Version 1 Last updated 20 December 2018

ab243390 Mitochondrial Stress Test Companion Assay

View Mitochondrial Stress Test Companion Assay datasheet:
www.abcam.com/ab243390

For the measurement of extracellular oxygen consumption in whole cell populations, isolated mitochondria, permeabilized cells, a wide range of 3D cultures, isolated enzymes, bacteria, yeasts and molds.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Mitochondrial Stress Test Companion Assay (ab243390) is used iin conjunction with Mitochondrial Stress Test Complete Assay Kit (ab232857) or Extracellular Oxygen Consumption Assay (ab197243). The companion assay contains all metabolic modulators required to characterise the main parameters of mitochondrial function in live cells. The Oxygen Consumption Assay (HS Method) is a highly flexible 96- or 384-well fluorescence plate reader-based approach, for the direct, real-time analysis of cellular respiration and mitochondrial function.

The easy-to-use assay allows measurement of extracellular oxygen consumption rates (OCR) with whole cell populations (both adherent and suspension cells), isolated mitochondria, permeabilized cells and a wide range of 3D cultures including: tissues, small organisms, spheroids, scaffolds and matrixes. The assay is also suitable for measurement of isolated enzymes, bacteria, yeasts and molds.

The Extracellular O_2 probe is chemically stable and inert, water-soluble and cell impermeable, making it the ideal and scalable mix-and-measure reagent for use in a wide range of cell culture conditions - all measured using a fluorescence plate-reader. In this assay, Extracellular O_2 probe is quenched by O_2, through molecular collision, and thus the amount of fluorescence signal is inversely proportional to the amount of extracellular O_2 in the sample. Rates of oxygen consumption are calculated from the changes in fluorescence signal over time.

The reaction is non-destructive and fully reversible (neither Extracellular O_2 probe nor O_2 are consumed), facilitating measurement of time courses and drug treatments.

This is a companion kit to be used in combination with Mitochondrial Stress Test Complete Assay Kit (ab232857) or Extracellular Oxygen Consumption Assay (ab197243); or with Extracellular Oxygen Consumption Reagent (ab197242) together with Mineral Oil High sensitivity (ab243855).

2. Protocol Summary

Plate desired cells and return to culture overnight.

Prepare all controls and Extracellular O_2 probe (from ab232857 or ab197243) as instructed.

Aspirate spent media, add fresh media and Extracellular O_2 probe.

Add control compounds at pre-optimised concentrations. Overlay plate with HS mineral oil (from ab232857 or ab197243).

Measure on fluorescence plate reader using recommended settings. Analyse kinetic data output as recommended.

3. General guidelines, precautions, and troubleshooting

- Please observe safe laboratory practice and consult the safety datasheet.
- For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide: www.abcam.com/assaykitguidelines
- For typical data produced using the assay, please see the assay kit datasheet on our website.

4. Materials Supplied, and Storage and Stability

- Store kit at -20°C in the dark immediately upon receipt and check below in Section 6 for storage for individual components.
- Aliquot components in working volumes before storing at the recommended temperature.
- Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage condition
Oligomycin	10 µg	-20°C
FCCP	4 µg	-20°C
Antimycin A	4 µg	-20°C
Glucose Oxidase	112.5 µg	-20°C

5. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Mitochondrial Stress Test Complete Assay Kit (ab232857) or Extracellular Oxygen Consumption Assay (ab197243); or Extracellular Oxygen Consumption Reagent (ab197242) together with Mineral Oil High sensitivity (ab243855).
- Standard clear 96- well TC+ plates OR 96- well black wall clear bottom TC+ plates.
- Fluorescence plate reader, with suitable filter(s) and plate temperature control.
- Plate block heater for plate preparation.

6. Reagent Preparation

- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- Prepare only as much reagent as is needed on the day of the experiment.
- Any components not listed here are ready to use as supplied.

6.1 Oligomycin:

6.1.1 To prepare 300 µM Oligomycin stock (200X) dissolve vial contents in 35 µL of DMSO.

6.1.2 Aliquot into two equal volumes and store at -20°C, each aliquot is sufficient for a minimum of 25 wells (1.5 µM final concentration/well).

6.2 FCCP:

6.2.1 To prepare 500 µM stock dissolve vial contents in 35 µL (200X) of DMSO.

6.2.2 Aliquot into two equal volumes and store at -20°C, each aliquot sufficient for a minimum of 25 wells (2.5 µM final concentration/well).

6.3 Antimycin A:

6.3.1 To prepare 200 µM Antimycin stock, dissolve vial contents in 35 µL (200X) of DMSO.

6.3.2 Aliquot into two equal volumes and store at -20°C, each aliquot is sufficient for a minimum of 25 wells (1 µM final concentration/well).

6.4 Glucose Oxidase:

6.4.1 Reconstitute in 70 µL distilled water to obtain 1.5 mg/mL stock solution.

6.4.2 Aliquot into two equal volumes and store at -20°C. Each aliquot is sufficient for at least 2 wells (10 µL per well).

7. Plate Reader Set-Up

7.1 Measurement Parameters

The Extracellular O_2 probe is a chemically stable and inert, biopolymer-based, cell impermeable oxygen-sensing fluorophore.

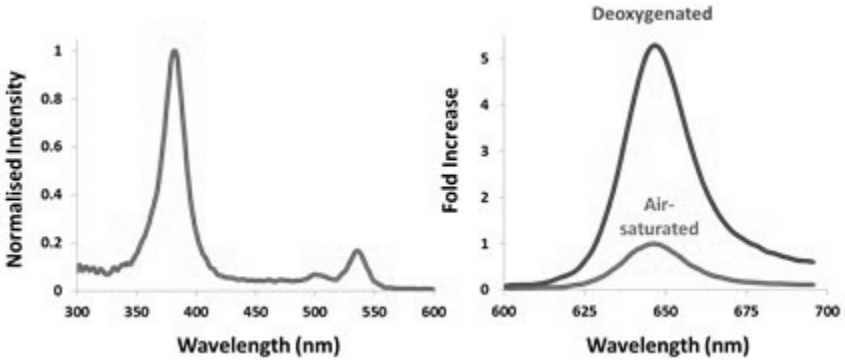

Figure 1. Excitation and emission spectra of the Extracellular O2 probe. Left panel shows normalized excitation (Ex = 360 – 400 nm; Peak 380 nm). Right panel shows emission (Em = 630 – 680 nm; Peak 650 nm) in deoxygenated and oxygenated (air-saturated) conditions.

	Peak Maxima (nm)	Peak (nm)
Excitation*	380	360 – 400
Emission	650	630 – 680

*Excitation at 532 ± 7.5 nm is also possible

- This assay is measured with prompt or time-resolved fluorescence (TR-F) readers, monochromator or filter-based. Optimal wavelengths are λ = 380 nm for excitation (λ = 532 nm can also be used) and λ = 650 nm for emission.
- Probe signals should be at least 3 times above blank signal.
- The O_2 probe response is temperature dependent, so good temperature control of the plate during the measurement is important.

7.2 Instruments and Settings

Outlined below are three fluorescence modalities that can be used with this assay, depending on the plate reader type and instrument setup.

7.2.1 Basic: Intensity Measurement

Measurement of Signal Intensity (sometimes referred to as Prompt) provides flexibility to use wide range of commonly available fluorescence-, monochromator or filter-based plate readers. Optimal wavelengths are λ = 380 nm for excitation and λ = 650 nm for emission, with detection gain parameters (PMT) typically set at medium or high.

Δ Note: Extracellular O_2 probe should return Signal to Blank ratio (S:B) ≥ 3.

7.2.2 Standard: TR-F Measurement

Using time-resolved fluorescence (TR-F) will increase performance levels. TR-F measurement reduces non-specific background and increases sensitivity.
Optimal delay time is 30 µs and gate (integration) time is 100 µs.

Δ Note: Extracellular O_2 probe should return Signal to Blank ratio (S:B) ≥ 3. S:B ~ 10 are typical.

7.2.3 Advanced: Dual-Read TR-F (Lifetime calculation)

Optimal performance can be achieved using dual-read TR-F in combination with subsequent ratiometric Lifetime calculation, to maximize dynamic range. See Figure 2.

Δ Note: Extracellular O_2 probe should return Signal to Blank ratio (S:B) ≥ 3. S:B up to 60 are possible.

Dual-read TR-F and subsequent Lifetime calculation allows measurement of the rate of fluorescence decay of the Extracellular O_2 probe, and can provide measurements of oxygen consumption that are more stable and with a wider dynamic range than measuring signal intensity.

Optimal dual-delay and gate (integration) times:

1. Integration window 1: 30 µs delay (D1), 30 µs measurement time (W1)
2. Integration window 2: 70 µs delay (D2), 30 µs measurement time (W2)

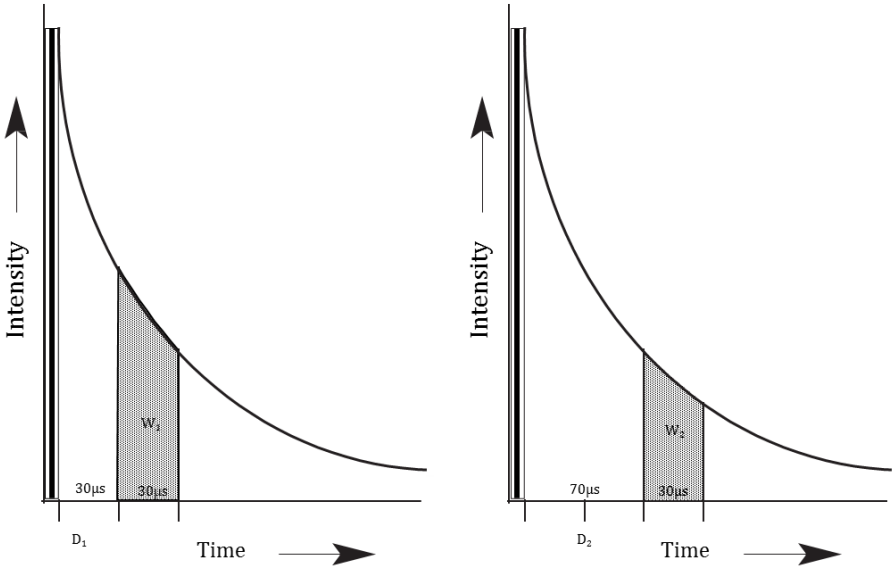

Figure 2. Illustrating dual read TR-F measurement.

Use the dual intensity readings to calculate the corresponding Lifetime (µs) using the following transformation:

Lifetime (µs) [T] = (D2-D1)/ln(W1/W2)

Where W1 and W2 represent the two (dual) measurement windows and D1 and D2 represent the delay time prior to measurement of W1 and W2 respectively. This provides Lifetime values in µs at each measured time point for each individual sample (Figure 2).

Δ Note: S:B for Integration window 2 is recommended to be ≥ 10 to allow accurate Lifetime calculation. Range of Lifetime values should be 22 – 68 µs, and should only be calculated from samples containing Extracellular O2 probe. Lifetime values should not be calculated from blank wells.

See Instrument and Measurement Setting table below for instrument-specific setting and filters. Readers equipped with a TR-F mode, may achieve improved performance using delay and gate time of 30 µs and 100 µs.

Table 1. Recommended Instrument and Measurement Settings

Instrument	Optical Configuration	Intg1 (D_1/W_1) Intg2 (D_2/W_2)	Optimum Mode	Ex (nm) Em (nm)
BioTek: Cytation 3 / 5	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 645 ± 15nm
BioTek: Synergy H1, Neo, 2	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 645 ± 15nm
BMG Labtech: CLARIOstar	Filter-based Bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EX) Em 665 ± 50nm or Em 645± 10nm With LP-TR Dichroic
BMG Labtech: FLUOstar Omega / POLARstar Omega	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EXL) Em 655 ± 25nm (BP-655)
Perkin Elmer: VICTOR series/ X4, X5	Filter-based Top read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 40nm (D340) Em 642 ± 10nm (D642)

Instrument	Optical Configuration	Intg1 (D_1/W_1) Intg2 (D_2/W_2)	Optimum Mode	Ex (nm) Em (nm)
Tecan: Infinite M1000Pro / F200Pro	Monochromator / Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 650 ±20nm or Em 670±40nm
BioTek: Synergy HTx / Mx	Monochromator / Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 380±20nm Em 650±15nm
BMG Labtech: PHERAstar FS	Filter-based Top or bottom read	40 / 100µs n/a	TR-F	Ex 337 nm (HTRF Module) Em 665 nm (HTRF Module)
BMG Labtech: FLUOstar Optima / POLARstar Optima	Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 340 ± 50nm (TR-EXL) Em 655 ± 50nm (BP-655)
Perkin Elmer: EnVision	Filter-based Top read	40 / 100µs n/a	TR-F	Ex 340 ±60nm (X340) Em 650 ± 8nm (M650)
Perkin Elmer: EnSpire	Monochromator based Top read	40 / 100µs n/a	TR-F	Ex 380 ±20nm Em 650±20nm
Tecan:	Monochromator	30 / 100µs	TR-F	Ex 380±20nm

Infinite M200Pro / Saffire / Genios Pro	/ Filter-based Top or bottom read	n/a		Em 650±20nm
Mol. Devices: SpectraMax / Flexstation / Gemini	Monochromator based Top or bottom read	n/a n/a	Intensity (Prompt)	Ex 380nm Em 650nm

8. Signal Optimization

- This step is recommended for first time users.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature.

8.1 Prepare 8 replicate wells of a 96-well plate, by adding 90 µL pre-warmed culture medium to each well (A1-A4, B1-B4).

8.2 Add 10 µL reconstituted Extracellular O_2 probe to 4 of the replicate wells (A1-A4) and 10 µL water, PBS or media to the remaining replicates wells (B1-B4).

8.3 Promptly add two drops (or 100 µL) pre-warmed High sensitivity mineral oil to all eight replicate wells, taking care to avoid air bubbles.

8.4 Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).

8.5 Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) readings (linear phase) and calculate Signal to Blank (S:B) ratio using the last reading at 30 minutes.

Δ Note: For dual read TR-F, calculate S:B for each measurement window.

Δ Note: For most fluorescence plate readers, set up according to Section 7. Extracellular O_2 probe should return a S:B ≥ 3. Higher readings are expected with TR-F and dual read TR-F measurement.

	1	2	3	4
A	Media + O_2 Probe + Oil	Media + O_2 Probe + Oil	Media + O_2 Probe + Oil	Media + O_2 Probe + Oil
B	Media + Oil	Media + Oil	Media + Oil	Media + Oil

8.6 The following options may be helpful to improve S:B if the determine ratio is not as high as expected:

8.6.1 Increase Gain (PMT) setting or flash energy/number
8.6.2 Adjust TR-F focal height
8.6.3 Repeat without Phenol red or serum
8.6.4 Measure as bottom read as available
8.6.5 Increase volume of Extracellular O2 Consumption Reagent
8.6.6 Contact instrument supplier for further options

9. Sample preparation

9.1 Cell Culture and Plating

9.1.1 Count cells and adjust to the desired plating density in culture medium (typically ~4-8×10^4 cells per well for adherent cells, depending on cell type).

9.1.2 Return the plate to culture overnight (typically >14 h). For suspension cells, seed ~4x10^5 cells in 100 µL medium on the day of the assay.

ΔNote: Typically, cells are seeded at a density to achieve full confluence on the day of measurement. Plating density, cell type, and basal metabolic rate will determine the oxygen consumption rate measured. If edge effects are observed, we recommend allowing the plate to stand for 30 min at RT after plating before returning to culture of cells or to omit the outer wells of the plate.

10. Pre-Assay Preparation

ΔNote: Prepare Working Stocks from Oligomycin, FCCP and Antimycin A aliquoted as described under Section 6 by diluting them 1/20 in fresh assay medium.

- FCCP exhibits a bell-shaped dose-response which can vary between cell types. The concentration which delivers maximum respiratory activity should be determined for each cell type. This can be achieved by running an FCCP serial dilution (typically between 15 – 0.5 µM).
- Oligomycin and Antimycin A are typically used at 1.5 µM or 1 µM, respectively. However, the optimal concentration can be cell-type dependent. Optimum concentrations can be determined by measuring OCR at varying Oligomycin and Antimycin A concentrations.

ΔNote: Test compounds/drugs (optional, not provided): Dilute so that volume of added compound is kept low (1-5 µL) to minimize any potential vehicle effects. We recommend the use of triplicate wells for each treatment.

- Drugs or test compounds are typically added immediately prior to measurement. Longer treatment times can be used as required.

ΔNote: Plate reader, plate block heater and buffers should be pre-warmed to 37°C prior to use. Set up plate reader as described. For first time users, performing the signal optimization step is advised.

11. Assay Protocol

11.1 Remove spent culture medium from all assay wells and replace with 80 µL of fresh culture media (see Figure 3 for recommended plate map), being careful not to dislodge cells. Add 90 µL of pre-warmed fresh culture medium to the wells designated as Signal and Glucose Oxidase Control wells (typically H 7-10).

ΔNote: We recommend always leaving two wells free (H11 and H12) from the addition of Extracellular O_2 probe, for use as Blank Controls. Add 100 µL of fresh culture media to these Blank Control wells.

11.2 Add 10 µL of reconstituted Extracellular O_2 probe to each well, except those wells for use as Blank Controls.

ΔNote: If plating a full 96-well plate, we recommend combining Step 10.1 and Step 10.2 by adding the 1 mL of reconstituted Extracellular O_2 probe to 10 mL pre-warmed fresh culture media and using a multi-channel pipette add, 90 µL of Extracellular O2 probe in media stock to each well. Add 100 µL of fresh culture media only (no Extracellular O_2 probe) to the Blank Control wells.

11.3 **(optional):** The Mitochondrial Stress Test can be used to determine the impact of different treatments (drug treatment, signaling pathway modulation, genetic manipulation, the addition of alternative nutrients etc.) on mitochondrial respiration. Add test compound, vehicle or other desired treatment to test wells and return the plate to culture for the desired period or test immediately. For a complete characterization, all controls (Vehicle Control, Oligomycin, FCCP, and Antimycin A) should be applied for each condition.

ΔNote: Keeping volume of added compound low (1-5 µL) and use of a vehicle control is recommended to minimize any potential vehicle effects.

11.4 Add 10 µL of Oligomycin working stock (prepared as described above) to wells to measure ATP-Coupled

Respiration (typically wells A-G 4-6, see Fig. 3), 10 µL FCCP working stock to wells to measure Maximal Respiration (typically wells A-G 7-9, see Fig. 3) and 10 µL Antimycin A working stock to wells to measure Non-Respiratory Oxygen Consumption (typically wells A-G 10-12, see Figure. 3). Add 10 µL of fresh culture medium to wells designated 'Probe only' (H 9-10).

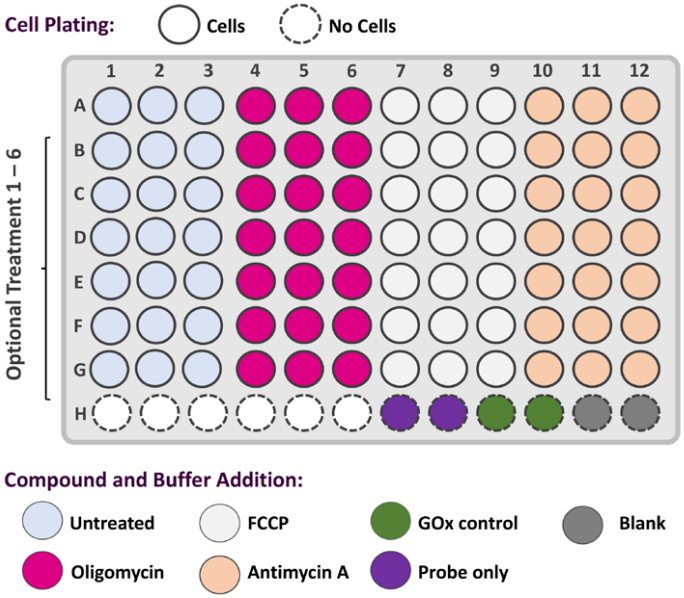

Figure 3: Recommended plate map for the Mitochondrial Stress Test with optional different treatments. Using this plate map, up to 7 different conditions can be tested simultaneously in triplicate across all treatment conditions.

11.5 Add 10 µL reconstituted Glucose Oxidase to the Signal Control wells (typically H 7-8).

11.6 Seal each well with 100 µL of pre-warmed HS Mineral Oil, taking care to avoid bubbles. Read the plate immediately on a fluorescence plate reader.

12. Calculations

12.1 Plot the Blank Control well-corrected Extracellular O_2 probe Intensity or Lifetime values versus Time (mins; Figure 4).

12.2 Select the linear portion of each signal profile (avoiding any initial lag or subsequent plateau) and apply linear regression to determine the slope (OCR) and correlation coefficient for each well.

13. Typical Data

Typical data provided **for demonstration purposes only**.

The slopes (m) derived from the linear part of the Signal Profile of cells treated with the kit components are indicative of oxygen consumption rates (OCR); an established measure of mitochondrial function.

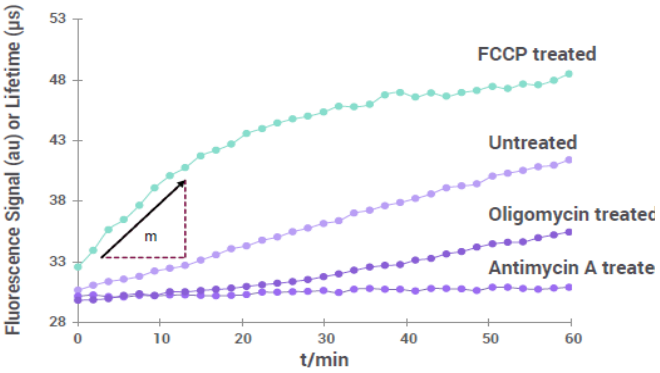

Figure 4. Signal Profiles of HepG2 oxygen consumption obtained using the Mitochondrial Stress Test Complete Assay. The slopes (m) derived from the linear part of the Extracellular O_2 probe Signal Profile of cells treated with the kit components are indicative of oxygen consumption rates (OCR); an established measure of mitochondrial function.

Non-Respiratory Oxygen Consumption Rate (OCR): $m_{\text{Antimycin A}}$

Slope (m) of Signal Profile after Antimycin A treatment. This value can be subtracted from all other rates in many experimental designs.

In most cell types, treatment with Antimycin A will result in diminished OCRs. However, in some cases oxygen consumption is observed even though ETC has been fully blocked. The remaining oxygen consumption may be attributed to detoxifying or desaturase enzyme activity or non-respiratory NADPH oxygenase activity. If unsure, if ETC blockage with Antimycin A was achieved, optimise Antimycin A concentrations by running a serial dilution or co-treatment with Rotenone. In most cases, Non-Respiratory Oxygen Consumption can be regarded as constant and subtracted from all other values for further analysis.

Basal Respiration Rate: $m_{untreated} - m_{Antimycin\ A}$

Slope (m) of Signal Profile of untreated cells. Impacted by factors such as current cellular ATP demand, metabolic phenotype and substrate availability.

Maximal Respiration Rate: $m_{FFCP} - m_{Antimycin\ A}$

Slope of Signal Profile after FCCP treatment. When using optimised FCCP concentrations, this value characterises the theoretical maximal mitochondrial capacity under given conditions. FCCP dissipates the mitochondrial membrane potential, uncoupling oxygen consumption from ATP production and allowing the respiratory chain to work at maximal capacity.

Spare Respiratory Capacity: – $m_{FFCP} - m_{Untreated}$

Difference between Signal Profile of FCCP treated cells (Maximal Respiration Rate) and untreated cells (Basal Respiration Rate). Spare Respiratory Capacity is a measure of the cells ability to respond to sudden increases in energy demand or other metabolic stressors. Reduced Spare Respiratory Capacity can be important indicator of mitochondrial dysfunction.

Non ATP-Coupled Oxygen Consumption ('Proton Leak'): – $m_{Oligomycin} - m_{Antimycin\ A}$

Slope of Signal Profile of Oligomycin treated cells indicates dissipation of the mitochondrial proton-gradient by means other than F1/Fo-ATPase activity (see Appendix A for details). Elevated levels of Non ATP-Coupled Oxygen Consumption can be a sign of impaired mitochondrial membrane integrity. In most cases, however, dissipation is a controlled process, caused for example by ion or substrate cycling or by specialized uncoupling proteins and can make up to 40% of the basal OCR in some tissues.

ATP-Coupled Oxygen Consumption: – $m_{Untreated} - m_{Oligomycin}$

Difference between Signal Profile slopes of untreated cells (Basal Respiration Rate) and Oligomycin treated cells (Non ATP-Coupled Oxygen Consumption). Reflects the portion of Basal Respiration that is linked to ATP production.

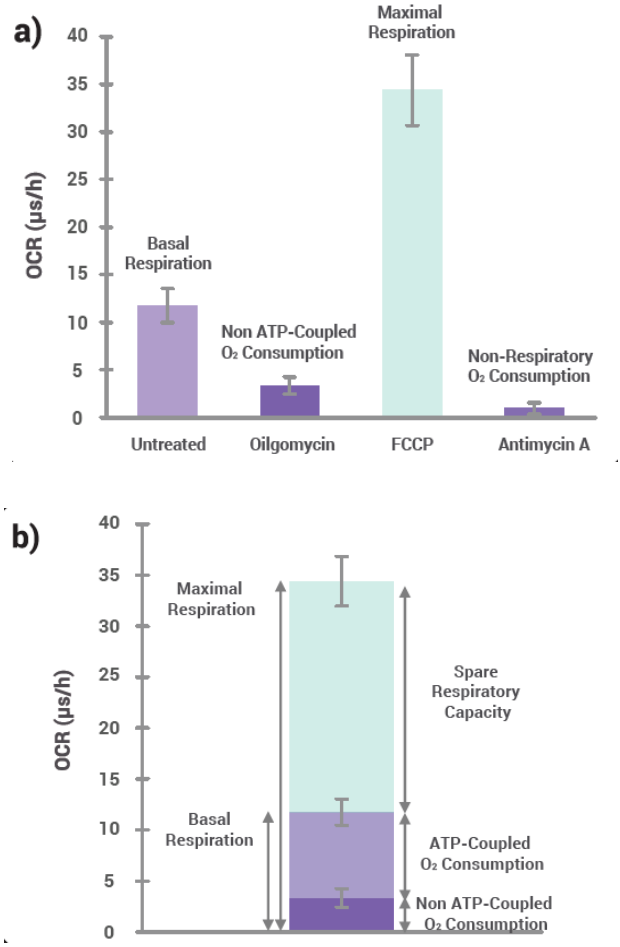

Figure 5. Full characterisation of mitochondrial function using the Mitochondrial Stress Test Complete Assay. a) The OCR (m) from the linear portion of the Signal Profile from each Stress Test condition reflect Basal Respiration, Non ATP-Coupled Oxygen Consumption, Maximal Respiration and Non Respiratory Oxygen Consumption. ATP-Coupled Oxygen Consumption and Spare Respiratory Capacity are calculated from these values as described in detail in the text. b) The contribution of each of these discrete metabolic processes to the Maximal Respiration can be conveniently visualised producing a detailed picture of aerobic metabolism.

Substrate depletion limits maximal respiration:

Substrate availability is an important contributor to Spare Respiratory Capacity. Figure 6 shows the Respiration of HepG2 cells measured in high glucose, low glucose and in low glucose plus oleate. Limited Spare Respiratory Capacity (Maximal Respiration minus Basal Respiration) is observed in the low glucose condition due to a restriction in the supply of reducing equivalents to the ETC. The provision of the dietary long chain fatty acid oleate significantly increases Spare Respiratory Capacity, as beta oxidation now provides the necessary reducing equivalents. Similar increases are achieved by increasing glucose concentration.

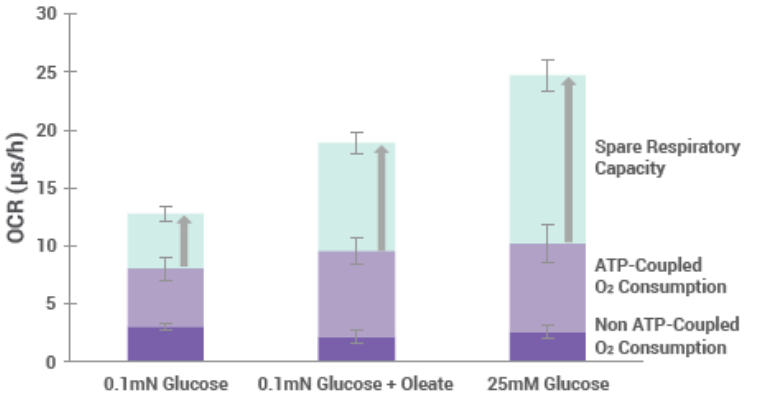

Figure 6. Basal Respiration and Maximal Respiration in HepG2 cells measured in different nutrient conditions. In low glucose medium, Spare Respiratory Capacity is low, but significantly increases in the presence of high glucose concentrations or 150 µM Oleate, due to the provision of reducing equivalents to fuel ETC activity.

14. Notes

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