ROS Detection
Cell-Based Assay Kit (DHE)

Item No. 601290

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

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

Item Number	Item	Quantity/Size	Storage
10009322	Cell-Based Assay Buffer Tablet	1 ea	RT
601291	Dihydroethidium Assay Reagent	2 vials/20 µl	-20°C
701176	Antimycin A Assay Reagent	1 vial/100 µl	-20°C
601292	N-acetyl Cysteine Assay Reagent	2 vials/10 mg	-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.

NOTE: It is recommended that gloves be worn at all times when working with mitochondrial inhibitors.

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

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, 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 480-520 nm and emission wavelength between 570-600 nm. Alternatively, a flow cytometer equipped with a blue laser (488 nm) and a filter for measuring PE (575/26 nm).
2. A black, clear-bottom tissue culture-treated 96-well plate
3. A V-bottom, 96-well dilution plate (suspension cell protocol)
4. A centrifuge with microplate adapter capable of >400 × g
5. A source of UltraPure water (≥18 MΩ)
6. Microcentrifuge tubes

INTRODUCTION

About This Assay

Dihydroethidium (hydroethidine or DHE) is a widely used ethidium-based, redox-sensitive fluorescent probe. DHE has been shown to be oxidized by superoxide to form 2-hydroxyethidium (2-OH-E^+) (ex 500-530 nm/em 590-620 nm) or by non-specific oxidation by other sources of reactive oxygen species (ROS) to form ethidium (E^+) (ex 480 nm/em 576 nm).[1,2] Given their narrow spectral range, distinguishing between the two species using filter-based optical systems is often difficult and can lead to misreporting of the species of ROS being generated. This assay kit uses DHE as a fluorescent probe for the detection of ROS generation. Antimycin A, an inhibitor of complex III of the mitochondrial electron transport chain, is included as a positive control for ROS generation. N-acetyl Cysteine is included as an antioxidant control.

PRE-ASSAY PREPARATION

Reagent Preparation

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

   Dissolve tablet in 100 ml of UltraPure water to make Cell-Based Assay Buffer. Unused Cell-Based Assay Buffer can be stored at 4°C for up to one year.

2. **Dihydroethidium Assay Reagent - (Item No. 601291)**

   This reagent is supplied as a 5 mM solution of DHE in DMSO. Thaw and use as detailed below to make ROS Staining Buffer. Once opened, Dihydroethidium Assay Reagent can be stored at -20°C for up to one month. Avoid freeze thaw cycles.

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

   This reagent is supplied as a 10 mM solution of antimycin A in ethanol and should be kept on ice. Once opened, unused Antimycin A Assay Reagent can be stored at -20°C for up to 6 months.

4. **N-acetyl Cysteine Assay Reagent - (Item No. 601292)**

   This reagent is supplied as a 10 mg powder of N-acetyl cysteine. Store unused vials at -20°C until kit expiration.

Pre-Assay Preparation

1. **ROS Staining Buffer**

   In a separate tube, dilute Dihydroethidium 1:1,000 in Cell-Based Assay Buffer to obtain a working concentration of 5 µM (15 µl Dihydroethidium into 15 ml of Cell-Based Assay Buffer). Any unused ROS Staining Buffer should be discarded immediately after use.

2. **Antimycin A Working Reagent**

   Make Antimycin A Working Reagent by diluting 15 µl of Antimycin A Assay Reagent into 1 ml of Cell-Based Assay Buffer in a separate tube. This will yield a 150 µM Working Reagent of Antimycin A. Unused Antimycin A Working Reagent should be discarded immediately after use.

3. **N-Acetyl Cysteine Working Reagent**

   Dissolve 10 mg vial of N-acetyl cysteine in 200 µl of Cell-Based Assay Buffer to make a 300 mM N-Acetyl Cysteine Working Reagent. Any unused N-Acetyl Cysteine Working Reagent should be discarded immediately after use.

ASSAY PROTOCOL

For Adherent Cells

1. Plate cells at desired concentration in a black, tissue culture-treated 96-well plate and culture cells, per desired protocol, in the media best suited for your cell line. Ensure that cells are healthy and not overgrown. *NOTE: When working with unfamiliar cell lines, we recommend performing a seeding titration as cell types can vary in size and volume.*
2. Designate wells as positive (Antimycin A) and negative controls (N-acetyl Cysteine) (we recommend a minimum of two replicates for each condition).
3. Carefully aspirate off the culture media and carefully add ~150 µl of Cell-Based Assay Buffer.
4. Carefully aspirate Cell-Based Assay Buffer, leaving a small amount (~10-20 µl) of liquid in the well.
5. Add 130 µl of ROS Staining Buffer to each well.
6. Add 10 µl N-Acetyl Cysteine Assay Reagent to designated negative control wells.
7. Cover plate and incubate for 30 minuntes at 37°C - *protected from light*.
8. Following the 30 minute incubation, add 10 µl of the Antimycin A Working Reagent to designated positive control wells and incubate for an additional hour at 37°C - *protected from light*.
9. Carefully aspirate ROS Staining Buffer and add 100 µl of Cell-Based Assay Buffer.
10. Place Assay Plate on fluorescent plate reader and measure the fluorescence using an excitation wavelength between 480-520 nm and an emission wavelength between 570-600 nm.

For 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. *NOTE: When working with unfamiliar cell lines, we recommend performing a seeding titration as cell types can vary in size and volume.*
3. Designate wells as positive (Antimycin A) and negative controls (N-acetyl Cysteine). We recommend a minimum of two replicates for each condition.
4. Centrifuge the plate at 400 x g to pellet cells.
5. Without disrupting the cell pellet, carefully remove the culture media and wash with ~150 µl of Cell-Based Assay Buffer.
6. Centrifuge the plate at 400 × g to pellet cells.
7. Without disrupting the cell pellet, carefully remove off the Cell-Based Assay Buffer, leaving a small amount (~10-20 µl), and add 130 µl of ROS Staining Buffer to each well.
8. Add 10 µl N-Acetyl Cysteine Assay Reagent to designated negative control wells.
9. Cover plate and incubate for 30 minutes at 37°C - *protected from light*.
10. Following the 30 minute incubation, add 10 µl of the Antimycin A Working Reagent to designated positive control wells and incubate for an additional hour at 37°C - *protected from light*.
11. Centrifuge the plate at 400 × g to pellet cells.
12. Carefully remove ROS Staining Buffer and add 100 µl of Cell-Based Assay Buffer.
13. Readout:
    a. **Plate reader -** Transfer cells to a black, tissue culture-treated 96-well plate. Place Assay Plate on fluorescent plate reader and measure the fluorescence using an excitation wavelength between 480-520 nm and an emission wavelength between 570-600 nm.
    b. **Flow cytometer readouts -** Transfer cells to tubes appropriate for your flow cytometer. DHE is typically excited with a 488 nm laser and emits in the PE channel. Collect at least 20,000 events.

**NOTE: If a V-bottom 96-well plate or centrifuge microplate adapter is unavailable, volumes found in this protocol are applicable to 1.5 ml microfuge tubes.*

Performance Characteristics

ROS generation is represented as total DHE fluorescence. It is important that positive and negative controls be included in every experiment for all cell types tested. Data can be expressed as total fluorescence, geometric mean fluorescence (flow cytometry), or as % of controls.

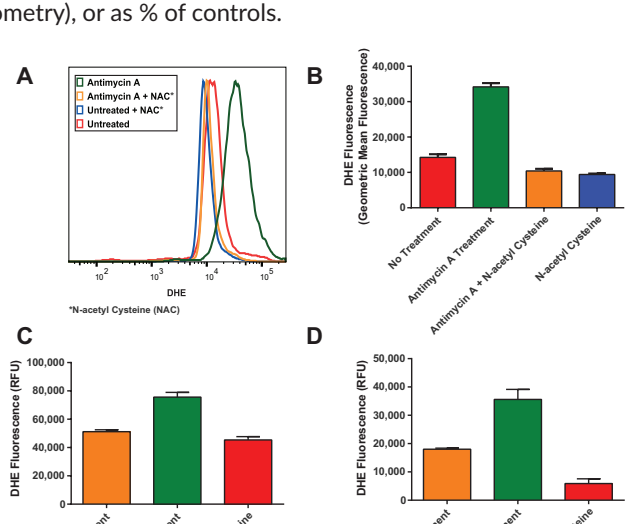

Figure 1. *Panels A-C* show example data of THP-1 cells treated with controls and stained using the protocol for suspension cells. *Panel D* shows example data for HepG2 cells treated with control compounds and stained using the protocol for adherent cells. It is important to conduct optimization experiments for every cell type and experimental condition as cell types often vary.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No fluorescence or minimal fluorescence is detected	A. Cells are not at sufficient density B. Gain is not optimized	A. Conduct seeding titrations to determine optimal cell density before performing experiment B. Adjust gain to optimize signal

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 Redic. Biol. Med.* **67**, 278-284 (2014).
2. Zhao, H., Kalivendi, S., Zhang, H., *et al.* Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: Potential implications in intracellular fluorescence detection of superoxide. *Free Radic. Biol. Med.* **34(11)**, 1359-1368 (2003).

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.