Oxygen Consumption/Glycolysis Dual Assay Kit

Item No. 601060

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	100 Tests Quantity/Size	Storage
600801	MitoXpress® - Xtra	1 vial	4°C
660910	HS Mineral Oil Assay Reagent	1 vial/15 ml	Room Temperature in the dark
600803	Cell-Based Assay Antimycin A	1 vial/200 µl	-20°C
600451	Glycolysis Assay Substrate	1 vial/250 µl	-20°C
600452	Glycolysis Assay Enzyme Mixture	1 vial/lyophilized	-20°C
600453	Glycolysis Assay L-Lactate Standard	1 vial/120 µl	4°C
600454	Glycolysis Assay Cofactor	1 vial/250 µl	4°C
10009322	Cell-Based Assay Buffer Tablet	1 tablet	Room Temperature

NOTE: MitoXpress® - Xtra is a product of Luxcel Biosciences.

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 laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

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

Hours: M-F 8:00 AM to 5:30 PM EST

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 MitoXpress® - Xtra 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 having plate temperature control and capable of measuring excitation and emission wavelengths of MitoXpress® - Xtra at 380 nm and 650 nm, respectively, and measuring absorbance at 490 nm
2. Adjustable pipettes and a repeating pipettor
3. 96-well (black) clear bottom TC+ plates or standard clear PS plates for culturing cells and 96-well assay plates for glycolysis assay

INTRODUCTION

Background

Mitochondria are the powerhouses of eukaryotic cells. They are organelles in which biochemical energy, ATP, is generated. In addition to supplying cellular energy for various physiological processes, mitochondria are involved in cell signaling, growth, differentiation, and cell death.[1] Dysfunction of mitochondria leads to diseases such as stroke and Alzheimer's disease. Furthermore, metabolic pathway changes in the mitochondria reflect pathological development in cells. For example, cancer cells preferentially obtain energy from aerobic glycolysis without consuming oxygen, rather than through oxidative phosphorylation, to produce ATP inside mitochondria.[2] Due to their central role in energy metabolism, mitochondria have been a pharmacological target for decades.[3]

Assessment of mitochondrial function is essential in studies where mitochondria are therapeutic targets. However, single parameter assays give limited information regarding the mechanism of drug action. On the other hand, a multi-parameter approach that measures cellular oxygen consumption and glycolysis provides a more complete metabolic picture of the cellular response to drug treatment.[4]

In the past, the measurement of oxygen consumption has been achieved by using an oxygen electrode. Recently, a phosphorescent oxygen probe, MitoXpress® - Xtra, developed by Luxcel Biosciences, has proven to be useful in analyzing oxygen consumption in whole cells. The phosphorescence of MitoXpress® - Xtra is quenched by oxygen, and thus the phosphorescent signal is inversely proportional to the amount of oxygen present. The oxygen consumption rate of cells can then be calculated from the change in MitoXpress® - Xtra probe signal over time.[5]

Lactate is the end product of glycolysis and is released into the extracellular environment. Extracellular lactate levels are proportionally correlated with intracellular glycolytic activity. In cultured cells, lactate released into the culture medium can be measured by an assay using lactate dehydrogenase to catalyze the oxidation of lactate to pyruvate, in which the formed NADH reduces a tetrazolium substrate (INT) to a highly-colored formazan derivative which absorbs strongly at 490-520 nm.

About This Assay

Cayman's Oxygen Consumption/Glycolysis Dual Assay Kit is a multi-parameter approach to measure cellular oxygen consumption and glycolysis in living cells. This assay utilizes MitoXpress® - Xtra to measure oxygen consumption rate and quantifies extracellular lactate as a readout for glycolysis. Antimycin A, an inhibitor of the mitochondrial electron transport chain, is included as a control. The kit can be used for efficient screening of compounds that modulate mitochondrial and glycolytic function in cultured cells.

Measurement Parameters

MitoXpress® - Xtra probe is a chemically stable and inert, biopolymer-based, cell impermeable probe. The probe is excitable between 360-400 or 535 nm and emits at 630-680 nm.

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

*Excitation at 532 ±7.5 nm is also possible.

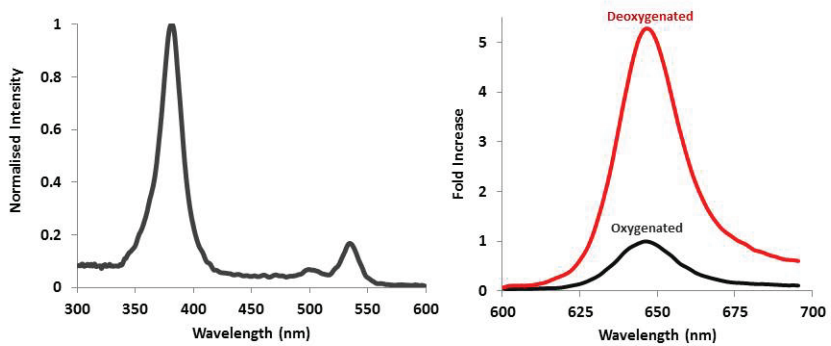

Figure 1. Excitation and Emission spectrums of MitoXpress® - Xtra
Left panel: shows normalized excitation spectrum of MitoXpress® - Xtra, with emission at 650 nm. Excitation maxima are observed at 340-400 or 525-545 nm. *Right panel:* shows emission spectrum of MitoXpress® - Xtra in oxygenated (black line) and deoxygenated (red 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 MitoXpress® - Xtra fluorescence:

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

The MitoXpress® - Xtra probe can be measured with standard or TR-F measurements, using monochromator or filter based plate-readers. TR-F measurement reduces non-specific background and increases probe sensitivity. Ratiometric measurement is used to maximize dynamic range and assay performance.

1. Standard Measurement

Optimal wavelengths are 380 nm for excitation and 650 nm for emission. Gain parameter (PMT) is typically set at medium or high. MitoXpress® - Xtra probe signals should be at least three times above the Blank (Background) signal.

2. TR-F Measurement

Optimal delay time is 30 microsecond units (µs) and gate (integration) time is 100 µs. MitoXpress® - Xtra probe signal should be greater than 3-fold that of the Blank (Background) signal. Signals of ~10-fold greater than blank are typical.

3. Ratiometric TR-F (Lifetime) Measurement

Optimal dual-delay and gate (integration) times:

Integration window 1 (W_1): 30 µs delay, 30 µs gate time

Integration window 2 (W_2): 70 µs delay, 30 µs gate time

The MitoXpress® - Xtra probe Signal to Blank ratio (S/B) for W_2 measurement is recommended to be >10/1 to allow accurate Lifetime calculation.

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

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

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 measured time point for each individual sample.

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 of ~22 to ~68 µs. Lifetime values can only be calculated from samples containing MitoXpress® - Xtra probe. S/B should be greater than 10 for W_2. Lifetime values should not be calculated from blank wells.

Instrument	Optical Config^{n}	Mode	Ex (nm) Em (nm)	Integn 1 (D1/W1) Integn 2** (D2/W2)
BMG Labtech: FLUOStar Omega/ POLARstar Omega	Filter-based Top or bottom read	*dual-read TR-F (Lifetime)	Ex 340 ± 50 nm (TR-EX L) Em 655 ± 50 nm (BP-655)	30 / 30 µs 70 / 30 µs
BMG Labtech: PHERAstar FS	Filter-based Top read	TR-F	Ex 337 nm (HTRF module) Em 665 nm (HTRF module)	40 / 100 µs n/a
BMG Labtech: FLUOStar Optima/ POLARstar Optima	Filter-based Top or bottom read	TR-F	Ex 340 ± 50 nm (TR-EX L) Em 655 ± 50 nm (BP-655)	30 / 100 µs n/a
Perkin Elmer: VICTOR series/ X4, X5	Filter-based Top read	* dual-read TR-F (Lifetime)	Ex 340 ± 40 nm (D340) Em 642 ± 10 nm (D642)	30 / 30 µs 70 / 30 µs
Perkin Elmer: ENVISION	Filter-based Top read	TR-F	Ex 340 nm Em 650 nm	40 / 100 µs n/a
BioTek: Synergy H1, H4, 2	Filter-based Top or bottom read	* dual-read TR-F (Lifetime)	Ex 380 ± 20 nm Em 645 ± 15 nm	30 / 30 µs 70 / 30 µs
Tecan: Infinite/Safire/ Genios Pro	Filter-based Top or bottom read	TR-F	Ex 380 ± 20 nm Em 650 ± 20 nm	30 / 100 µs n/a
Mol. Devices: SpectraMax/ Flexstation/Gemini	Monochromator-based Top or bottom read	Intensity (Prompt)	Ex 380 nm Em 650 nm	n/a n/a
Thermo: Varioskan/ Fluoroscan Ascent	Monochromator/ Filter-based Top or bottom read	TR-F	Ex 380 nm Em 650 nm	30 / 100 µs n/a

Table 1. Recommended Instrument and Measurement settings for MitoXpress® - Xtra
*TR-F attachment installed in instrument
**Applicable to ratiometric TR-F measurement only.

NOTE: Preset Protocol Files for BMG instruments are available from www.luxcel.com and BMG Technical Support.

PRE-ASSAY PREPARATION

Reagent Preparation

1. MitoXpress® - Xtra Solution

Prior to use, reconstitute the contents in the vial (Item No. 600801) with 1 ml of distilled water (sterile). The reconstituted MitoXpress® - Xtra solution will be stable prior to use on the day of preparation, when stored at 4°C. For long term storage, aliquot and store at -20°C. The MitoXpress® - Xtra will be stable for one month when stored at -20°C.

2. Antimycin A Stock Solution

Prior to use, thaw the Cell-Based Assay Antimycin A vial (Item No. 600803) and warm to room temperature. The Antimycin A will be stable for at least one year if stored at -20°C.

3. Assay Buffer Preparation

Dissolve the Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of distilled water. This Buffer should be stable for approximately one year at room temperature.

4. Glycolysis Assay Enzyme Mixture

The vial contains a lyophilized powder of enzymes. Immediately prior to use, reconstitute the contents of the vial with 150 µl of Assay Buffer. If you are not using all of the mixture at one time, make small aliquots and store at -80°C. Freezing and thawing of this solution should be limited to a single time.

5. Reaction Solution

To make 12 ml of Reaction Solution sufficient for use on one 96-well plate, add 120 µl of each of the following mixtures to 11.64 ml of the Assay Buffer:

Glycolysis Assay Substrate (Item No. 600451)

Glycolysis Assay Cofactor (Item No. 600454)

Reconstituted Glycolysis Assay Enzyme Mixture (prepared in Step 5)

The Reaction Solution is stable for approximately one hour at room temperature.

ASSAY PROTOCOL

Instrument Set Up for Oxygen Consumption Rate Measurement

1. Set the plate reader temperature control to 37°C.
2. Set the excitation filter to 380 ±20 nm and emission filter to 650 ±20 nm.
3. Set delay/gate time to 30 µs and integration/measurement time to 100 µs.
4. Set gain to a range from 80 to 100.
5. Z' height is typically set to ~8 mm.
6. Select kinetic measurement protocol to read the plate at three minute intervals for 2.5-3.5 hours.

Instrument Signal Optimization: Assessing the S/B ratio on plate reader.

1. Set plate reader parameters for suitable measurement of MitoXpress® - Xtra as advised in Table 1 on page 12.
2. Prepare a 96-well microwell plate with wells containing medium and wells containing medium with MitoXpress® - Xtra Stock Solution added, 150 µl sample volume/well.
3. Seal all wells with 100 µl of HS Mineral Oil Assay Reagent.
4. Measure this plate on a plate reader for a short 30 minute kinetic test.
5. Adjust instrument parameters of interest to increase/decrease measurement sensitivity as required in order to achieve maximum S/B ratio.

Plate Set Up

There is no specific pattern for using the wells on the plate, but it is important to include wells for background control signal (medium + oil only) and probe control signal (medium + MitoXpress® - Xtra + oil). A typical experimental plate will include wells without cells, wells with cells treated with experimental compounds, and wells of untreated cells. 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 27).

NOTE: All wells of the 96-well plate can be used for cell adhesion except the designated Control wells.

	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	MX	MX	MX	AA	AA	AA	29	29	29

1-29 = Sample Wells
Blk = Blank/Background Wells, containing no cells
MX = MitoXpress® - Xtra Wells, containing no cells
AA = Antimycin A Wells

Figure 2. Sample plate format

Performing the Oxygen Consumption Assay

Pipetting Hints

- It is recommended that a repeating pipettor 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 plate (a regular clear 96-well cell culture plate can also be used) at a density of 40,000-80,000 cells/well in 200 µl of culture medium. Leave six wells with culture medium only, for different controls, as indicated in Figure 2, on page 15. Incubate the cells overnight in a carbon dioxide incubator at 37°C.
2. To assess the effect of a compound on oxygen consumption and/or glycolysis, cells are treated immediately prior to measurement (Steps 7-9 below). Long term incubation with compound/vehicle can also be performed. We recommend the use of triplicate wells for each treatment.
3. Remove the culture medium from all wells and replace with 150 µl of fresh medium containing treated compounds or vehicle.
4. Add 20 µl of culture medium to 3 Blk (Blank/Background) wells, as indicated in Figure 2, sample **Plate Set Up**.
5. Add 10 µl of Antimycin A Stock Solution (Item No. 600803) to the AA (Antimycin A) wells, as indicated in Figure 2, sample **Plate Set Up**.
6. Add 10 µl of culture medium to all the sample wells and to the MX (MitoXpress® - Xtra wells containing no cells) wells.
7. Add 10 µl of MitoXpress® - Xtra solution, as prepared above, to each well except three Blk wells.
8. Using a repeating pipettor, slowly pick up HS Mineral Oil Assay Reagent (Item No. 660910) from the supplied bottle (avoid pipetting up and down) and gently dispense 100 µl to overlay each well. Ensure HS mineral oil is pre-warmed to measurement temperature in advance.
9. Read the plate immediately with the set up described on page 14. The plate should be measured kinetically for >120 minutes for oxygen consumption rate.
10. Remove the plate from the plate reader and follow the procedure on page 19 to measure lactate levels in each well.

Wells	Culture Medium (µl)	Antimycin A (µl)	Extra Culture Medium (µl)	MitoXpress® - Xtra (µl)
Sample	150	-	10	10
Blk	150	-	20	-
MX	150	-	10	10
AA	150	10	-	10

Table 2. Pipetting summary

Gycolysis Assay Standard Preparation

To prepare the standards for use in the glycolysis assay, obtain eight clean test tubes or microcentrifuge tubes and label them #1 through #8. Aliquot 450 µl of Assay Buffer into tube #1 and 250 µl into tubes #2-#8. Transfer 50 µl of the Glycolysis Assay L-Lactate Standard (Item No. 600453) into tube #1 and mix thoroughly. The lactic acid concentration of this standard, the first point on the standard curve, is 1 mM. Serially dilute the standard by removing 250 µl for tube #1 and place into tube #2, mix thoroughly. Next, remove 250 µl from tube #2 and place into tube #3; mix thoroughly. Repeat this procedure for tubes #4 through tubes #7. Do not add any standard to tube #8. This tube will be your blank.

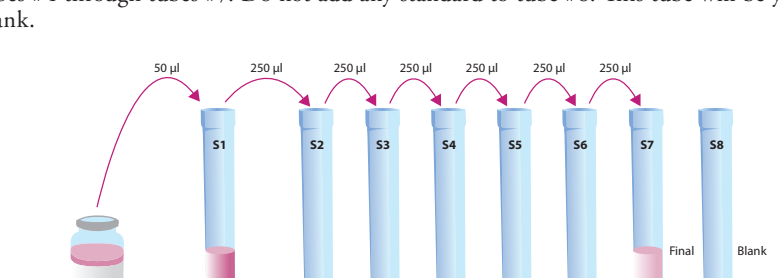

Figure 3. Preparation of the L-Lactic Acid Standards

Performing the Glycolysis Assay

The assay can be performed with the same samples for Oxygen Consumption measurement performed above. Alternatively, the assay can be performed with a different set of samples having identical treatments as those for oxygen consumption measurement. However, this different set of samples should be on the same plate as those for Oxygen Consumption measurement except that the wells will not be sealed with oil.

The following protocol is for measurement of lactate on the same samples for Oxygen Consumption measurement.

1. Using a new 96-well plate, transfer 100 µl of the standards prepared above into appropriate wells. We recommend that the standards be run in duplicate.
2. Add 90 µl of Assay Buffer to each well except standard wells.
3. Transfer 10 µl of culture medium from each sample well to corresponding wells on the new plate. To ensure that only culture medium, not oil, is transferred, quickly push the pipette tip to the bottom of the well and suck up 10 µl of the culture medium. Wipe off the oil attached to the tip before releasing the culture medium into wells.
4. Add 100 µl of Reaction Solution (prepared in Step 5, on page 13) to each well, including the standard wells, using a repeating pipettor.
5. Incubate the plate with gentle shaking on an orbital shaker for 30-60 minutes at room temperature.
6. Read the absorbance at 490 nm with a plate reader.

ANALYSIS

Calculations

Assessing Oxygen Consumption

Plot the MitoXpress® - Xtra Signal, Intensity, or Lifetime *versus* Time (mins) (see Figure 4 on page 21). Select the linear portion of the signal profiles and apply a linear regression to determine the slope and correlation coefficient 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/Background Wells (sample without cells) should be subtracted from all test values.

Assessing Glycolysis

1. Determine the average fluorescence of each standard and sample.
2. Subtract the absorbance value of the blank from itself and all other standards and samples. This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of the final concentration of lactate. See Figure 5, on page 22, for a typical standard curve.
4. Calculate the L-lactate concentration of the samples using the corrected absorbance of each sample and the equation below.

$$\text{L-Lactate } (\mu M) = \left[\frac{\text{Absorbance - (y-intercept)}}{\text{Slope}} \right] \times 10$$

Performance Characteristics

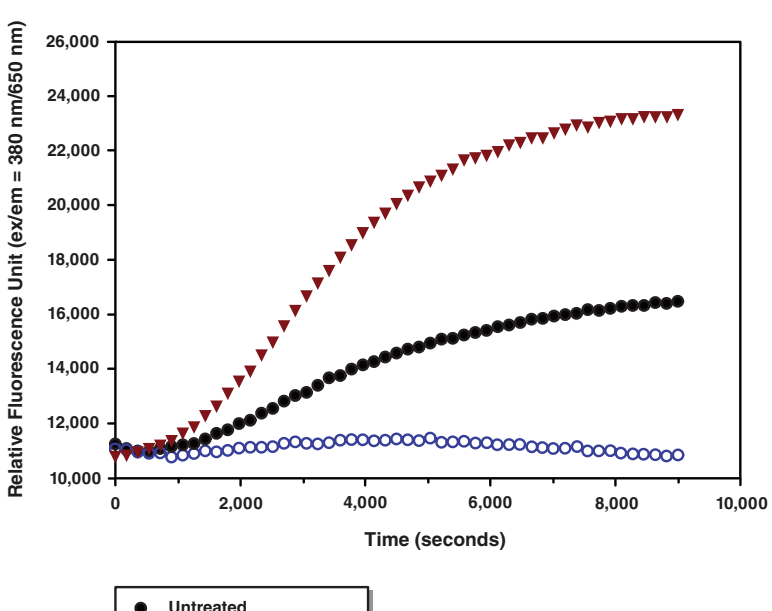

Figure 4. Typical relative fluorescence unit profiles of MitoXpress® - Xtra measuring the effect of mitochondrial inhibition (Antimycin treatment) and uncoupling (FCCP treatment) on cell respiration. Measurement made immediately post treatment.

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You **must** run a new standard curve. Do not use the data below to determine the value of your samples. Your results could differ substantially.

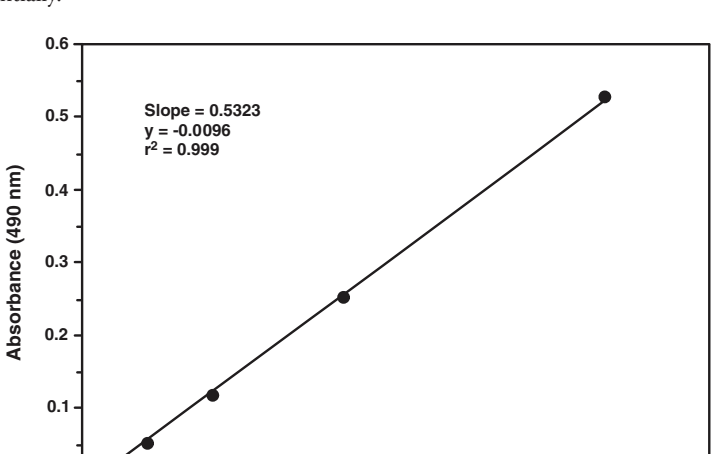

Figure 5: Typical standard curve.

Specificity

To assess substrate specificity, the assay was performed with L-lactate replaced by structurally similar compound D-lactate. No reaction occurred when D-lactate is used at a concentration up to 10 mM.

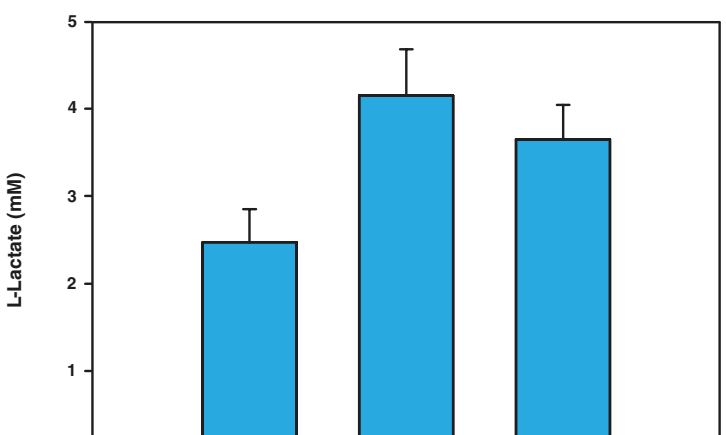

Figure 6. Disruption of electron transport chain by antimycin or FCCP results in increases in glycolystic rate, evidenced by increased lactate production. HCT116 cells were seeded in a 96-well plate at a density of 50,000 cells/well in culture medium containing 10% FBS and grown in a 5% carbon dioxide incubator at 37°C. The next day, cells were treated with vehicle, 1 µM Antimycin A, or 2.5 µM FCCP, and analyzed using a plate reader for a kinetic measurement of oxygen consumption at 37°C for three hours (shown in figure 4). The L-lactate levels in the culture medium from each well were then measured according to the protocol described in the booklet.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Sample signal 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)
Signal 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; optimize 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

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4. Perry, S.W., Norman, J.P., Barbieri, J., *et al.* Mitochondrial membrane potential probes and the proton gradient: A practical usage guide. *Biotechniques* **50(2)**, 98-115 (2011).
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Related Products

Annexin V FITC Assay Kit - Item No. 600300
Caspase-3 Fluorescence Assay Kit - Item No. 10009135
Glucose Colorimetric Assay Kit - Item No. 10009582
Glucose Uptake Cell-Based Assay Kit - Item No. 600470
Glycolysis Cell-Based Assay Kit - Item No. 600450
JC-1 Mitochondrial Membrane Potential Assay Kit - Item No. 10009172
LDH Cytotoxicity Assay Kit - Item No. 10008882
MTT Cell Proliferation Assay Kit - Item No. 10009365
Multi-Parameter Apoptosis Assay Kit - Item No. 600330
NAD^+/NADH Cell-Based Assay Kit - Item No. 600480
Oxygen Consumption/MitoMembrane Potential Dual Assay Kit - Item No. 600880
Oxygen Consumption Rate Assay Kit (MitoXpress® - Xtra HS Method) - Item No. 600800
WST-1 Cell Proliferation Assay Kit - Item No. 10008883

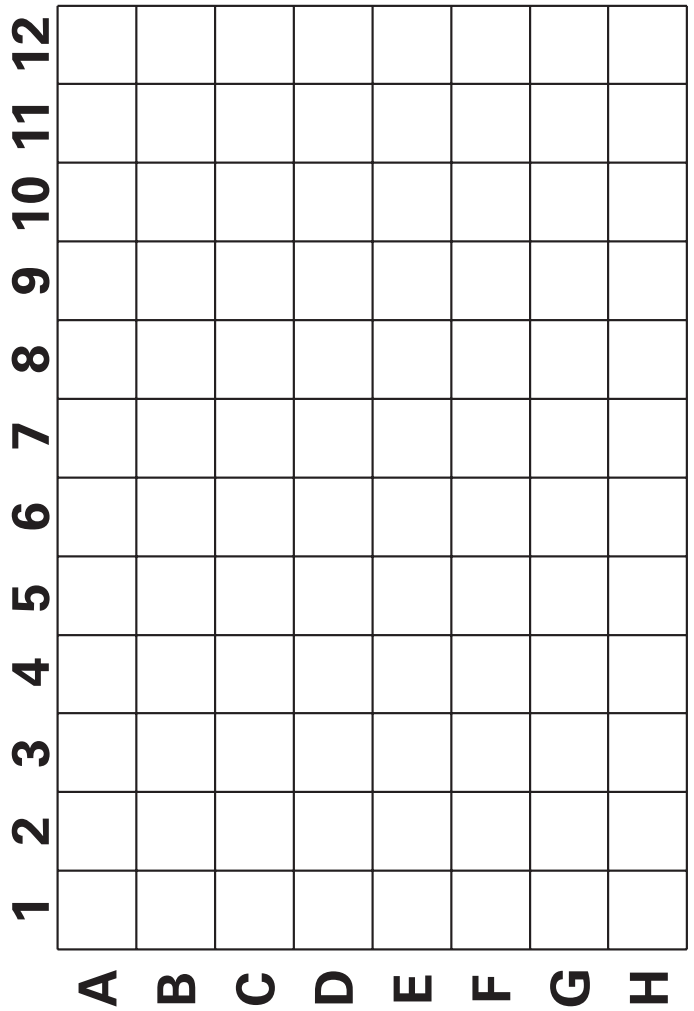
Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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