of Helsinki and approved by the ethics committee of the Landesärztekammer Baden-Württemberg, Germany. Furthermore, all volunteers were informed in advance and gave their written consent to the use of their blood samples.

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