Oxygen Consumption/MitoMembrane Potential Dual Assay Kit

Item No. 600880

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
600802	Cell-Based Assay Glucose Oxidase	1 vial/2 mg	-20°C
600803	Cell-Based Assay Antimycin A	1 vial/200 µl	-20°C
10009908	JC-1 Reagent	1 vial/500 µl	-20°C

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 JC-1 red aggregates at excitation/emission = 650 nm/690 nm with band width at 10 nm and JC-1 green monomers at excitation/emission = 485 nm/535 nm with band width at 10 nm.
2. Adjustable pipettes and a repeating pipettor
3. 96-well (black) clear bottom TC+ plates or standard clear PS plates for culturing cells

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. Assays ranging from measuring oxygen consumption to mitochondrial membrane potential have been used to assess mitochondrial function. In order to obtain a more complete picture on mitochondrial metabolism and function, multiplex assays will have an advantage in offering the most comprehensive picture of mitochondrial performance.[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]

JC-1 is a cationic dye that is capable of entering into mitochondria and reversibly changing color as the membrane potential increases. In cells with high mitochondrial $\Delta\Psi m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in cells where mitochondrial $\Delta\Psi m$ is low, JC-1 remains in a monomeric form with pronounced green fluorescence.

About This Assay

Cayman's Oxygen Consumption/Mitochondrial Membrane Potential Dual Assay Kit utilizes MitoXpress® - Xtra, and JC-1 to measure oxygen consumption rate and mitochondrial membrane potential simultaneously in living cells. Antimycin A, an inhibitor of the mitochondrial electron transport chain, is included to be used as a positive control. Glucose oxidase is also included in the kit to be use as a reference for oxygen depletion. The kit is easy to use and can be easily adapted to high throughput screening for compounds which modulate mitochondrial functions.

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.

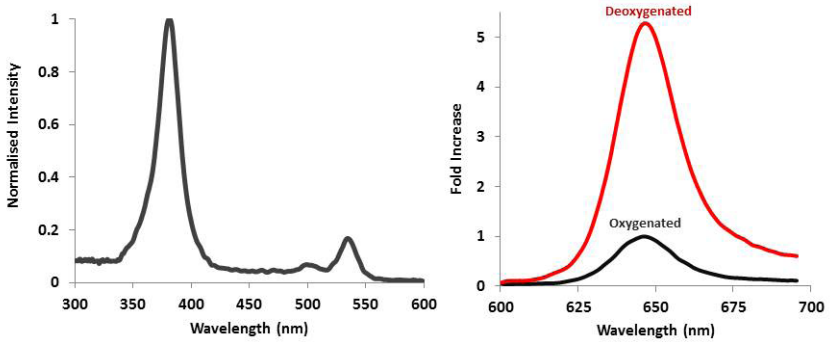


Figure 1. Excitation and Emission spectrums of MitoXpress® - Xtra
Left panel: shows normalized excitation spectrum of MitoXpress® - Xtra, with emission at 650 nm. Excitation maxima are observed at 340-400 or 525-545 nm. *Right panel:* shows emission spectrum of MitoXpress® - Xtra in oxygenated (black 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. Ratio-metric measurement is used to maximize dynamic range and assay performance.

1. Standard Measurement

Optimal wavelengths are 380 nm 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 microsecound 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. Ratio-metric 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 ~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.

	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
Gate time 1	30 µs	30 µs	100 µs	100 µs	N/A
**Gate time 2	30 µs	30 µs	N/A	N/A	N/A

Table 1. Recommended Instrument and Measurement settings for MitoXpress® - Xtra
TR-F, time-resolved fluorescence
*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. Glucose Oxidase Stock Solution

Prior to use, reconstitute the contents in the vial (Item No. 600802) with 0.2 ml of distilled water. 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 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.

4. JC-1 Staining Solution

Thaw the JC-1 Reagent (Item No. 10009908) at room temperature. Prepare a staining solution by diluting the reagent 1:10 in the culture medium you are using for your cells. Mix well to make sure there are no particles or flakes in the solution. Prepare just enough staining solution for each experiment. If you are not using all the JC-1 Reagent at one time, we recommend that you make small aliquots and store them at -20°C.

NOTE: JC-1 Staining Solution is difficult to prepare due to its low solubility in aqueous medium and tendency to form particulates that are difficult to remove. Make sure JC-1 Reagent is completely thawed and warmed to room temperature before diluting it into culture medium. Do not centrifuge the reagent.

NOTES

- JC-1 is light sensitive. All staining procedures must be performed without direct exposure to intense light. Therefore, incubations need to be done in the dark.

ASSAY PROTOCOL

Typical 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 minutes intervals for 2.5-3.5 hours.

For Mitochondrial Membrane Potential Measurement

1. Set the excitation to 485 ±10 nm and emission to 535 ±10 nm. This is to measure JC-1 monomers.
2. Set the excitation to 560 ±10 nm and emission to 590 ±10 nm. This is to measure JC-1 aggregates.

Instrument Signal Optimization for Oxygen Consumption Rate Measurement

Instrument Signal Optimization: Assess 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 9.
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 19).

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	GO	GO	GO	AA	AA	AA

1-28 = Sample Wells
Blk = Blank/Background Wells, containing no cells
MX = MitoXpress® - Xtra Wells, containing no cells
GO = Glucose Oxidase Wells, containing no cells
AA = Antimycin A Wells

Figure 2. Sample plate format

Performing the 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 nine wells with culture medium only for different controls, as indicated in Figure 2, on page 13. 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 mitochondrial membrane potential, cells are treated immediately prior to measurement (Step 6 to 8 below). Long term incubation with compound/vehicle can also be performed. We recommend the use of triplicate wells for each treatment.
3. Add 10 µl of JC-1 Staining Solution prepared above to each well. Gently shake the plate to mix the staining solution with the medium in the wells and incubate the cells with JC-1 Staining Solution for 30 minutes at 37°C.
4. Remove the culture medium from all wells and wash the cells with 200 µl of fresh culture medium.
5. Remove the culture medium from all wells and replace with 150 µl of fresh medium with or without treating compounds.
6. Add 20 µl of culture medium to 3 Blk (Blank/Background) wells, as indicated in figure 2, sample **Plate Set Up**.
7. Add 10 µl of Glucose Oxidase Stock Solution prepared above to the + (Glucose Oxidase Positive Control) Wells, 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**. Add 10 µl of culture medium to all the sample wells.
8. Add 10 µl of MitoXpress® - Xtra solution prepared above to each well except three Blk wells. Add 10 µl of culture medium to the Blk wells.
9. Use a repeat pipettor, slowly pick up HS Mineral Oil (Item No. 600910) from supplied bottle (avoid pippetting up and down) and gently dispense 100 µl to overlay each well. Ensure HS oil is pre-warmed to measurement temperature in advance.
10. Read the plate immediately with the set up described on page 13. The plate should be measured kinetically for >120 minutes for oxygen consumption rate.
11. Measure J-aggregates from JC-1 staining with excitation and emission at 560 nm and 590 nm, respectively with a bandwidth of 10 nm.
12. Measure green monomers from JC-1 staining with excitation and emission at 485 nm and 535 nm, respectively with a bandwidth of 10 nm.

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

Table 2. Pipetting summary

Calculations

Assessing Oxygen Consumption

Plot the MitoXpress® - Xtra Signal, Intensity, or Lifetime *versus* Time (mins) (see Figure 3 below). 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.

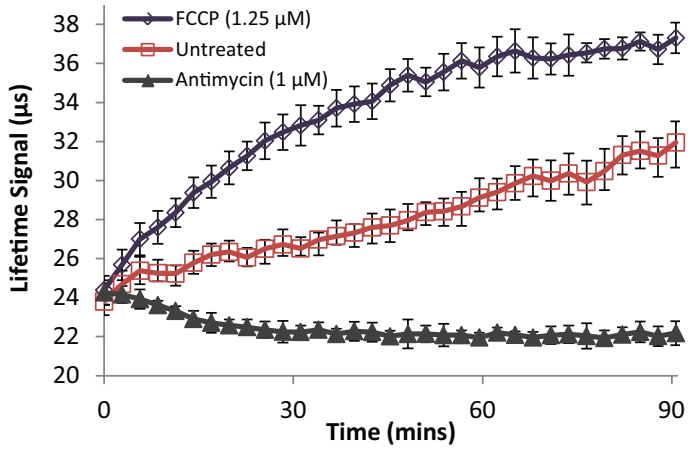


Figure 3. Typical Lifetime profiles of MitoXpress® - Xtra measuring the effect of mitochondrial inhibition (Antimycin treatment) and uncoupling (FCCP treatment) on cell respiration. Measurement made immediately post treatment.

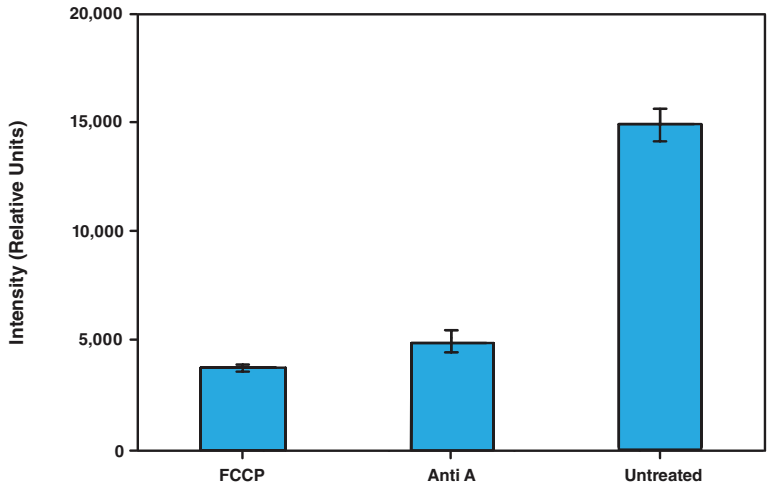


Figure 4. The effect of Antimycin and FCCP treatment on MMP as measured using JC-1. Data presented as J-aggregate intensity (595 nm). Readings are made immediately after oxygen measurements.

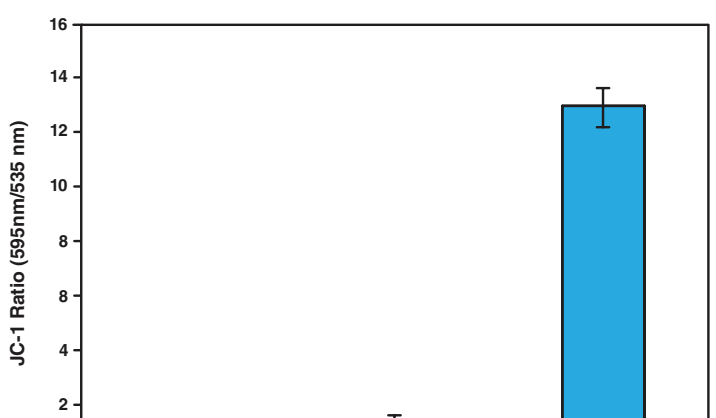


Figure 5. The effect of of Antimycin and FCCP treatment on MMP as measured using JC-1. Data presented as a ratio of J-aggregate intensity (595 nm) to monomer intensity (535 nm). Readings are made immediately after oxygen measurements.

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
Control cells without treatment show low ratio of red to green signal	Control cells are not healthy	Use only healthy cells
Staining is too strong	JC-1 Staining Solution is too concentrated for this cell type	Dilute JC-1 Staining Solution

Reference

1. Gogvadze, V., Orrenius, S., and Zhivotovsky, B. Mitochondria in cancer cells: What is so special about them? *Trends Cell Biol.* **18(4)**, 165-173 (2008).
2 Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. Understanding the Warburg effect: The metabolic requirements of cell proliferation. *Science* **324**, 1029-1033 (2009).
3 Szewczyk, A. and Wojtczak, L. Mitochondria as a pharmacological target. *Pharmacol. Rev.* **54(1)**, 101-127 (2002).
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).
5. 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).

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/Glycolysis Dual Assay Kit - Item No. 601060
Oxygen Consumption Rate Assay Kit (MitoXpress® - Xtra HS Method) - Item No. 600800
WST-1 Cell Proliferation Assay Kit - Item No. 10008883

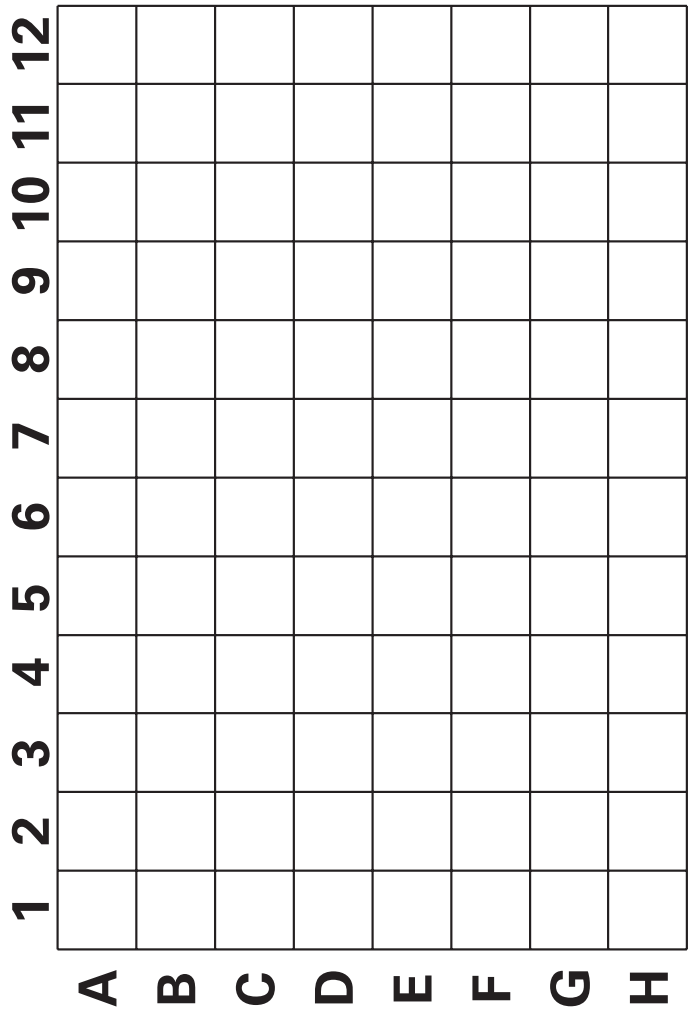
Warranty and Limitation of Remedy

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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