Oxygen Consumption Rate Assay Kit

Item No. 600800

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
600801	Phosphorescent Oxygen Probe	1 vial	-20°C
660910	HS Mineral Oil Assay Reagent	1 vial/15 ml	Room temperature in the dark
600802	Cell-Based Assay Glucose Oxidase	1 vial/2 mg	-20°C
600803	Cell-Based Assay Antimycin A	1 vial/200 µl	-20°C

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.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

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

The Phosphorescent Oxygen Probe vial may be stored in the following manner:

Dry material: store between +2 to +8°C (until the indicated expiration date). **Reconstituted product:** can be stored aliquoted at -20°C. Avoid freeze/thaw cycles and use within one month. Protect products from prolonged exposure to light.

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

Materials Needed But Not Supplied

1. A plate reader capable of measuring fluorescence using excitation and emission wavelengths of 380 and 650 nm, respectively, and having plate temperature control.
2. Adjustable pipettes and a repeating pipette.
3. 96-well (black) clear bottom tissue culture plates for culturing cells.

INTRODUCTION

Background

Cellular homeostasis is maintained through the generation of ATP. 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 (O_2) dependent, takes place in the mitochondrion and is the most efficient and preferred means of ATP synthesis by mammalian cells. Thus, the oxygen consumption rate (OCR) of cells is an important indicator of normal cellular function. Unhealthy cells with dysfunctional mitochondria show a lower oxygen consumption rate compared to healthy cells.

Measurement of oxygen consumption has classically been achieved through the use of a Clark-type oxygen electrode. However, this method has limitations, as it requires specialized equipment and has a low sample throughput. A Phosphorescent Oxygen Probe has proven to be useful in measuring oxygen consumption rates in whole cells.[1] The signal of the Phosphorescent Oxygen Probe is quenched by oxygen, resulting in a signal that is inversely proportional to the amount of oxygen present. Additionally, the signal lifetime is also quenched by molecular O_2. The probe can be used in a time resolved mode, minimizing background florescence. Using the data obtained, the cellular OCR can then be calculated from the changes in the Phosphorescent Oxygen Probe signal over time.[2]

About This Assay

Cayman's cell-based Oxygen Consumption Rate Assay Kit utilizes the Phosphorescent Oxygen Probe to measure OCRs in living cells. The Phosphorescent Oxygen Probe functions best in samples containing oxygen concentrations from 0-21%. Antimycin A, an inhibitor of the mitochondrial electron transport chain, is included and is to be used as a control for zero oxygen consumption (low signal). Glucose oxidase is also included in the kit to be used as a reference for oxygen depletion (high signal).

Properties of Phosphorescent Oxygen Probe

Phosphorescent Oxygen Probe is a chemically stable and inert biopolymer-based cell-impermeable probe. Excitation and emission information can be found in Figure 1. Phosphorescent Oxygen Probe lifetime signal increases as the oxygen concentration decreases. These properties make the probe ideal for time resolved fluorescence measurements which can offer an increased signal under conditions where background is high.

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

*Excitation at 532 ±7.5 nm is also possible.

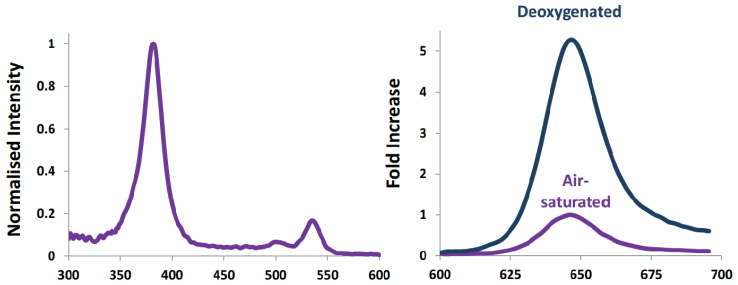


Figure 1. Excitation and Emission spectrums of Phosphorescent Oxygen Probe
Left panel: shows normalized excitation spectrum of Phosphorescent Oxygen Probe, with emission at 650 nm. Excitation maxima are observed at 380 or 532 nm. *Right panel:* shows emission spectrum of Phosphorescent Oxygen Probe in oxygenated (purple line) and deoxygenated (black line) conditions with excitation at 380 nm. Under the conditions of measurement, signal increased 5-fold on deoxygenation.

Fluorescence Measurements

There are three available options for measuring fluorescence:

1. Standard fluorescence intensity measurement
2. Time-resolved fluorescence (TR-F) measurement
3. Ratiometric TR-F measurement (subsequent Lifetime calculation)

The Phosphorescent Oxygen Probe can be measured with standard fluorescence intensity or TR-F measurements, using monochromator or filter based plate-readers. TR-F measurement reduces non-specific background and increases probe sensitivity, offering a more stable reading and wider dynamic range than measuring fluorescence intensity. Ratiometric TR-F measurement can be used to maximize dynamic range and assay performance. To determine which measurement mode is best suited for your instrument please refer to Table 1 on page 10.

1. Standard Measurement

Optimal wavelengths are 380 nm for excitation and 650 nm for emission. Please refer to Table 1 on page 10 for instrument specific settings. *NOTE: This option can often result in a lower signal to background. Time resolved measurements be performed to improve signal to background.*

2. TR-F Measurement

Optimal wavelengths are 380 nm for excitation and 650 nm for emission with a recommended delay time of 30 µs. Please refer to Table 1 on page 10 for instrument specific settings.

3. Ratiometric TR-F (Lifetime) Measurement

Ratiometric TR-F allows for the calculation of lifetime using dual time resolved measurements. In this mode, two separate time resolved readings (W_1 and W_2) are taken. From these values, a lifetime is calculated using the equation below. *NOTE: For accurate calculation of lifetime, ensure that gain values for W_1 and W_2 are identical. Please refer to table 1 on page 10 for instrument specific settings.*

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

$$\text{Lifetime (µs) } [\tau] = (70\text{-}30)/\ln(W_1/W_2)$$

Where W_1 and W_2 represent window 1 and 2, respectively, for the measured intensity readings at each time point, and 70 and 30 represent the delay time of W_2 and W_1, respectively. This provides Lifetime values in µs at each measurement.

Example calculation:

W_1 = 75,629 counts and W_2 = 14,654 counts

Lifetime = (70-30)/ln(75,629/14,654)

Lifetime = 24.4 µs

Lifetime Signal should be in the range ~22 to ~68 µs. Lifetime values should only be calculated from samples containing Phosphorescent Oxygen Probe. Lifetime values should not be calculated from blank wells.

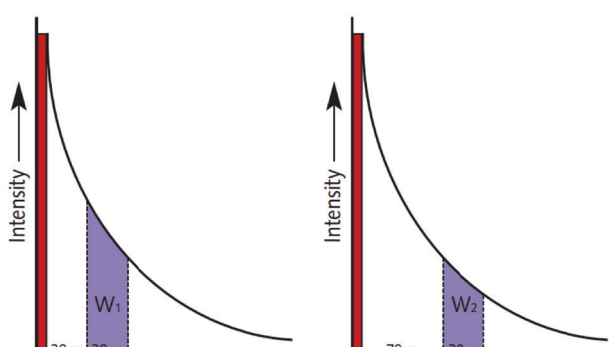


Figure 2. Illustration of ratiometric TR-F measurement

	FLUOStar & POLARstar Omega (BMG Labtech)	Victor series X3, X4, X5 (Perkin Elmer)	FLUOStar & POLARstar Optima (BMG Labtech)	Infinite/Safire/ Genios Pro (Tecan)	SpectraMax/ Flexstation/ Gemini (Mol. Devices)
Light source	Xe-flashlamp	Xe-flashlamp	Xe-flashlamp	Xe-flashlamp	Xe-flashlamp
Optical Configuration	Filter-based Top/ Bottom reading	Filter-based Top reading	Filter-based Top/ Bottom reading	Filter-based Top/ Bottom reading	Monochromator-based Top/Bottom reading
Measurement mode	*Ratiometric TR-F	*Ratiometric TR-F	TR-F	TR-F	Standard
Excitation	380 ±20 nm (TR-EX L)	340 ±40 nm (D340)	380 ±20 nm (TR-EX L)	380 ±20nm	380 nm
Emission	650 ±50 nm (BP-650)	642 ±10 nm (D642)	650 ±50 nm (BP-650)	650 ±20 nm	650 nm
Delay time 1	30 µs	30 µs	30 µs	30 µs	N/A
**Delay time 2	70 µs	70 µs	N/A	N/A	N/A
Read time 1	30 µs	30 µs	100 µs	100 µs	N/A
Read time 2	30 µs	30 µs	N/A	N/A	N/A

Table 1. Recommended Instrument and Measurement Settings.
TR-F, time-resolved fluorescence
*TR-F attachment installed in instrument
**Applicable to ratiometric TR-F measurement only.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Phosphorescent Oxygen Probe Solution**

   Prior to use, reconstitute the contents of the Phosphorescent Oxygen Probe vial (Item No. 600801) with 1 ml of distilled water or assay medium. The reconstituted Phosphorescent Oxygen Probe solution is stable for one day when stored at 4°C. For long term storage, aliquot the reconstituted solution and store at -20°C. The reconstituted Phosphorescent Oxygen Probe will be stable for one month when stored at -20°C.

2. **Glucose Oxidase Stock Solution**

   Prior to use, reconstitute the contents of the Cell-Based Assay Glucose Oxidase vial (Item No. 600802) with 0.2 ml of distilled water. For long term storage, aliquot the reconstituted solution and store at -20°C. The reconstituted stock solution will be stable for two months when stored at -20°C.

3. **Antimycin A Stock Solution**

   Prior to use, thaw the Cell-Based Assay Antimycin A vial (Item No. 600803) and warm to room temperature. To make the Antimycin A Stock Solution, dilute the Cell-Based Assay Antimycin A 1:10 in culture medium. Unused Cell-Based Assay Antimycin A will be stable for at least one year of stored at -20°C. *NOTE: Please ensure that proper personal protective equipment is worn when handling Antimycin A.*

ASSAY PROTOCOL

Typical Instrument Set Up

NOTE: Instrument settings will vary between manufacturers.

1. Set the plate reader temperature control to 37°C.
2. Optimal wavelengths are 380 ±20 nm for excitation and 650 ±20 nm for emission.
3. For TR-F or ratiometric TR-F, delay and mesurment times refer to Table 1 on page 10 for the parameters best suited for your plate reader.
4. Gain should be optimized so that the fluorescent signal of Phosphorescent Oxygen Probe in 21% O_2 (air saturated) buffer is equal to 20% of the maximum detectable signal.

Instrument Signal Optimization

To optimize the signal, the following steps should be performed. For standard measurements or TR-F measurements, a signal to blank ratio ≥3 is required. For ratiometric TR-F (lifetime) measurement, a signal to blank ratio ≥10 is required for W_2 (see Ratiometric TR-F (Lifetime) Measurement on page 8).

1. In a spare black, clear bottom 96-well tissue culture treated plate, add 140 µl of culture medium to six wells.
2. Add 10 µl of culture medium to three wells. These are your blank signal wells.
3. Add 10 µl of Phosphorescent Oxygen Probe (Page 11) to three wells. These are your signal wells.
4. Gently overlay each well with 100 µl of HS Mineral Oil (Item No. 660910). The use of a repeating pipette is preferred.
5. Read the plate immediately with the set up described on page 12. The plate should be measured kinetically for 30 minutes to ensure the fluorescent signal is stable.
6. If required, adjust the instrument parameters to increase measurement sensitivity in order to achieve maximal S/B ratio. The following options may be helpful:

   -Increase gain (or PMT) settings or flash energy

   -Adjust TR-F focal height

   -Repeat without phenol red or serum

   -Repeat as a top, or bottom read (plate reader dependent)

   -Increase volume of Phosphorescent Oxygen Probe from 10 µl to 15 µl

   -Contact Instrument supplier for further options

Plate Set Up

There is no specific pattern for using the wells on the plate, but it is important to include the following control wells containing no cells:

Blk - Background Wells containing culture medium overlaid with oil.

POP - Phosphorescent Oxygen Probe Wells containing culture medium plus Phosphorescent Oxygen Probe Solution overlaid with oil.

GO - Glucose Oxidase Wells containing culture medium, Glucose Oxidase Solution, and Phosphorescent Oxygen Probe Solution overlaid with oil.

Antimycin A (**AA**) wells contain cells treated with Antimycin A Stock Solution. Sample wells contain cells treated with experimental compounds or vehicle. We recommend that each treatment be performed in triplicate and that you record the contents of each well on the template sheet provided (see page 22).

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

1-28 = Sample Wells
Blk = Blank/Background Wells, containing no cells
POP = Phosphorescent Oxygen Probe Wells, containing no cells
GO = Glucose Oxidase Wells, containing no cells
AA = Antimycin A Wells

Figure 3. Sample plate format

Performing the Assay

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver 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.

1. Seed cells in a black, clear bottom 96-well tissue culture treated plate at a density of 40,000-80,000 cells/well in 200 µl of culture medium. *NOTE: Optimal seeding density will vary based on aerobic capacity of the cell line. We recommend trying a range of cell densities to optimize oxygen consumption rates.* Incubate the cells overnight using appropriate culture conditions for the experimental cell type. It is important to have nine wells with no cells for the controls described in **Plate Set Up**.
2. Remove spent culture medium from all wells and replace with 150 µl of fresh medium.
3. Add test compounds or the appropriate vehicle in 10 µl to Sample Wells. *NOTE: To assess the effect of a compound on mitochondrial function, cells are typically treated immediately prior to measurement. Prolonged incubations with test compounds can be performed if required.* After prolonged treatment remove spent culture medium from all wells and replace with 150 µl of fresh medium. Ensure that solvent concentration does not exceed 0.5% of the total volume in the well.
4. Add 20 µl of culture medium to the three Blk Wells.
5. Add 10 µl of Glucose Oxidase Stock Solution (Page 11) to the **GO** Wells.
6. Add 10 µl of Antimycin A Stock Solution (Page 11) to the **AA** Wells.
7. Add 10 µl of culture medium to the **POP** Wells.

8. Add 10 µl of Phosphorescent Oxygen Probe Solution (Page 11) to every well ***except*** the three **Blk** Wells.
9. Gently overlay every well with 100 µl of HS Mineral Oil (Item No. 660910). The use of a repeating pipette is preferred. *NOTE: Ensure the HS Mineral Oil is pre-warmed to the measurement temperature.*
10. Read the plate immediately with the set up described on page 12. The plate should be measured kinetically for ≥120 minutes.

Wells	Culture Medium (µl)	Glucose Oxidase (µl)	Antimycin A (µl)	Test Compounds (µl)	Extra Culture Medium (µl)	Phosphorescent Oxygen Probe (µl)
Sample	150	-	-	10		10
Blk	150	-	-		20	-
MX	150	-	-		10	10
GO	150	10	-		-	10
AA	150	-	10		-	10

Table 2. Pipetting summary

ANALYSIS

Calculations

Assessing Oxygen Consumption

Plot the Phosphorescent Oxygen Probe Signal, Intensity, or Lifetime *versus* Time (mins) (see Figure 4 on page 18). Select the linear portion of the signal profiles and apply linear regression to determine the slope for each of the signal profiles. (This approach is preferable to calculating a slope from averaged profiles.)

Tabulate the slope values for each sample and calculate appropriate average and standard deviation values. The slope obtained for the **Blk** Wells (sample without cells) should be subtracted from all test values.

Plotting the Dose Response Curve

To generate dose response data, plot the data generated as outlined above against the corresponding compound concentration, see Figure 5 on page 19.

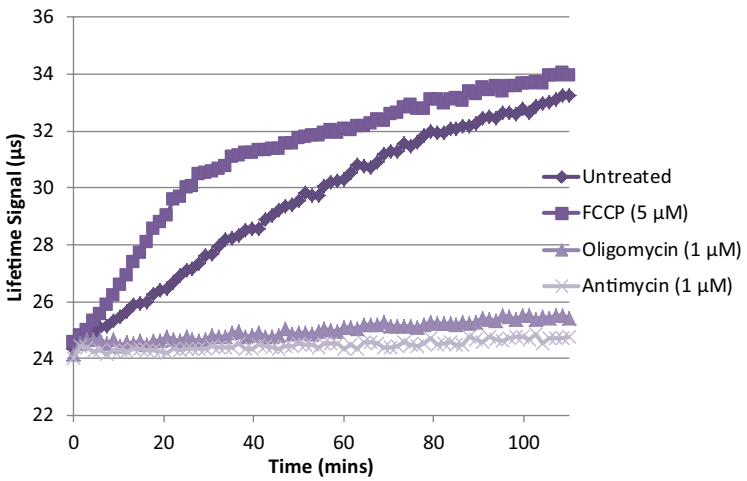


Figure 4. Typical lifetime signal profile of Phosphorescent Oxygen Probe for cell samples which have been treated with different classical electron transport chain inhibitor or activator compounds.

Performance Characteristics

The dose response curve presented here is an example of the data typically produced with the assay.

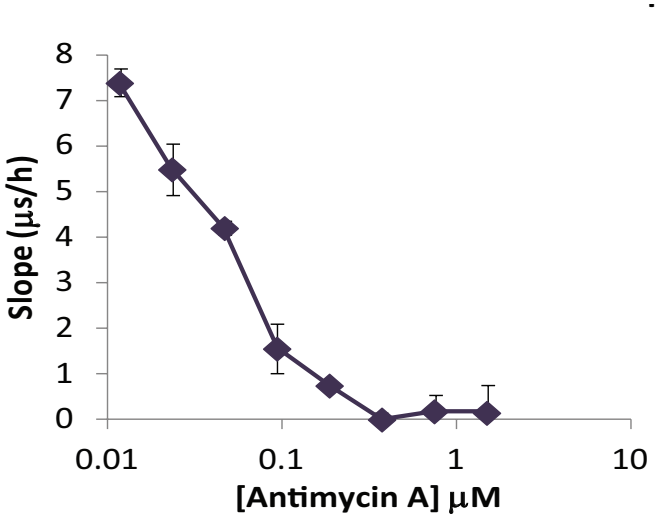


Figure 5. Dose response curve
Antimycin A concentration (µM) *versus* calculated slope (µs/hour), showing Antimycin A causes an inhibitory response on cellular oxygen consumption.

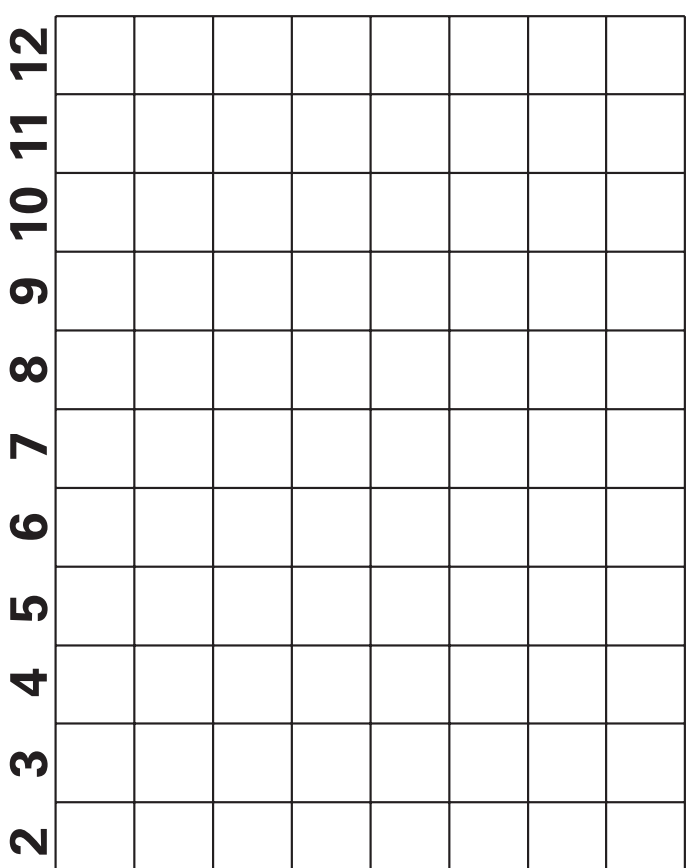
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Signals indistinguishable from blanks	Incompatible instrument or incorrect instrument settings	Check instrument suitability and setup and run proper controls without cells (S/B test) (probe/no probe)
Signals detectable, but signal changes too small	Instrument performance is poor (low S/B ratio); monolayer cell density used is too low	Check the instrument and run proper controls; use greater cell density; optimise assay conditions
There is a drop in signal over the initial minutes	Plate temperature equilibration; baseline drift	Use plate block heater during plate preparation; pre-warm all solutions
Initial intensity is inconsistent	Long plate preparation times	Reduce plate preparation time to <10 minutes; use plate heater during plate preparation

References

1. Hynes, J., Marroquin, L.D., Ogurtsov, V.I., *et al*. Investigation of drug-induced mitochondrial toxicity using fluorescence-based oxygen-sensitive probes. *Toxicol. Sci.* **92(1)**, 186-200 (2006).
2 Hynes, J., Natoli, E., Jr., and Will, Y. Fluorescent pH and oxygen probes of the assessment of mitochondrial toxicity in isolated mitochondria and whole cells. *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.