

Version 12 Last updated 20 November 2018

ab197243

Extracellular O₂

Consumption Assay

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

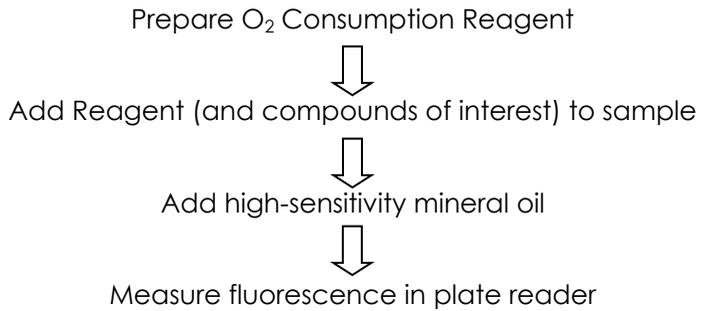
Extracellular O₂ Consumption Assay (ab197243) measures extracellular oxygen consumption rate (OCR) in a variety of samples.

Mitochondrial dysfunction is implicated in numerous disease states and is also a major mechanism of drug-induced toxicity. Oxygen consumption is one of the most informative and direct measures of mitochondrial function. Traditional methods of measuring oxygen consumption are hampered by the limitations of low throughput and high complexity. The Extracellular O₂ Consumption Assay (ab197243) solves these limitations by providing a direct, real-time measurement of extracellular oxygen consumption rate (OCR) to analyze cellular respiration and mitochondrial function.

The assay is based on the ability of oxygen to quench the excited state of Extracellular O₂ Consumption reagent present in the kit. As the test material respire, oxygen is depleted in the surrounding environment, which is seen as an increase in phosphorescence signal. The addition of a high-sensitivity mineral oil is used to limit back diffusion of ambient oxygen. Measured on standard fluorescence plate readers (96- or 384- well), with standard cell culture microplates, the Extracellular O₂ Consumption Assay (ab197243) is suitable for use 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 flexible plate reader format, allows multiparametric or multiplex combination with other similar products. For example, in combination with Glycolysis Assay (ab197244), the Extracellular O₂ Consumption Assay (ab197243) allows simultaneous real-time measurement of mitochondrial respiration and glycolysis and the analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

Δ Note: Reconstituted reagent is stable for 1 month.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity		Storage condition (before prep)	Storage condition (after prep)
	96 tests	4 x 96 tests		
Extracellular O ₂ Consumption Reagent	1 vial	4 x 1 vial	4°C	-20°C
High Sensitivity Mineral Oil	1 dropper bottle (15 mL)	4 x 1 dropper bottle (15 mL)	4°C	RT

7. Materials Required, Not Supplied

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

- Microplate reader capable of measuring fluorescence, with suitable filter and plate temperature control – see Instrument and Measurement Settings section for suitable plate readers
- Double distilled water (ddH₂O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Sterile 96 well plate (black wall with clear flat bottom), or standard clear plates for cell culture

For cells:

- Cell culture medium

For isolated mitochondria:

- Measurement buffer (250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM MgCl₂, 30 mM, K₂HPO₄, pH 7.4)
- Mitochondrial substrate (succinate, glutamate or malate)
- ADP

8. Technical Hints

- **This kit is sold based on number of tests. A “test” simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.
- Refer to Instrument and Measurement Settings table (Table 1) for recommended settings for your plate reader.
- While compatible with all plate types, black border clear bottom plates give optimal signal-to-noise ratios.
- For first time users, we recommend performing a Signal Optimization Step (see Section 11).

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Extracellular O₂ Consumption Reagent:

Prepare a stock solution of the Extracellular O₂ Consumption Reagent by adding 1 mL of ddH₂O, PBS, culture media or buffer to the vial. Mix by gently aspirating 3 – 4 times.

Recommended working dilution = 1/15 (10 µL per 150 µL of sample for 1x 96-wp).

Reconstituted probe stock can be stored in the dark between +2 to +8 °C for two days or stored as aliquots in water at -20 °C for use within one month (avoid freeze thaw).

9.2 High Sensitivity Mineral Oil:

Ready to use as supplied. Pre-warm to 37°C prior to use.

Although mineral oil is provided in a dropper bottle for convenience, we recommend using a repeater pipette for routine use. To apply oil using a repeater pipette, trim 3 – 4 mm off the tip at a 45° angle. Remove internal nozzle cap from the dropper bottle and slowly pick up the pre-warmed mineral oil.

Store mineral oil at room temperature in the dark.

10. Plate Reader Set-Up

10.1 Measurement Parameters

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

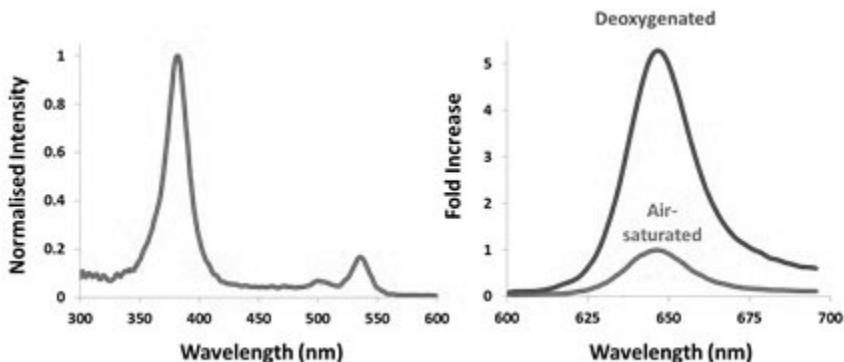


Figure 1. Excitation and emission spectra of the Extracellular O₂ Consumption Reagent. Left panel shows normalized excitation (Ex = 360 – 400 nm; Peak 380 nm). Right panel shows emission (Em = 630 – 680 nm; Peak 650 nm) in deoxygenated and oxygenated (air-saturated) conditions.

	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 $\lambda = 380$ nm for excitation ($\lambda = 532$ nm can also be used) and $\lambda = 650$ nm for emission.
- Probe signals should be at least 3 times above blank signal.
- The O₂ probe response is temperature dependent, so good temperature control of the plate during the measurement is important.

10.2 Fluorescence measurements

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

10.2.1. 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 $\lambda = 380$ nm for excitation and $\lambda = 650$ nm for emission, with detection gain parameters (PMT) typically set at medium or high.

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

10.2.2. 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 μ s and gate (integration) time is 100 μ s.

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

10.2.3. 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.

Δ Note: Extracellular consumption reagent 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 consumption reagent, 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:

- Integration window 1: 30 μ s delay (D1), 30 μ s measurement time (W1)
- Integration window 2: 70 μ s delay (D2), 30 μ s measurement time (W2)

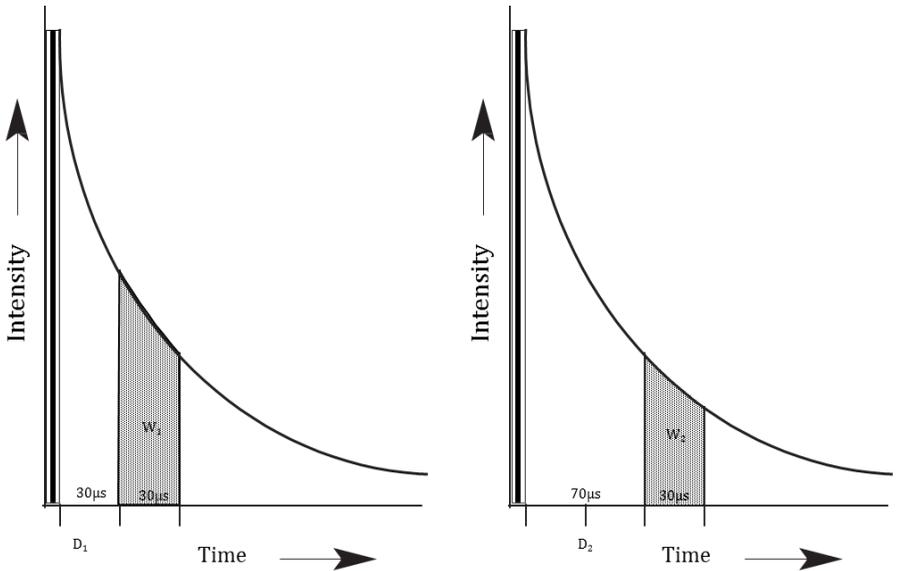


Figure 2. Illustrating dual read TR-F measurement.

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

$$\text{Lifetime } (\mu\text{s}) [T] = (D_2 - D_1) / \ln(W_1 / W_2)$$

Where W_1 and W_2 represent the two (dual) measurement windows and D_1 and D_2 represent the delay time prior to measurement of W_1 and W_2 respectively. This provides Lifetime values in μ s 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 μ s, and should only be calculated from samples containing reagent. 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 μ s and 100 μ s.

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 / 30 μ s 70 / 30 μ s	Dual-read TR-F (Lifetime)	Ex 380 \pm 20nm Em 645 \pm 15nm
BioTek: Synergy H1, Neo, 2	Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual-read TR-F (Lifetime)	Ex 380 \pm 20nm Em 645 \pm 15nm
BMG Labtech: CLARIOstar	Filter-based Bottom read	30 / 30 μ s 70 / 30 μ s	Dual-read TR-F (Lifetime)	Ex 340 \pm 50nm (TR-EX) Em 665 \pm 50nm or Em 645 \pm 10nm With LP-TR Dichroic
BMG Labtech: FLUOstar Omega / POLARstar Omega	Filter-based Top or bottom read	30 / 30 μ s 70 / 30 μ s	Dual-read TR-F (Lifetime)	Ex 340 \pm 50nm (TR-EXL) Em 655 \pm 25nm (BP-655)
Perkin Elmer: VICTOR series/ X4, X5	Filter-based Top read	30 / 30 μ s 70 / 30 μ s	Dual-read TR-F (Lifetime)	Ex 340 \pm 40nm (D340) Em 642 \pm 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)

Instrument	Optical Configuration	Intg1 (D ₁ /W ₁) Intg2 (D ₂ /W ₂)	Optimum Mode	Ex (nm) Em (nm)
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: Infinite M200Pro / Saffire / Genios Pro	Monochromator / Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 380±20nm Em 650±20nm
Mol. Devices: SpectraMax / Flexstation / Gemini	Monochromator based Top or bottom read	n/a n/a	Intensity (Prompt)	Ex 380nm Em 650nm

11. 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.
- 11.1 Prepare 8 replicate wells of a 96-well plate, by adding 150 μL pre-warmed culture medium to each well (A1-A4, B1-B4).
 - 11.2 Add 10 μL reconstituted Extracellular O_2 Consumption Reagent to 4 of the replicate wells (A1-A4) and 10 μL water, PBS or media to the remaining replicates wells (B1-B4).
 - 11.3 Promptly add two drops (or 100 μL) pre-warmed High sensitivity mineral oil to all eight replicate wells, taking care to avoid air bubbles.
 - 11.4 Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes).
 - 11.5 Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) readings (linear phase) and calculate Signal to Blank (S:B) ratio.

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

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

- 11.6 The following options may be helpful to improve S:B if the determine ratio is not as high as expected:
 - 11.6.1. Increase Gain (PMT) setting or flash energy/number
 - 11.6.2. Adjust TR-F focal height
 - 11.6.3. Repeat without Phenol red or serum
 - 11.6.4. Measure as bottom read as available
 - 11.6.5. Increase volume of Extracellular O_2 Consumption Reagent
 - 11.6.6. Contact instrument supplier for further options

12. Sample Preparation

General Sample Information:

- Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.
- Prepare test compounds for sample treatment as desired. Example of typical compounds that can be used as assay control are shown in the table below.

Typical control	Stock concentration
Antimycin A (Complex III inhibitor)	150 μ M in DMSO
FCCP (ETC uncoupler)	Titration recommended to establish best concentration
Glucose Oxidase (positive signal control)	1 mg/mL in ddH ₂ O

12.1 Adherent cells:

12.1.1. Seed cells in a 96-well plate at a density of $4 - 8 \times 10^4$ cells/well in 200 μ L culture medium.

12.1.2. Incubate overnight in a CO₂ incubator at 37°C.

Δ Note: Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

12.2 Suspension cells:

Seed cells in a 96-well plate at a density of 4×10^6 cells/well in 150 μ L culture medium.

Δ Note: Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

12.3 Isolated mitochondria:

Δ Note: Mitochondria should be freshly prepared as per user's protocol and should not be left on ice longer than recommended in the literature.

Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

Initial isolated mitochondria assay optimization: prepare a six-point dilution series of mitochondrial preparation in respiration buffer in 1.5 mL total volume for each concentration.

- 12.3.1. Prepare measurement buffer as follows: 250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM MgCl₂, 30 mM, K₂HPO₄; adjust to pH 7.4.
- 12.3.2. Dilute isolated mitochondria to the desired concentration (typical range = 0.125 – 1.5 mg/mL final concentration) in measurement buffer, depending on the substrate(s) used and which respiration state is being measured.

13. Assay Procedure

- We recommend that you assay all controls and samples in duplicate.
- Prepare all controls and samples as directed in the previous sections.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C; 30°C for mitochondria).
- Sufficient cell numbers are required to produce measurable signal changes. Oxygen consumption rate is cell-type dependent – highly glycolytic cells may need to be trypsinized and concentrated prior to measurement.

PROTOCOL FOR CELLS:

13.1 Plate loading:

- 13.1.1. Adherent cells: remove culture media from all assay wells and replace with 150 μ L of fresh culture media.
Suspension cells: ready to use as prepared in Step 12.2.
- 13.1.2. Blank controls (we suggest using wells H11 and H12): add 150 μ L fresh culture media.

13.2 Assay set up:

- 13.2.1. Add 10 μ L reconstituted Extracellular O₂ Consumption Reagent to each sample well.
- 13.2.2. Add 10 μ L of fresh culture media to blank control wells.
- 13.2.3. Add 1 – 10 μ L test compound (vehicle control and/or stock) to the wells.

Δ Note: we recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.

- 13.2.4. Promptly seal each well by adding 100 μ L (or 2 drops) of pre-warmed High Sensitivity mineral oil, taking care to avoid air bubbles.

Δ Note: plate preparation time should be kept to a minimum.

13.3 Measurement:

- 13.3.1. Insert the prepared plate into a fluorescence plate reader pre-set to the measurement temperature (typically 37°C).
- 13.3.2. Measure Extracellular O₂ Consumption signal at 1.5 min intervals for 90 – 120 minutes (longer for more glycolytic cells) at Ex/Em = 380/650 nm.

PROTOCOL FOR ISOLATED MITOCHONDRIA:

- 13.4 Dilute reconstituted Extracellular O₂ Consumption Reagent (Step 9.1) 1:10 in measurement buffer.
- 13.5 Add 100 µL reconstituted probe to each sample well.
- 13.6 Add 1 µL test compound in appropriate solvent to the wells.
- 13.7 Add 50 µL of diluted isolated mitochondria (Step 12.3) to each test well. For blank control wells (wells H11 and H12), add 200 µL fresh culture media.
- 13.8 Dissolve substrate in measurement buffer and add 50 µL of solution to test wells (see table below for suggested concentrations). Do not add substrate to blank control wells.

Substrate	Mitochondria Concentration (mg/mL)	Typical final substrate concentration (mM)
Basal state [State 2]		
Glutamate/Malate	1.5	12.5 / 12.5
Succinate	1.0	25
ADP-stimulated respiration rate [State 3]		
Glutamate/Malate/ADP	1.0	12.5 / 12.5 / 1.65
Succinate/ADP	0.5	25 / 1.65

- 13.9 Promptly seal each well by adding 100 µL (or 2 drops) of High Sensitivity mineral oil, pre-warmed at 30°C, taking care to avoid bubbles.
- 13.10 Insert the prepared plate into a fluorescence plate reader pre-set to 30°C.
- 13.11 Measure Extracellular O₂ Consumption signal at 1.5 min intervals for 10 – 30 minutes at Ex/Em = 380/650 nm.

14. Assay Procedure for 384 well plate

This kit provides enough reagent to perform 200 tests in 384-wp format (half plate).

Follow the same recommendations outlined in Section 13.

- Adherent cells: seed cells in a 384-wp at a density of $2 - 4 \times 10^5$ cells/well in 100 μ L culture medium overnight.

14.1 Cell preparation:

- Adherent cells: seed cells in a 384-wp at a density of $2 - 4 \times 10^5$ cells/well in 100 μ L culture medium overnight.
- Suspension cells: prepare a cell concentration stock of 4×10^6 cells/mL. Add 75 μ L cells per well.

Δ Note: Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

14.2 Plate loading:

- 14.2.1. Adherent cells: remove culture media from all assay wells and replace with 75 μ L of fresh culture media.
Suspension cells: ready to use as prepared in Step 14.1.
- 14.2.2. Blank controls (we suggest using wells H11 and H12): add 75 μ L fresh culture media (for cell-based assay).

14.3 Assay set up:

- 14.3.1. Add 5 μ L reconstituted Extracellular O_2 Consumption Reagent to each sample well.
- 14.3.2. Add 5 μ L of fresh culture media to blank control wells.
- 14.3.3. Add 1 – 5 μ L test compound (vehicle control and/or stock) to the wells.

Δ Note: we recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.

- 14.3.4. Promptly seal each well by adding 50 μ L (or 1 drops) of pre-warmed High Sensitivity mineral oil, taking care to avoid air bubbles.

Δ Note: High Sensitivity mineral oil is very viscous and it might be difficult to plate into smaller wells.

Δ Note: plate preparation time should be kept to a minimum.

14.4 Measurement:

- 14.4.1. Follow instructions described in page 18.

15. Calculations

- 15.1 Plot the Blank control well-corrected Extracellular O₂ consumption assay Intensity or Lifetime values versus Time (min).
- 15.2 Select the linear proportion of the signal profile (avoiding any initial lag or subsequent plateau) and apply linear regression to determine the slope (OCR) and correlation coefficient for each well.

Δ Note: this approach is preferable to calculating a slope from averaged profiles.

- 15.3 Tabulate the slope values for each test sample, calculating appropriate average and standard deviation values across replicate wells. If optional Signal Control wells are included, the slope obtained for the Signal Control (sample without cells) should be subtracted from all test values.

16. Typical Data

Data provided for demonstration purposes only.

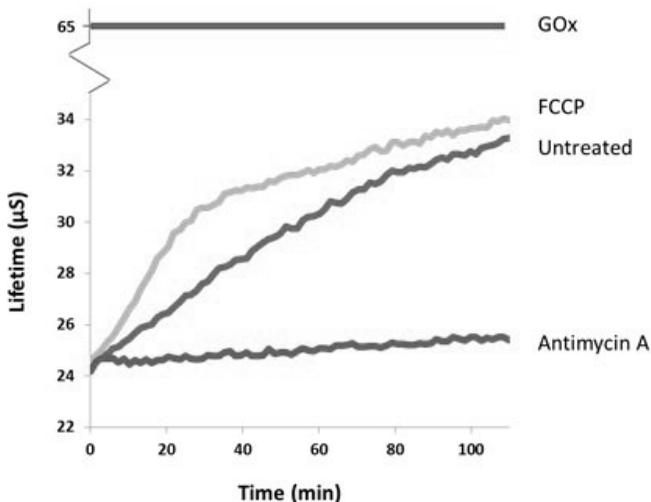


Figure 3. Typical Lifetime profile of Extracellular O₂ Consumption Assay for adherent cells, treated with different electron transport chain (ETC) modulator compounds, including antimycin A (recommended negative control). Effect of glucose oxidase (GOx) as positive signal control is illustrated schematically.

17. Assay Throughput and Performance

Data output from the Extracellular O₂ Consumption Assay (96 samples) was compared to a polarographic analysis (one sample) of mitochondrial oxygen consumption in Figure 4.

Figure 4A shows typical polarographic analysis illustrating initiation of State 2 (basal) and State 3 (stimulated state) respiration through addition of substrate and ADP respectively. Figure 4B shows oxygen consumption measured using the Extracellular O₂ Consumption Assay, where glutamate/malate- (left panel) and succinate- (right panel) driven oxygen consumption is measured at decreasing mitochondrial protein concentrations in both State 2 (top panel) and State 3 (bottom panel).

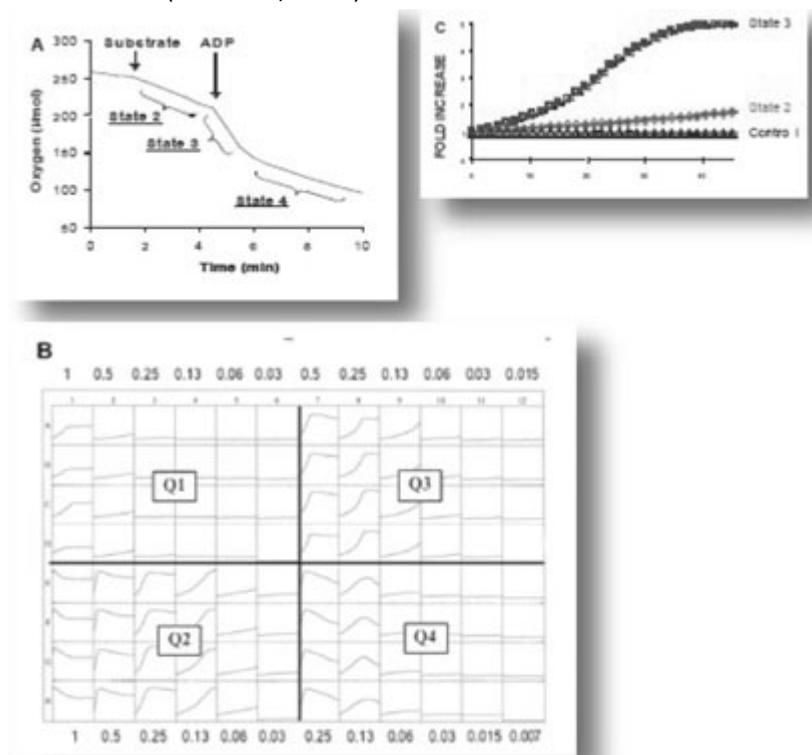


Figure 4. Analysis of isolated mitochondria using conventional polarography (A) and Extracellular O₂ Consumption Assay (n = 4, %CV < 3%) (B). Figure 4C illustrates activation of succinate-driven mitochondrial oxygen consumption measured in figure 4B.

The compatibility of this assay with the microplate format allows analysis under 96 or 384 discrete conditions. The effectiveness of this level of throughput in analyzing isolated mitochondria is highlighted in Figure 4B, which examines increasing mitochondrial protein concentrations on glutamate/malate- and succinate-driven respiration in both basal (State 2) and ADP activated (State 3) states, all tested in quadruplicates.

The performance of the assay is highlighted in Figure 4C, with a coefficient of variance (%CV) below 3%.

18. Additional Assays/Data

18.1 Monitoring cell respiration

The ability of this product to assess cellular respiration is illustrated in Figure 4. Dilutions curves for HepG2 cells (panel A) and primary rat hepatocytes (panel B) are presented.

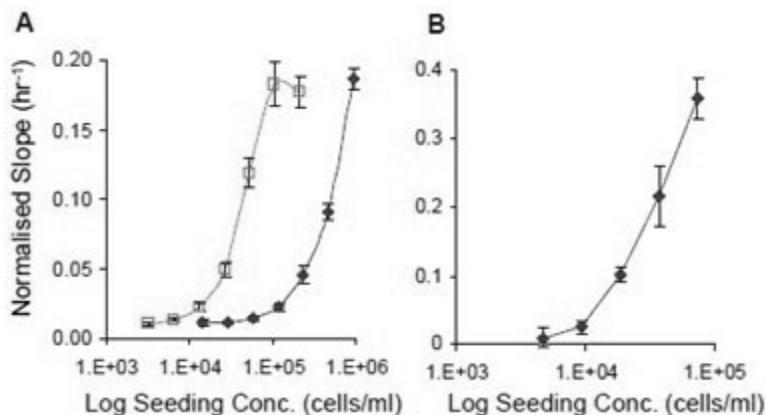


Figure 5. Cell dilutions measured on 96-well plates using Extracellular O₂ Assay. HepG2 cells (A) were assayed after an overnight (open squares) or 2-day culture period (dark squares); primary rat hepatocytes (B) were assayed after an overnight culture period. Rates of probe signal change (slope of fluorescence signal) were normalized against initial intensity.

18.2 Extracellular O₂ Consumption Assay and ATP analysis of cells

To contrast the sensitivities of oxygen consumption and cellular ATP concentrations as indicators of mitochondrial dysfunction, HepG2 cells were treated with a panel of classical ETC modulators. Both cellular oxygen consumption and cellular ATP concentrations were measured.

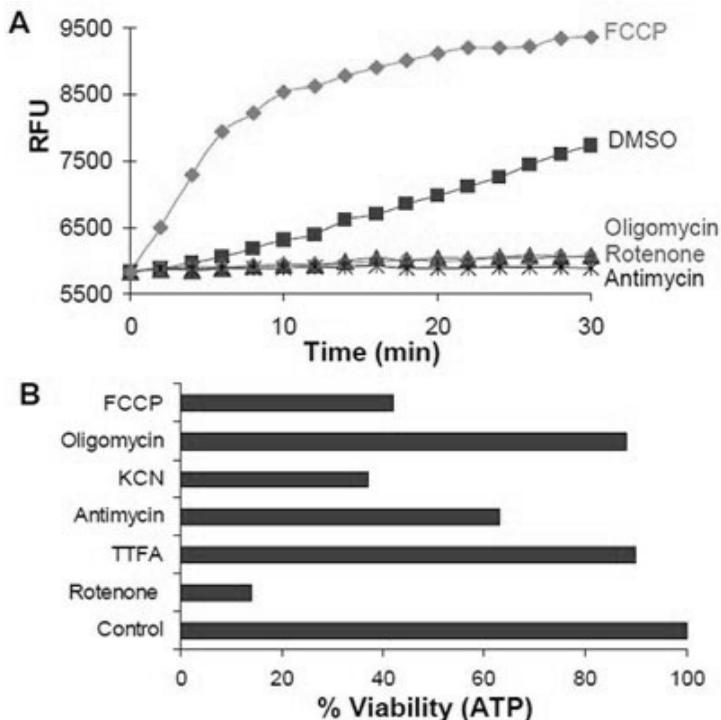


Figure 6. Parallel analysis of classical ETC inhibitors on HepG2 cells. 80,000 cells/well were plated and allowed to adhere overnight before performing the assay. Oxygen consumption was measured immediately after treatment, whereas ATP concentration was measured 24 hr post-treatment. Extracellular O₂ consumption data (panel A) illustrates that drug induced mitochondrial dysfunction is evident immediately post-treatment with both inhibition (induced by oligomycin, rotenone or antimycin) and uncoupling (induced by FCCP) being detected. Despite this dysfunction and an additional 24 hr exposure, analysis of cellular ATP concentration (panel B) showed high levels of viability in most scenarios.

This pattern is also replicated in other cell lines and when using other assays.

18.3 Cellular energy flux analysis

Multiparametric (or multiplex) combination of Extracellular O₂ Consumption Assay together with Glycolysis Assay (ab197244) allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis, leading to the analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states.

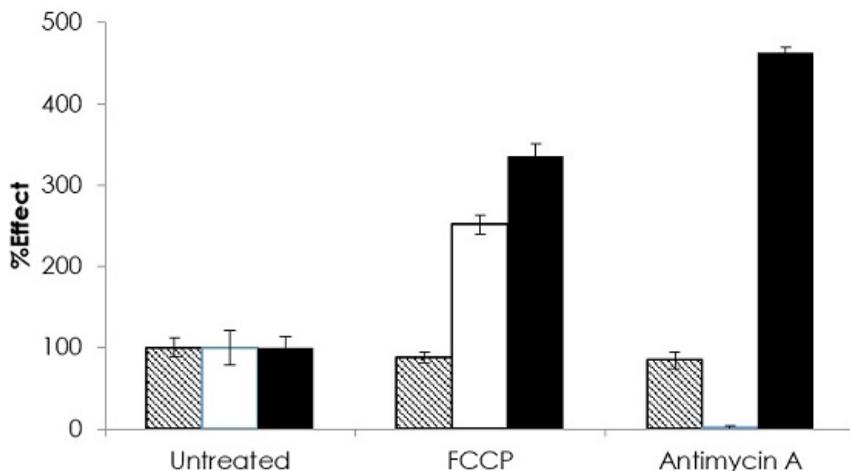


Figure 7. Cellular Energy Flux for HepG2 cells (seeded at 65,000 per well), treated with a combination of drug compounds modulating the ETC (Antimycin A [1 μ M] and FCCP [2.5 μ M]), shown as a percentage relative to untreated control cells. Comparative measurements were taken with Extracellular Oxygen Consumption Assay (ab197243) (white column) and Glycolysis Assay [Extracellular acidification] (ab197244) (black column) show the shift between mitochondrial respiration and glycolysis and the cellular control of energy (ATP; measured 1h post-treatment using Luminescent ATP Detection Assay kit (ab113849) (striped column)).

18.4 Assessment of classical mitochondrial effects

Validation of Extracellular O₂ Consumption Assay for assessment of mitochondria is illustrated in the figure below. The data illustrate the inhibition of mitochondrial function using a panel of classical mitochondrial inhibitors and highlight the dose dependence of this inhibition for KCN (potassium cyanide).

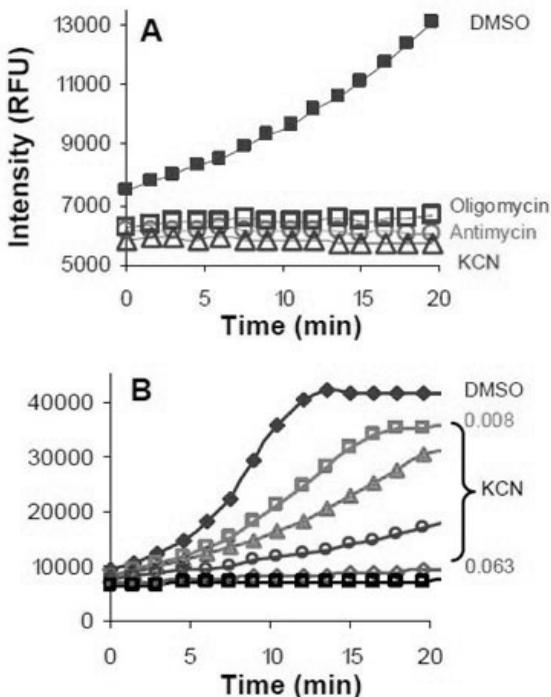


Figure 8. Effect of a panel of classical ETC inhibitors on mitochondrial function (A) and dose response inhibition of mitochondrial function by KCN (B).

18.5 Compound Screening

Extracellular O₂ Consumption Assay allows screening of compounds at multiple concentrations and in multiple conditions in a single microtiter plate as illustrated in Figure 9. Such data can be processed further to generate dose response data.

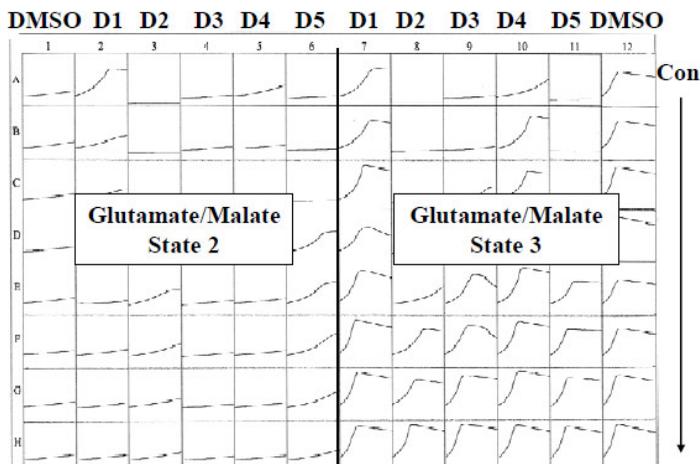


Figure 9. Effect of test compounds (D01 – D05), on both State 2 and State 3 isolated mitochondrial function using Extracellular O₂ Consumption Assay. Some compounds uncouple in a dose dependent manner while other inhibit.

A 96-well plate format allows screening of 200 compounds a day at a single dose, or acquisition of dose response characteristics for 25 compounds per day. This capability represents a fundamental shift in the capacity for mitochondrial toxicity testing in drug discovery programs, without compromising data quality or information content.

19. Notes

Technical Support

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