Mitochondrial ROS Detection Assay Kit

Item No. 701600

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. Once opened, please remove components and store as stated below

Item Number	Item	Quantity/Size	Storage
10009322	Cell-Based Assay Buffer Tablet	1 ea	RT
701601	Mitochondrial ROS Detection Reagent	100 tests	-20°C
701176	Antimycin A Assay Reagent	100 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

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 at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorescent plate reader with a monochromator, or with filter sets capable of measuring an excitation wavelength between 480-515 nm and emission wavelength between 560-600 nm. Alternatively, a flow cytometer equipped with a blue laser (488 nm) and a filter for measuring PE (575/26).
2. A black, clear-bottom tissue culture-treated 96-well plate (adherent cell protocol).
3. A V-bottom, 96-well dilution plate (suspension cell protocol).
4. A centrifuge with microplate adapter capable of >400 × g (suspension cell protocol).
5. A source of pure, glass distilled, or HPLC-grade water. *NOTE: Ultra-Pure water is available for purchase from Cayman (Item No. 400000).*
6. Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+}

INTRODUCTION

About This Assay

As one of the major sources of reactive oxygen species (ROS) in the cell, mitochondrial ROS has often been difficult to single out from the other sources of ROS within the cell. While some ROS dyes such as DHE and DCFDA tend to be less location-specific and dyes such as ADHP are only extracellular, Mitochondrial ROS Detection Reagent is targeted primarily to the mitochondrion making Mitochondrial ROS Detection Reagent an ideal reagent to study mitochondrial ROS.[1-3]

Cayman's Mitochondrial ROS Detection Assay Kit utilizes Mitochondrial ROS Detection Reagent to measure the production of mitochondrial ROS under specific conditions. When properly titrated, Mitochondrial ROS Detection Reagent accumulates in the mitochondrial matrix and fluoresces in the presence of ROS. When used in excess, Mitochondrial ROS Detection Reagent can lead to non-specific staining resulting in cytosolic or nuclear staining. Antimycin A, which causes superoxide production from complex III of the mitochondrial electron transport chain is included as a positive control in this kit.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Cell-Based Assay Buffer Tablet - (Item No. 10009322)**

   This vial contains one Cell-Based Assay Buffer tablet. Dissolve the tablet in 100 ml of pure water to make Cell-Based Assay Buffer. Unused Cell-Based Assay Buffer can be stored at room temperature for up to one year.

2. **Mitochondrial ROS Detection Reagent - (Item No. 701601)**

   This vial contains lyophilized Mitochondrial ROS Detection Reagent. Reconstitute the contents of the vial in 13 µl of DMSO to yield a 5 mM stock. Once reconstituted, Mitochondrial ROS Detection Reagent should be used within 24 hours.

3. **Antimycin A Assay Reagent - (Item No. 701176)**

   This vial contains a 10 mM solution of antimycin A in ethanol and should be kept on ice. Unused Antimycin A Assay Reagent can be stored at -20°C for up to 6 months.

Pre-Assay Preparation

1. **Mitochondrial ROS Detection Reagent Staining Solution**

   Mitochondrial ROS Detection Reagent should be diluted in HBSS. To avoid non-specific staining, it is recommended that an optimal staining concentration of Mitochondrial ROS Detection Reagent be determined empirically. Final concentrations of Mitochondrial ROS Detection Reagent should be in the range of 1.0 - 0.25 µM. For one 96-well plate, prepare 12 ml of Mitochondrial ROS Detection Reagent staining solution and warm to 37°C before use.

2. **Antimycin A Working Reagent**

   Prepare Antimycin A Working Reagent by diluting 10 µl of Antimycin A Assay Reagent into 1 ml of Cell-Based Assay Buffer. The concentration of this reagent will be 100 µM.

ASSAY PROTOCOL

Performing the Assay

For extended treatment with unknown compounds, test to ensure that compounds do not compromise the mitochondrial membrane potential or mitochondrial membrane integrity. To do this, we recommend JC-1 Mitochondrial Membrane Potential Assay Kit (Item No. 10009172) to measure membrane potential in a plate reader, JC-1 Mitochondrial Membrane Potential Flow Cytometry Assay Kit (Item No. 701560) to measure membrane potential by flow cytometry, or Mitochondrial PT Pore Assay Kit (Item No. 601430) to measure mitochondrial permeability.

Note: For compounds with known or unknown effects on mitochondrial membrane potential, treatment is recommended in parallel with Antimycin A.

For Adherent Cells

This protocol is written for 96 well plates. Adjust accordingly for different vessel types.

1. Plate cells at desired concentration in a black, tissue culture treated 96-well plate and culture cells as required in the media best suited for your cell line. Ensure cells are healthy and not overgrown.
2. Designate wells for positive and vehicle controls. We recommend a minimum of two replicates for each.
3. Carefully aspirate off the culture media and add 120 µl of pre-warmed Cell-Based Assay Buffer.
4. Carefully aspirate off the pre-warmed Cell-Based Assay Buffer leaving a small amount (~10-20 µl) of liquid in the well.
5. Add 100 µl of pre-warmed Mitochondrial ROS Detection Reagent Staining Solution to each well and incubate at 37°C, protected from light for 20 minutes.
6. Carefully aspirate off the Mitochondrial ROS Detection Reagent Staining Solution leaving a small amount (~10-20 µl) of liquid in the well.
7. Carefully add 120 µl of pre-warmed HBSS.
8. Repeat steps 6 and 7 two additional times for a total of 3 washes.
9. Carefully add 100 µl of pre-warmed HBSS.
10. Add Antimycin A Working Reagent to positive control wells. We recommend using a 10 µM maximal concentration of antimycin A. This concentration, however, should be determined empirically for individual cell types.
11. Incubate for one hour at 37°C.
12. Read on a plate reader, microscope, or imager using an excitation wavelength of 480-515 nm and an emission wavelength of 560-600 nm.

Suspension Cells (V-bottom 96-well plate)

1. Culture cells, per desired protocol, in media best suited for your cell line.
2. Add cell suspension at the desired concentration to a V-bottom 96-well plate. Ensure that cells are healthy and not overgrown.
3. Designate wells for positive and vehicle controls. We recommend a minimum of two replicates for each.
4. Centrifuge the plate at 400 x g for 1-2 minutes to pellet cells.
5. Without disrupting the cell pellet, carefully remove the spent culture media from each well.
6. Add 100 µl of pre-warmed Cell-Based Assay Buffer to each well.
7. Centrifuge the plate at 400 x g for 1-2 minutes to pellet cells.
8. Without disrupting the cell pellet, carefully remove the Cell-Based Assay Buffer from each well.
9. Add 100 µl of pre-warmed Mitochondrial ROS Detection Reagent Staining Solution to each well and incubate at 37°C, protected from light for 20 minutes.
10. Centrifuge the plate at 400 x g for 1-2 minutes to pellet cells.

11. Carefully remove the Mitochondrial ROS Detection Reagent Staining Solution and add 100 µl of pre-warmed HBSS.
12. Repeat steps 10 and 11 two additional times for a total of 3 washes.
13. Carefully add 100 µl of pre-warmed HBSS to each well for all wells being used.
14. Add Antimycin A Working Reagent to positive control wells. We recommend using a 10 µM maximal concentration of antimycin A. This concentration, however, should be determined empirically for individual cell types.
15. Incubate for one hour at 37°C.
16. Read on a plate reader using an excitation wavelength between 480-515 nm and an emission wavelength between 560-600 nm or a flow cytometer equipped with a 488 laser and PE filter.

ANALYSIS

Performance Characteristics

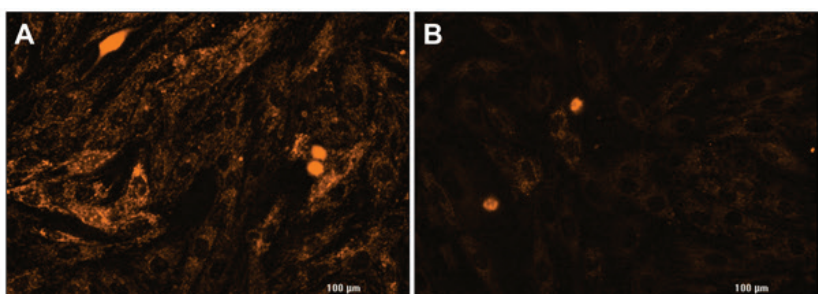

Figure 1. H9c2 cells were stained with 0.62 µM Mitochondrial ROS Detection Reagent following the Adherent Cells Protocol treated with 3 µM antimycin A (A) or vehicle (B). Images were captured one hour after treatment with antimycin A using Biotek's Cytation™ 5 Multi-Mode Reader.

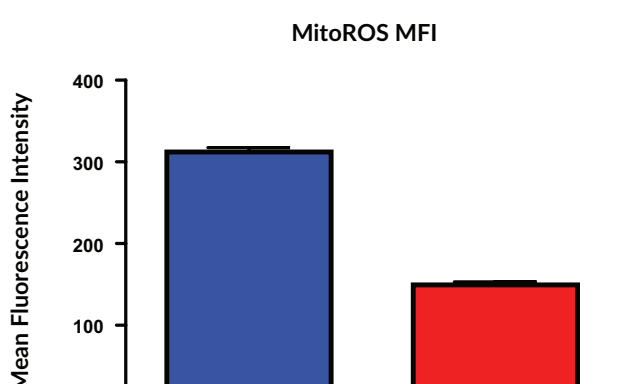

Figure 2. THP-1 cells were stained with 0.5 µM Mitochondrial ROS Detection Reagent following the Suspension Cells Protocol. Cells were treated with 1 µM antimycin A or vehicle and incubated for 1 hour before measuring on a flow cytometer.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No signal detected	Dye concentration or cell density is too low	Perform a cell seeding and dye titration to ensure that cell density and dye concentration is optimal
Non-mitochondrial signal is detected	Dye concentration is too high	Perform dye titration to ensure dye concentration is optimal

References

1. Michalski, R., Michalowski,B., Sikora, A., *et al.* On the use of fluorescence lifetime imaging and dihydroethidium to detect superoxide in intact animals and *ex vivo* tissues: A reassessment. *Free Radic. Biol. Med.* **67**, 278-284 (2014).
2. Polster, B.M., Nicholls, D.G., Ge, S.X., *et al.* Use of potentiometric fluorophores in the measurement of mitochondrial reactive oxygen species. *Methods Enzymol.* **547**, 225-250 (2014).
3. Zielonka, J., Zielonka, M., Sikora, A., *et al.* Global profiling of reactive oxygen and nitrogen species in biological systems: High-throughput real-time analyses. *J. Biol. Chem.* **287(5)**, 2984-2995 (2012).

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.