Extracellular Oxygen Consumption Assay Kit

Item No. 502880

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
600802	Cell-Based Assay Glucose Oxidase	1 vial/2 ml	-20°C
401131	Extracellular OCR Probe	1 vial	-20°C
401159	OCR Mineral Oil	1 vial/15 ml	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Extracellular Oxygen Consumption Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of heating to 37°C and measuring fluorescence with excitation and emission wavelengths of 380 nm (360-400 nm) and 650 nm (630-670 nm), respectively
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. 96-well black-walled plate with clear bottom (use tissue culture-treated plates for cell experiments)
4. Materials needed for cell culture or mitochondria isolation
5. A source of pure water; glass-distilled water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
6. Culture medium containing glucose (see **Calibration Procedure** on page 13)

INTRODUCTION

Background

Cellular homeostasis is maintained through the generation of ATP.[1] The generation of ATP can be accomplished through glycolysis alone (anaerobic respiration) or through the coupling of glycolysis to oxidative phosphorylation. Oxidative phosphorylation, which is oxygen-dependent, occurs in mitochondria and is the most efficient and preferred means of ATP synthesis by mammalian cells. Due to this, the oxygen consumption rate (OCR) of cells can be used as an indicator of cellular function, with a lower OCR indicating mitochondrial dysfunction. Historically, OCR measurement has been performed via polarography, a chamber-based method using a Clark-type oxygen platinum electrode.[2,3] However, it is a low-throughput method that requires large sample volumes and the suspension and stirring of cells, which disrupts cellular structures and connections with extracellular matrix proteins.[2] In contrast, a multi-well format using a fluorescent oxygen probe is high-throughput, uses smaller sample volumes, and can be used to assess adherent cells as well as cells in suspension.

About This Assay

Cayman's Extracellular Oxygen Consumption Assay Kit uses a phosphorescent oxygen-sensitive probe. Because of its inability to cross the membrane, the probe selectively reports on changes in oxygen levels in the medium. The probe's signal is quenched upon binding to oxygen, resulting in a signal that is inversely proportional to the amount of dissolved oxygen present in culture medium. As mitochondria consume oxygen during respiration, the extracellular oxygen concentration decreases, leading to an increase in signal intensity over time. This assay is compatible with standard fluorescence plate readers and provides a simple and sensitive method for determining OCR in living cells and isolated mitochondria.[4]

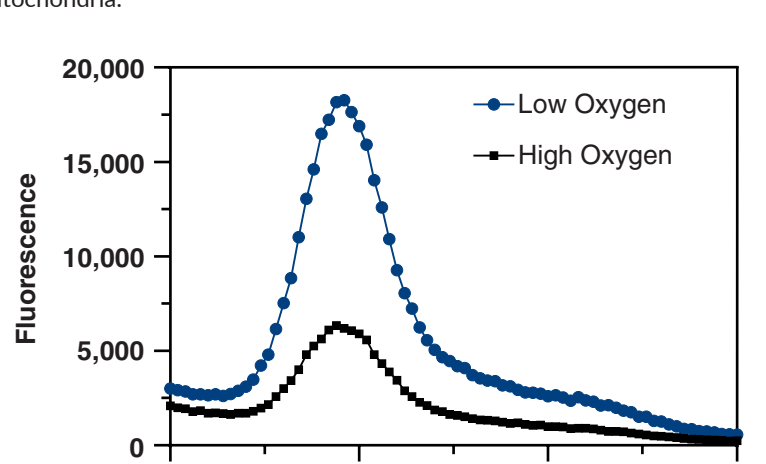


Figure 1. Emission wavelength spectrum of the Extracellular OCR Probe in low and high oxygen conditions upon excitation at 380 nm

PRE-ASSAY PREPARATION

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is important to include one glucose oxidase (GO) and two background (BG) wells in the assay. It is suggested that the samples be assayed in triplicate. A typical layout is shown in Figure 2, below. It is suggested that the contents of each well be recorded on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	GO	BG	BG	8	8	8	16	16	16	24	24	24
B	1	1	1	9	9	9	17	17	17	25	25	25
C	2	2	2	10	10	10	18	18	18	26	26	26
D	2	2	2	11	11	11	19	19	19	27	27	27
E	4	4	4	12	12	12	20	20	20	28	28	28
F	5	5	5	13	13	13	21	21	21	29	29	29
G	6	6	6	14	14	14	22	22	22	30	30	30
H	7	7	7	15	15	15	23	23	23	31	31	31

GO = Glucose Oxidase Well
BG = Background Wells
1-31 = Sample Wells

Figure 2. Sample plate format

Sample Preparation

NOTE: Leave three wells empty for background and glucose oxidase wells.

Cell Culture

1. Seed cells in a black, clear-bottom 96-well tissue culture-treated plate at a density of 2-8 x 10^4 cells per well in 200 µl of culture medium. *NOTE: Seeding density will vary based on the aerobic capacity of the cell line and will need to be optimized. Avoid using edge wells.*
2. Incubate under optimal culture conditions specific to the cell line and according to the experimental design.
3. Carefully remove existing culture medium and add 90 µl of fresh culture medium prior to performing the assay.

Isolated Mitochondria

1. Isolate mitochondria using a protocol compatible with respirometry analysis. **Do not freeze isolated mitochondria.**

   NOTE: Mitocheck® Mitochondria (Cell Culture) Isolation Kit (Item No. 502871) and Mitocheck® Mitochondria (Tissue) Isolation Kit (Item No. 701010) are available for purchase from Cayman.
2. Resuspend isolated mitochondria in a sample buffer suitable for respiration assays, such as Mitochondrial Assay Solution (MAS) or 10 mM HEPES pH 7.4, 250 mM sucrose, and 1 mM EGTA.

   NOTE: Substrates, such as glutamate, malate, pyruvate, or succinate, may be included depending on the experimental design.
3. Add 90 µl of mitochondrial suspension per well. *The total amount of mitochondria loaded into each well will need to be optimized. Determine protein concentration using a BCA or Bradford assay before loading.*

Reagent Preparation

*NOTE: The **reconstitution solution** for the Extracellular OCR Probe should match the sample preparation medium/buffer.*

1. **Cell-Based Assay Glucose Oxidase - (Item No. 600802)**

   This vial contains 2 mg of lyophilized glucose oxidase. Reconstitute in 200 µl of pure water. Store on ice. If not using all at once, prepare aliquots and store at -20°C, where it will be stable for two months. Limit freeze-thaw cycles to one.
2. **Extracellular OCR Probe - (Item No. 401131)**

   This vial contains lyophilized Extracellular OCR Probe. Reconstitute the contents of the vial with 600 µl of the same solution used to prepare the samples. Keep on ice and protect from light. If not using all at once, prepare aliquots and store at -20°C, where it will be stable for one month. Limit freeze-thaw cycles to three.
3. **OCR Mineral Oil - (Item No. 401159)**

   This vial contains 15 ml of OCR Mineral Oil. One vial contains a sufficient volume to assay 96 wells. Store at room temperature, protected from light. Warm the OCR Mineral Oil to 37°C prior to use in the assay.

Test Compounds

To evaluate compound effects on oxygen consumption, prepare test compounds at 20X the desired final concentration in sample buffer or culture medium containing up to 10% organic solvent. This ensures that the final solvent concentration does not exceed 0.5% in the well. Include appropriate vehicle controls in each experiment.

Many common control compounds for mitochondrial function are available from Cayman (see Table 1, page 11). It is critical to titrate the control compound concentration based on the specific cell type and experimental design.

Category	Reagent	Item No.	Function
Substrates & Activators (Recommended for use with isolated mitochondria)	ADP	21121	Stimulates oxidative phosphorylation and State 3 respiration
	Pyruvate	--	Supports NADH production
	Malate	--	Supports NADH production
	Succinate	--	Complex II substrate
	L-Glutamic Acid	30377	Supports NADH production and Complex I activity
Uncouplers	FCCP	15218	Disrupts mitochondrial proton gradient
	CCCP	25458	
	BAM15	17811	ATP synthase-independent uncoupler
Inhibitors	Antimycin A Complex	34799	Complex III Inhibitor
	Oligomycin Complex	11341	ATP synthase Inhibitor
	Rotenone	13995	Complex I Inhibitor

Table 1. Control compounds for mitochondrial function

ASSAY PROTOCOL

Pipetting Hints

- It is recommended that a repeating pipettor be used to overlay the wells with OCR Mineral Oil.
- It is recommended that a multichannel pipette be used to deliver all other reagents to the wells. This saves time and helps maintain more precise incubation times
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- All reagents should be prepared as described in the **Reagent Preparation** section (see page 9). The reconstituted Extracellular OCR Probe and glucose oxidase should be kept on ice, and the OCR Mineral Oil should be pre-warmed to 37°C before beginning the assay.
- It is recommended that the assay be performed at 37°C.
- Read the fluorescence with excitation and emission wavelengths of 380 and 650 nm, respectively.

Calibration Procedure

NOTES:

- *Pre-warm the OCR Mineral Oil to 37°C and let air bubbles resolve prior to use.*
- *The GO well is used to establish the instrument's gain setting.*

1. Pre-heat the instrument to 37°C
2. Add 85 µl of culture medium, 5 µl of reconstituted extracellular OCR probe, and 10 µl of reconstituted glucose oxidase to the GO well. Mix thoroughly ensuring no bubbles are generated.
3. Gently overlay each control well with 100 µl of pre-warmed OCR Mineral Oil. *NOTE: It is critical that these wells be prepared and sealed without delay.*
4. Incubate at 37°C for 10 minutes and read fluorescence using excitation and emission wavelengths of 380 and 650 nm, respectively. The GO well should be used to set the instrument's *gain*.

Performing the Assay

NOTES:

- *Pre-heat instrument to 37°C.*
- *Pre-warm the OCR Mineral Oil to 37°C and let air bubbles resolve prior to use.*
- *Use a repeating pipettor to overlay samples with mineral oil.*
- *Sample wells should already contain 90 µl of sample (see* ***Sample Preparation*** *on page 8).*

1. Add reagents to the corresponding wells according to the table below. If desired, prepare master mixes, making at least 10% extra volume to account for pipetting loss. Mix thoroughly ensuring no bubbles are formed.

Reagent	Background Wells	Sample Wells		
		Vehicle	Treated	Untreated
Culture Medium or Sample Buffer (µl)	95	--	--	5
Vehicle Solution (µl)	--	5	--	--
Test Compound Solution (µl)	--	--	5	--
Reconstituted Extracellular OCR Probe (µl)	5	5	5	5

2. Gently overlay each well with 100 µl of pre-warmed OCR Mineral Oil and ensure that no bubbles are formed. The use of a repeating pipettor is recommended. *NOTE: It is critical that the entire surface is overlaid with mineral oil to minimize oxygen exchange.*
3. Using the *gain* setting identified in the Calibration Procedure, read fluorescence of sample and background wells kinetically every two minutes for at least 120 minutes at 37°C using excitation and emission wavelengths of 380 and 650 nm, respectively.

NOTE: It is normal to observe an initial decrease in fluorescence values at the start of the experiment as the contents of the well equilibrate. The signal then stabilizes, followed by a steady linear change in fluorescence over time.

ANALYSIS

Calculations

Determination of OCR

1. Plot fluorescence as a function of time in minutes for each replicate.
2. Identify and select the linear portion of the profile and perform linear regression to determine the slope. This slope is the OCR in RFU/min.
3. Calculate the average slopes. Subtract the average background slope from each sample's average values to determine the corrected OCR.
4. Optional: normalize corrected OCR values to the number of cells or protein content per well. For cell-based assays, divide OCR by the number of cells per well. Cell number may be determined using the seeding density or post-assay nuclear staining (*e.g.*, Hoecsht or DAPI). For mitochondrial assays, divide OCR by the µg of isolated mitochondrial protein loaded per well.

Example Data

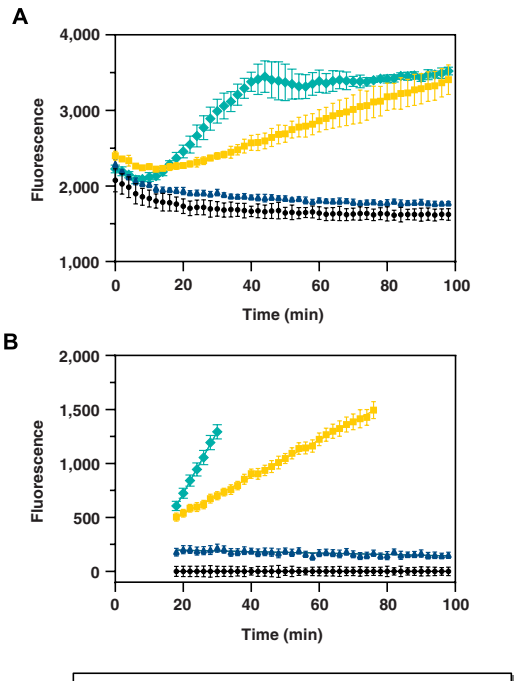


Figure 3. Oxygen consumption by HEK293T cells treated with Antimycin A and FCCP. HEK293T cells were seeded at 6.5 x 10^4 cells per well and allowed to adhere overnight prior to analysis using Cayman's Extracellular Oxygen Consumption Assay Kit. The following day, cells were treated with either 5 µM antimycin A or 1.5 µM FCCP, and OCRs were determined. Background wells (BG) represent culture medium-only control. Data are shown as mean raw fluorescence values ± standard deviation from 8 wells in (A). The linear portion each oxygen consumption profile was selected and corrected for background fluorescence in (B).

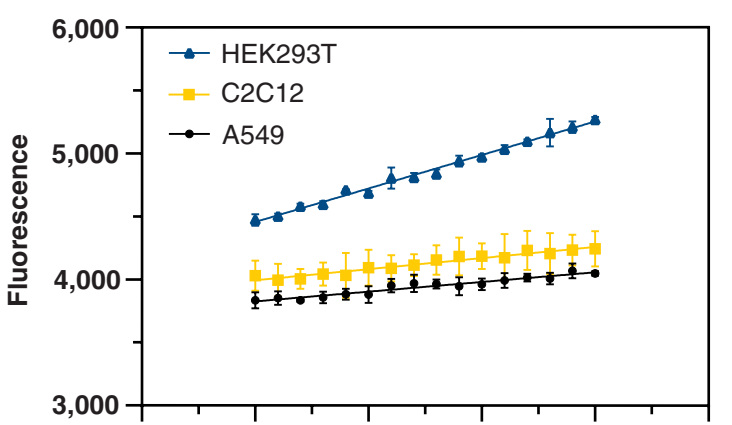


Figure 4. Oxygen consumption by various cell types. HEK293T, C2C12, and A549 cells were seeded at 2 x 10^4 cells per well and their respective OCRs were determined 24 hours later using this kit. Data are shown as mean raw fluorescence values ± standard deviation from 3 wells and are representative of 3 independent experiments.

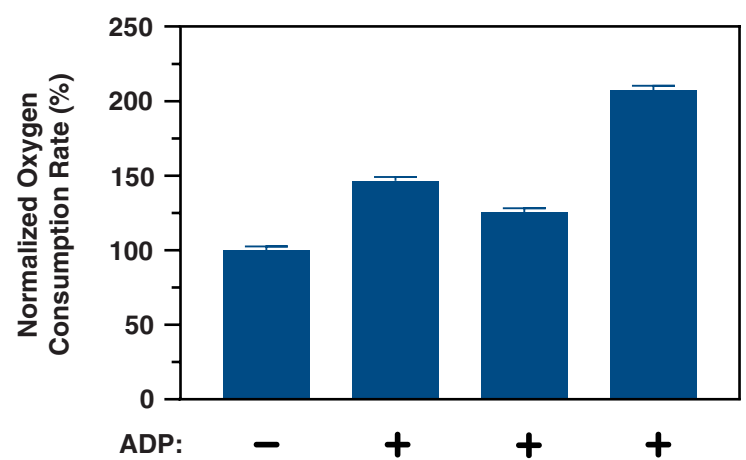


Figure 5. Oxygen consumption by isolated mitochondria. The oxygen consumption rate of mitochondria isolated from C2C12 cells (70 µg/well) was determined using this kit. Oxygen consumption increased upon addition of ADP, indicating state 3 respiration, and further stimulation was observed with FCCP, reflecting uncoupled respiration. Simultaneous addition of oligomycin A to ADP-treated mitochondria led to a decrease in oxygen consumption, consistent with inhibition of ATP synthase. Oxygen consumption rates were normalized to untreated mitochondria, which were set to 100%. Mitocheck® Mitochondria (Cell Culture) Isolation Kit, ADP, oligomycin A (Item No. 11342), and FCCP are available for purchase from Cayman.

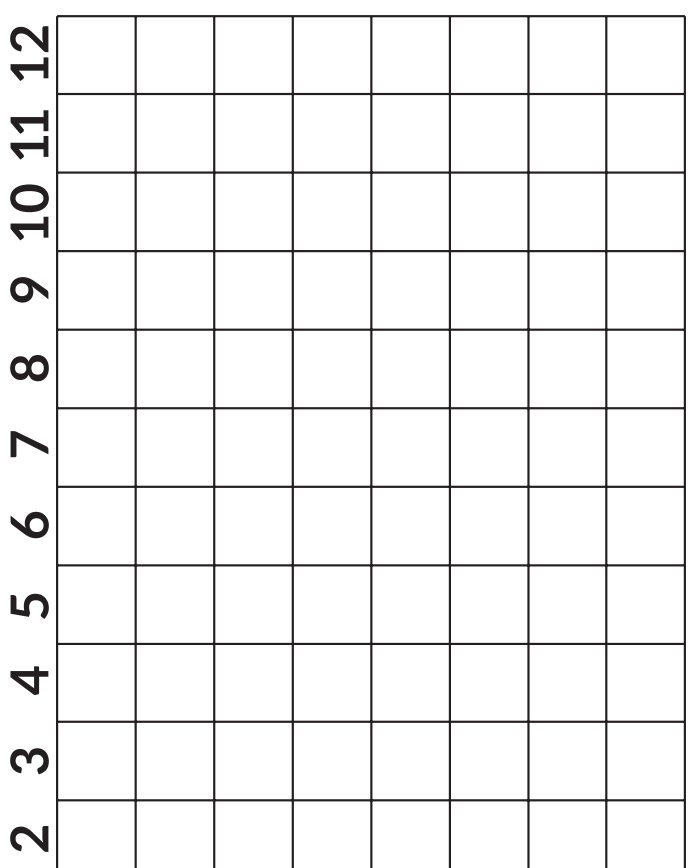
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Initial decrease in fluorescence before linear trend	This is normal and occurs due to oxygen redistribution at start of measurement	Exclude this phase from analysis as part of equilibration (see **Calculations** on page 16)
No change or minimal change in fluorescence over time	A. Cells are not metabolically active B. Oxygen reintroduced through poor sealing C. Improper instrument *gain*	A. Confirm cell viability and density B. Confirm oil overlay is intact and fully covers the media C. Calibrate instrument's *gain* using the **Calibration Procedure** on page 13
Non-linear signal throughout the time course	A. Oxygen leak B. Temperature variation	A. Ensure oil overlay is complete and no bubbles are present B. Pre-warm reagents and instrument to 37°C
Initial intensity is inconsistent	A. Uneven cell seeding B. Edge effects in multi-well culture plates C. Inconsistency in oil overlay technique	A. Ensure to seed cells evenly across wells B. Avoid using edge wells with cells C. Overlay oil carefully and consistently across wells; use a repeating pipettor

References

1. Berg, J.M., Tymoczko, J.L., and Stryer, L. *Biochemistry.* (2002).
2. Divakaruni, A.S. and Jastroch, M. *Nat. Metab.* **4(8)**, 978-994 (2022).
3. Hynes, J., Marroquin, L.D., Ogurtsov, V.I. *et al. Toxicol. Sci.* **92(1)**, 186-200 (2006).
4. Hynes, J., Natoli, E., Jr., and Will, Y. *Curr. Protoc. Toxicol.* 2.16.1-2.16.22 (2009).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.