Instructions for Use

For the measurement of extracellular oxygen consumption by isolated mitochondria, cell populations, 3D culture models, small organisms, tissues and enzymes

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

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 $O_2$ Consumption Reagent (ab197242), solves these limitations of low throughput and high complexity, 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 $O_2$ Consumption Reagent (ab197242). As the test material respires, oxygen is depleted in the surrounding solution/environment, which is seen as an increase in phosphorescence signal. The addition of a high-sensitivity mineral oil (not supplied, available in Extracellular $O_2$ Consumption Assay, ab197243) 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 $O_2$ Consumption Reagent (ab197242) is suitable for use with whole cell populations (both adherent and suspension cells), isolated mitochondria, a wide range of 3D culture models, tissues, small organisms, as well as 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 $O_2$ Consumption Reagent (ab197242) 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. **ASSAY SUMMARY**

**Figure 1:** Preparation and use of Extracellular O₂ Consumption Reagent (ab197242) flow diagram.

- **Step 1:** Mix reagent
- **Step 2:** Add reagent (and compounds of interest) to plated cells
- **Step 3:** Add high-sensitivity mineral oil
- **Step 4:** Read plate using fluorescence plate reader
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. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

- Store vial containing Extracellular O$_2$ Consumption Reagent at 4°C in dark.
- Reconstituted product can be aliquoted and stored at -20°C. Use within one month (avoid freeze thaw).

5. **MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>Components supplied</th>
<th>Quantity</th>
<th>Storage</th>
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<tr>
<td>Extracellular O$_2$ Consumption Reagent</td>
<td>1 vial</td>
<td>2-8°C</td>
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</table>
6. **MATERIALS REQUIRED, NOT SUPPLIED**

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

- Repeater pipette and multiple-channel pipette
- Plate heater
- 96-well (black wall) clear bottom TC+ plates or standard clear polystyrene plates for cell culture
- Compounds of interest, for example Antimycin A, FCCP or GOx
- Fluorescence plate reader
- Cell culture medium (for cells)
- Measurement buffer (for isolated mitochondria)
- Mitochondrial substrate (succinate, glutamate or malate) (for isolated mitochondria)
- ADP (for isolated mitochondria)

7. **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.
8. **TECHNICAL HINTS**

- 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.
- Refer to the guide in the Resources section, for recommended settings for your plate reader.
- Perform a Signal Optimization step (especially first time users).
- 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.
9. **MEASUREMENT PARAMETERS**

The Extracellular O$_2$ Consumption reagent is a chemically stable and inert, biopolymer-based, cell impermeable oxygen-sensing fluorophore.

**Figure 2:** Excitation and Emission spectra of Extracellular O$_2$ Consumption Reagent. Left panel shows normalized excitation (Ex = 360-400nm; Peak 380nm). Right panel shows emission (Em = 630 - 680nm; Peak 650nm) in oxygenated and deoxygenated conditions.

**Phosphorescence Measurements**

ab197242 is measured with prompt or time-resolved fluorescence (TR-F) readers, monochromator or filter-based. Optimal wavelengths are 380 nm for excitation and 650 nm for emission, excitation at 532 nm can also be used. For TR-F readers, optimal delay and gate times are 30 µs and 100 µs respectively. Probe signals should be at least 3 times above blank signal. While compatible with all plate types, black border clear bottom plates give optimal signal to noise ratios. The Extracellular O$_2$ probe response to oxygen is temperature dependent, so good temperature control of the plate during the measurement is important.
Fluorescence measurements

Three fluorescence modalities (outlined below) can be successfully used with the Extracellular O$_2$ Consumption Assay, depending on plate reader type and instrument setup.

1. **Basic: Intensity Measurement**
Measurement of Signal Intensity (sometimes referred to as Prompt) provides flexibility to use a very wide range of commonly available fluorescence, monochromator or filter-based plate readers. Optimal wavelengths are 380 nm excitation and 650 nm for emission, with detection Gain parameters (PMT) typically set at medium or high.
**Note:** Signal Optimization test may return a Signal to Blank ratio (S:B) $\geq$3

2. **Standard: TR-F Measurement**
Increased levels of performance can be achieved by using time-resolved fluorescence (TR-F). TR-F measurement reduces non-specific background and increases sensitivity. Optimal delay time is $\sim$30 µs and gate (integration) time is 100 µs.
**Note:** Signal Optimization test may return a S:B $\sim$10.

3. **Advanced: Dual-Read TR-F (Lifetime)**
Optimal performance can be achieved using dual-read TR-F in combination with subsequent ratiometric Lifetime calculation, to maximise dynamic range.
**Note:** Signal Optimisation test may return a S:B up to 60.

10. **DUAL-READ TR-F AND LIFETIME ILLUSTRATED**
Dual-read TR-F and subsequent Lifetime calculation allows measurement of the rate of fluorescence decay of the Extracellular O$_2$ Consumption Assay, and can provide measurements of oxygen consumption that are more stable and with a wider dynamic range than measuring signal Intensity.
GENERAL INFORMATION

Optimal dual-delay and gate (integration) times:
- Integration window 1: 30 µs delay (D1), 30 µs measurement time (W1)
- Integration window 2: 70 µs delay (D2), 30 µs measurement time (W2)

Note: S:B for Integration window 2 is recommended to be ≥10 to allow accurate Lifetime calculation.

Figure 3: Illustrating dual read TR-F measurement

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

\[ \text{Lifetime (µs)[r]} = \frac{(D2-D1)}{\ln(W1/W2)} \]
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 microsecond units (µs) at each measured time point for each individual sample (Figure 11).

**Note:** Lifetime values should be in the range ~22 to ~68 µs, and should only be calculated from samples containing Extracellular O₂ Consumption Reagent. Lifetime values should not be calculated from blank wells.
11. CELL CULTURE AND PLATING

1. For Adherent cells: Seed cells in a 96-well plate at a density (typically 40,000 – 80,000 cells/well) in 200 µL culture medium. Incubate overnight in a CO₂ incubator at 37°C.

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

1.2. For Suspension cells: Seed on the day of assay in 150 µL culture medium at a density of ~ 4 x 10⁶/mL.

1.3. For Isolated Mitochondria: Dilute to the desired concentration (typically in the range of 0.125-1.5 mg/mL final concentration, depending on the substrate(s) used and whether measuring Basal [state 2] or ADP-stimulated respiration rate [state 3]) in measurement buffer and add 150 µL to each test well.

   **Note:** Mitochondria should be freshly prepared as per user’s protocol and should not be left on ice longer than recommended in the literature. Measurement buffers should be prepared freshly on the day of measurement.

   **Note:** Always leave two wells (H11 and H12) free from the addition of Extracellular O₂ Consumption Reagent, as Blank Controls.
12. REAGENT PREPARATION

Prepare a stock solution of the Extracellular O$_2$ Consumption Reagent by adding 1 mL of water, PBS, culture media or buffer to the vial, gently aspirating 3-4 times. The recommended working dilution is 1:15 (i.e. 10 µL per 150 µL of sample for a 96-well plate).

Prepare test compounds, controls and dilutions as desired. Examples of typical controls are shown in the table below.

<table>
<thead>
<tr>
<th>Typical control</th>
<th>Stock concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimycin A - Complex III inhibitor</td>
<td>150 µM in DMSO</td>
</tr>
<tr>
<td>FCCP - ETC uncoupler</td>
<td>Titration recommended to establish best concentration</td>
</tr>
<tr>
<td>Glucose oxidase - positive signal control</td>
<td>1 mg/mL in water</td>
</tr>
</tbody>
</table>

For use with isolated mitochondria, prepare measurement buffer (pH 7.4) according to the following table:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>250</td>
</tr>
<tr>
<td>KCl</td>
<td>15</td>
</tr>
<tr>
<td>EGTA</td>
<td>1</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>5</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>30</td>
</tr>
</tbody>
</table>

Note: We recommend that all culture media and stock solutions to be used are pre-warmed to the assay temperature (typically 37°C). Use a plate block heater for plate preparation and pre-warm the fluorescence plate reader to measurement temperature.
13. INITIAL ISOLATED MITOCHONDRIA ASSAY OPTIMISATION

Prepare a six point dilution series of mitochondrial preparation in respiration buffer in 1.5 mL total volume for each concentration. Starting mitochondrial concentrations recommended for different substrates and respiration states are:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mg/mL)</th>
<th>Typical final concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate/malate</td>
<td>1.5</td>
<td>12.5/12.5</td>
</tr>
<tr>
<td>Glutamate/malate/ADP</td>
<td>1.0</td>
<td>12.5/12.5/1.65</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.0</td>
<td>25</td>
</tr>
<tr>
<td>Succinate/ADP</td>
<td>0.5</td>
<td>25/1.65</td>
</tr>
</tbody>
</table>
# ASSAY PROCEDURE

## 14. SIGNAL OPTIMISATION

**Note:** Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C).

13. Prepare 8 replicate wells of a 96-well plate, by adding 150 µL pre-warmed culture medium to each well (A1-A4, B1-B4).

14.2 Add 10 µL reconstituted Extracellular O\textsubscript{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).

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

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

14.5 Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) readings (linear phase) and calculate Signal to Blank (S:B) ratio.

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

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Media + O\textsubscript{2} Reagent + Oil</td>
<td>Media + O\textsubscript{2} Reagent + Oil</td>
<td>Media + O\textsubscript{2} Reagent + Oil</td>
<td>Media + O\textsubscript{2} Reagent + Oil</td>
</tr>
<tr>
<td>B</td>
<td>Media + Oil</td>
<td>Media + Oil</td>
<td>Media + Oil</td>
<td>Media + Oil</td>
</tr>
</tbody>
</table>
15. TYPICAL ASSAY

**Note:** Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C).

15.1 For adherent cells: remove spent culture medium from all assay wells and replace with 150 µL of fresh culture media. For Isolated Mitochondria: dissolve substrate (succinate or glutamate/malate) and ADP in measurement buffer and add 50 µL to test wells giving a final substrate concentration of 25 mM (succinate) or 12.5/12.5 mM (glutamate/malate) and a final ADP concentration of 1.65 mM.

15.2 Leave two wells (H11 and H12) free from the addition of Extracellular O₂ Consumption reagent, for use as Blank Controls. Add 150 µL fresh culture media (cell based assay) or 200 µL measurement buffer (isolated mitochondria assay) to these Blank Control wells.

15.3 Add 10 µL reconstituted Extracellular O₂ Consumption reagent to each well, except those wells for use as Blank Controls. Add 10 µL of fresh culture media to these Blank Control wells.

15.4 Test compound stock or vehicle (typically 1-10 µL) may be added at this point if desired. **Note:** We recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.

15.5 Promptly seal each well by adding two drops (or 100 µl) pre-warmed High Sensitivity mineral oil, taking care to avoid air bubbles. **Note:** Plate preparation time should be kept to a minimum.

15.6 Insert the prepared plate into a fluorescence plate reader pre-set to measurement temperature (typically 37°C).

15.7 Measure Extracellular O₂ Consumption signal at 1.5 min intervals for 90–120 minutes (longer for more glycolytic cells) using excitation and emission wavelengths of 380 nm and 650 nm respectively.
See Instrument and Measurement section – Section 17 – for instrument-specific settings and filters). Readers equipped with a time-resolved mode (TR-F), may achieve improved performance using delay and gate time of 30 and 100 μsec. **Note:** *Sufficient cell numbers are required to produce measurable signal changes. Oxygen consumption rate is cell-type dependant – highly glycolytic cells may need to be trypsinized and concentrated prior to measurement.*

For Isolated Mitochondria: measure Extracellular $O_2$ Consumption Assay at 1.5 min intervals for 10–30 minutes.
16. **ASSESSING OXYGEN CONSUMPTION**

- Plot the Blank Control well-corrected Extracellular $O_2$ Consumption Assay Intensity or Lifetime values versus Time (mins).
- Select the linear portion 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.

**Note:** *This approach is preferable to calculating a slope from averaged profiles.*

- 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.
Figure 4: Typical Lifetime profile of Extracellular $O_2$ Consumption Assay for adherent cells, treated with different ETC compounds, including Antimycin A (recommended as a Negative Control). The effect of Glucose Oxidase as a positive Signal Control is illustrated schematically.
### INSTRUMENT AND MEASUREMENT SETTINGS

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Optical Configuration</th>
<th>Integration 1 ((D_1/W_1))</th>
<th>Integration 2 ((D_2/W_2))</th>
<th>Optimum Mode</th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
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<tbody>
<tr>
<td>BioTek: Cytation 3 / 5</td>
<td>Filter-based</td>
<td>30 / 30μs 70 / 30μs</td>
<td>Dual-read TR-F (Lifetime)</td>
<td>Ex 380 ± 20nm</td>
<td>Em 645 ± 15nm</td>
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<tr>
<td>BioTek: Synergy H1 / Neo / 2</td>
<td>Filter-based</td>
<td>30 / 30μs 70 / 30μs</td>
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<td>Ex 380 ± 20nm</td>
<td>Em 645 ± 15nm</td>
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<td>Ex 340 ± 50nm (TR-EX)</td>
<td>Em 665 ± 50nm or Em 645± 10nm With LP-TR Dichroic</td>
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<tr>
<td>BMG Labtech: FLUOstar Omega / POLARstar Omega</td>
<td>Filter-based Top or bottom read</td>
<td>30 / 30μs 70 / 30μs</td>
<td>Dual-read TR-F (Lifetime)</td>
<td>Ex 340 ± 50nm (TR-EXL)</td>
<td>Em 655 ± 25nm (BP-655)</td>
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<td>Em 650 ±20nm or Em 670±40nm</td>
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<td>TR-F</td>
<td>Ex 337 nm (HTRF Module)</td>
<td>Em 665 nm (HTRF Module)</td>
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<td>Filter-based Top or bottom read</td>
<td>30 / 100μs n/a</td>
<td>TR-F</td>
<td>Ex 340 ± 50nm (TR-EXL)</td>
<td>Em 655 ± 50nm (BP-655)</td>
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18. **ADDITIONAL INFORMATION**

**Monitoring Cell Respiration**

The ability of the Extracellular $O_2$ Consumption Assay to assess cell respiration is illustrated in Figure 5. Dilutions curves for HepG2 cells (Fig. 5A) and primary rat hepatocytes (Fig. 5B) are presented.

**Figure 5:** Cell dilutions measured on 96-well plates using Extracellular $O_2$ Assay A) HepG2 cell dilution after an overnight (□) or 2 day culture period (■), B) primary rat hepatocyte cell dilution after an overnight culture period. Rates of probe signal change (slope of fluorescence signal) were normalised against initial intensity.

**Extracellular $O_2$ 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 electron transport chain (ETC) modulators and both cellular oxygen consumption and cellular ATP concentrations were measured.
Figure 6: Parallel analysis of classical inhibitors on HepG2 cells. A) Extracellular $O_2$ Consumption Assay assessment of mitochondrial function immediately post treatment, B) analysis of ATP concentrations 24 h post-treatment. For Extracellular $O_2$ Consumption Assay measurements, cells were plated at 80,000 cells/well, allowed to adhere overnight and then assayed. Extracellular $O_2$ consumption data presented in A illustrates that drug induced mitochondrial dysfunction is evident immediately post treatment with both inhibition (Oligomycin, Rotenone, Antimycin) and uncoupling (FCCP) being detected. Despite this dysfunction and an additional 24 hour exposure, analysis of cellular ATP concentrations indicated high levels of ‘viability’ (B). This pattern is also reflected using other assays and cell lines.
**Cellular Energy Flux Analysis**

Multiparametric (or multiplex) combination of Extracellular $O_2$ Consumption Assay together with Abcam’s Glycolysis Assay (Cat No: ab 197244) allows the simultaneous real-time measurement of mitochondrial respiration and glycolysis and analysis of the metabolic phenotype of cells and the shift (flux) between the two pathways under pathological states (Figure 7).

![Graph showing cellular energy flux](image)

**Figure 7:** Cellular Energy Flux for HepG2 cells, treated with a combination of drug compounds modulating the ETC or inhibiting lactate production, shown as a percentage relative to untreated control cells. Comparative measurements carried out with Extracellular $O_2$ Consumption Assay (ab197243) and Glycolysis Assay (ab197244), show the shift between mitochondrial respiration and glycolysis and the cellular control of energy (ATP).
**Extracellular O$_2$ Consumption Assay: comparison with other methods of cell screening**

The dose-response relationship for the ETC inhibitor rotenone using four different viability assays: Extracellular O$_2$ Consumption Assay, Bioluminescent ATP Measurement, LDH and Cellular DNA Measurement outlined in Figure 8. These data indicate that treatment suppresses mitochondrial respiration, leading to loss of function and reduced oxygen consumption prior to cell death.

![Graph showing the effects of rotenone treatment on H-4-II-E cells](image)

**Figure 8:** Effects of Rotenone treatment on H-4-II-E cells analysed using Extracellular O$_2$ Consumption Assay, Bioluminescent ATP Measurement, LDH and Cellular DNA Measurement (24h exposure).

These results demonstrate the effectiveness of Extracellular O$_2$ Consumption Assay in assessing mitochondrial function in whole cells as well as the importance of evaluating mitochondrial function in the context of drug toxicity. The specificity and sensitivity of the Extracellular O$_2$ Consumption Assay is demonstrated by its ability to detect mitochondrial toxicity more rapidly and at lower drug doses than other assays. When combined with other assays, Extracellular O$_2$ Consumption Assay allows detailed evaluation of mechanisms of drug toxicity, adding significantly to the portfolio of information available for compound evaluation.
Assay Throughput and Performance

The data output from the Extracellular \( \text{O}_2 \) Consumption Assay (96 samples), and polarographic (one sample) analysis of mitochondrial oxygen consumption is contrasted in Figure 9. Fig 9a shows typical polarographic analysis illustrating initiation of State 2 and State 3 respiration through addition of substrate and ADP respectively. Fig 9b shows oxygen consumption measured using Extracellular \( \text{O}_2 \) Consumption Assay, with glutamate/malate (left) and succinate (right) driven oxygen consumption is measured at decreasing mitochondrial protein concentrations in both State 2 (top) and State 3 (bottom) (n = 4).
**Figure 9:** Analysis of isolated mitochondrial using A) conventional polarography and B) Extracellular O$_2$ Consumption Assay. C) Data extracted from Fig. 9B illustrating activation of succinate driven mitochondrial oxygen consumption measured using Extracellular O$_2$ Probe (n = 4, % CV < 3 %).

The compatibility of the Extracellular O$_2$ Consumption Assay with the microplate format permits analysis under 96 (or 384) discrete conditions. The effectiveness of this level of throughput in analysing isolated mitochondria is highlighted in Fig. 9B 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 in quadruplicates). The performance of the Extracellular O$_2$ Consumption Assay is highlighted in Fig. 9C, with coefficient of variance below 3%.
**Assessment of Classical Mitochondrial Effectors**

Validation of Extracellular $O_2$ Consumption Assay for assessment of mitochondria is illustrated in Figure 10. These data illustrate the inhibition of mitochondrial function using a panel of classical mitochondrial inhibitors and highlight the dose dependence of this inhibition for KCN.

**Figure 10:** A) Monitoring the effect of a panel of classical ETC inhibitors on mitochondrial function using Extracellular $O_2$ Consumption Assay, and B) dose dependent inhibition of mitochondrial function by KCN.
Compound Screening

Extracellular O$_2$ Consumption Assay allows screening of compounds at multiple concentrations and in multiple conditions in a single microtitre plate as illustrated in Figure 11. Such data may be processed further to generate dose response data.

**Figure 11**: Effect of test compounds (D01-D05), on both State 2 and State 3 isolated mitochondrial function using Extracellular O$_2$ Consumption Assay. Some compounds uncouple in a dose dependant manner while others inhibit.

Overall, Extracellular O$_2$ Consumption Assay allows highly sensitive high-throughput detection of mitochondrial dysfunction in isolated mitochondria. Use of a 96-well plate format allows screening of 200 compounds per 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.
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