Signosis, Inc.
Innovative Plate Assay Solutions

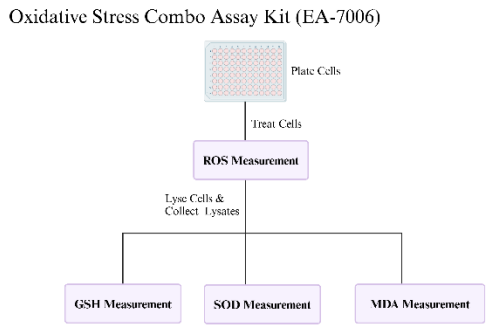
Oxidative Stress Combo Assay Cell Kit

Catalog # EA-7006 **(For Research Use Only)**

Introduction

The Signosis **Oxidative Stress Combo Assay Cell Kit** provides a comprehensive solution for measuring key oxidative stress and antioxidant markers in cells in a single, streamlined workflow. This kit enables the detection of **reactive oxygen species (ROS), lipid peroxidation (MDA), glutathione (GSH),** and **superoxide dismutase (SOD) activity** by integrating multiple assays into a single kit.

First, the **DCFDA assay** is used to quantify **ROS levels** in live cells in culture media. After the measuring the ROS levels, the cells are lysed, and the lysates are used to measure **MDA levels**, **GSH levels** and **SOD activity**.



Principle

DCFDA Assay

The DCFDA (H2DCFDA) Assay kit utilizes 2',7' dichlorofluorescin diacetate (DCFDA), a cell permeable reagent, to detect reactive oxygen species (ROS) in live cells. DCFDA is capable of measuring hydroxyl, peroxyl, and other ROS activity within the cell. When DCFDA enters the cell, it is deacetylated by cellular esterases into a non-fluorescent compound. Then, it is oxidized by ROS into 2', 7' dichlorofluorescein (DCF), which is highly fluorescent and can be detected by fluorescence spectroscopy at excitation and emission wavelengths of 485 nm/535 nm.

MDA Assay

The Lipid Peroxidation MDA Assay utilizes thiobarbituric acid (TBA) to detect malondialdehyde (MDA) in biological samples. MDA is a marker for oxidative stress and forms from the lipid peroxidation of polyunsaturated fatty acids. When TBA reacts with MDA, a fluorescent red product is formed that can be measured spectrophotometrically at an absorbance of 532 nm.

GSH Assay

The Glutathione (GSH) Assay utilizes Ellman's reagent (DTNB) to measure GSH in biological samples. DTNB reacts with GSH to form a yellow product that can be measured spectrophotometrically at an absorbance of 412 nm.

SOD Assay

The Superoxide Dismutase (SOD) Activity Assay utilizes WST-8 to assess SOD activity in biological samples. SOD is an enzyme that catalyzes the dismutation of the superoxide (O2-) anion radical into molecular oxygen (O2) and hydrogen peroxide (H2O2). This assay indirectly measures the activity of SOD by using WST-8 to detect superoxide O2- levels in samples, which is suppressed when SOD activity is high. WST-8 interacts with superoxide O2- to form a colored product which can be measured spectrophotometrically at an absorbance of 450 nm.

Materials Required but Not Provided

- PBS
- Cell culture incubator at 37°C
- Microplate reader for fluorescence and absorbance detection
- 96-well black microplate with clear bottom for measuring cell fluorescence
- 96-well clear microplate

Materials Provided

- KRPG buffer (4°C)
- 10mM DCFDA Reagent (-20°C)
- Lysis Buffer (4°C)
- 1 mM MDA Stock Solution (-20°C)
- TBA Solution (RT)
- 10 mM GSH Stock Solution (-20°C)
- DTNB Detection Reagent (-20°C)
- WST Reagent (-80°C)
- Substrate Reagent (-20°C)
- 5mM DTPA (-20°C)
- Oxidase Reagent (4°C)

Data Analysis

Conditions	Interpretation
High ROS, High MDA, Low GSH	Oxidative stress damage
High ROS, High SOD, High GSH	Active antioxidant response
Low SOD, Low GSH, High MDA	Severe oxidative damage, impaired defenses

****Spin down small tubes before starting experiment. ****

ROS Measurement

Adherent Cells

1. Seed cells the day before the experiment in a clear bottom 96-well black microplate at 20,000 – 50,000 cells per well, depending on cell type.
2. Incubate the cells overnight in a cell culture incubator at 37°C
3. Prepare a 10 µM DCFDA solution by diluting the 10 mM DCFDA reagent 1:1000 in KRPG buffer. Prepare enough 10 µM DCFDA solution for 100 µL per well used.
4. Optional: for toxicity assays, dilute compound(s) of interest in KRPG buffer to the desired concentration for the experiment (100 µL per well).
5. Carefully remove the media from the wells and wash the cells once with 100 µL of KRPG buffer.
6. Add 100 µL of the 10 µM DCFDA solution to each well.
7. Incubate the cells in a cell culture incubator at 37°C for 45 minutes to stain the cells.
8. Remove the DCFDA solution from each well and add 100 µL of KRPG buffer to each well.
9. Optional: Remove the KRPG buffer from each well and add 100 µL of the diluted compound(s) to each well. Incubate the cells in a cell culture incubator at 37°C for the desired time (1-4 hours).
10. Measure the fluorescence of the plate without removing compounds or buffer in a fluorescence plate reader at Ex/Em = 485/535 nm.

Suspension Cells

1. Grow suspension cells so that approximately 150,000 cells per well are available.
2. Collect cells in a conical tube and wash by centrifugation once in KRPG buffer.
3. Prepare a 10 µM DCFDA solution by diluting the 10 mM DCFDA reagent 1:1000 in KRPG buffer. Prepare enough 10 µM DCFDA solution for 100 µL per well used.
4. Stain the cells by resuspending in 10 µM DCFDA solution at a concentration of 1,000,000 cells/mL and incubate in a cell culture incubator at 37°C for 30 minutes.
5. Wash cells by centrifugation with KRPG buffer.
6. Resuspend cells in KRPG buffer at concentration of 1,000,000 cells/mL.
7. Seed a clear bottom 96-well black microplate with 100,000 stained cells/well.
8. Optional: Remove the KRPG buffer from each well and add 100 µL of the diluted compound(s) to each well. Incubate the cells in a cell culture incubator at 37°C for the desired time (1-4 hours).
9. Measure the fluorescence of the plate without removing compounds or buffer in a fluorescence plate reader at Ex/Em = 485/535 nm.

****Save the cells from the ROS Measurement for cell lysis and further analysis. ****

Cell Lysis

1. Remove the buffer from each well of the used cell culture plate and add 40 µL of Lysis Buffer to each well.
2. Place the cell culture plate on an orbital shaker and gently shake for 20 minutes at room temperature.
3. Pipette the cell lysates up and down to fully detach the cells from the wells and transfer to a 1.5 mL tube.
4. The cell lysates may be assayed directly or stored at -80°C.

MDA Measurement

1. Using the provided 1 mM MDA stock solution, prepare a standard curve dilution in PCR tubes as described in the table below:

MDA Dilution Table

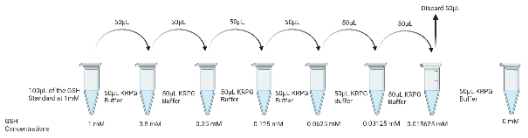
Standard #	1	2	3	4	5	6
MDA Stock Solution Volume (µL)	5	4	3	2	1	0
ddH_2O (µL)	45	46	47	48	49	50
MDA Final Concentration (µM)	100	80	60	40	20	0
Standard Final Volume (µL)	50	50	50	50	50	50

Note: **Ensure to include a blank well as a negative control. **

2. Prepare samples in PCR tubes by diluting 10 µL of the cell lysates in 40 µL of ddH_2O.
3. Add 50 µL of the TBA solution to each sample or standard in the PCR tubes.
4. Heat the PCR tubes in a thermal cycler at 95°C for 1 hour.
5. After heating, cool the PCR tubes on ice or in a 4°C fridge for 10 minutes.
6. Transfer the heated samples from the PCR tubes to 1.5mL centrifuge tubes and centrifuge at >10,000RPM for 1 minute to pellet any precipitate. (The standards do not contain any precipitate and do not need to be centrifuged.)
7. Transfer 60 µL of the standards and the liquid supernatant from the samples without disturbing the pellet to a 96-well clear plate.
8. Measure the absorbance of the plate at 532 nm using a plate reader.

GSH Measurement

1. Prepare a GSH standard curve in a 96-well clear plate using an 8-well serial dilution. In the first well, dilute 10 µL of the 10 mM GSH stock solution in 90 µL of PBS to make a 1 mM GSH standard. Next, add 50 µL of PBS to the next 7 wells. Then, transfer 50 µL of the first well to the next well to make a two-fold dilution. Perform six additional two-fold serial dilutions and leave the last, 8th well untouched as the blank buffer well.



2. Prepare samples in each well of the plate by diluting 10 µL of the cell lysates in 40 µL of PBS.
3. Prepare GSH detection solution by diluting DTNB detection reagent 1:20 in PBS.
4. Add 50 µL of the GSH detection solution to each sample or standard in the 96-well plate.
5. Incubate the plate at 37°C for 10 minutes.
6. Measure the absorbance of the plate at 412 nm using a plate reader. Alternatively, measure the fluorescence of the plate in a fluorescence plate reader at Ex/Em = 340/460 nm.

SOD Measurement

1. Begin preparation of 10 mL of WST working solution by diluting 200 µL WST reagent, 100 µL Substrate reagent, and 100 µL 5mM DTPA in 9.6 mL of PBS.
2. Right before loading the samples, complete the WST working solution by adding 10 µL of the Oxidase reagent to the WST working solution and mixing. **Make sure the Oxidase reagent is evenly resuspended by pipetting up and down before using.**
3. In a clear 96-well plate, add 100 µL of the WST working solution to each well. Add 10 µL of cell sample to each well with WST working solution and mix thoroughly. **Be sure to load the samples quickly, since the WST reaction is active when the Oxidase reagent is added. Use a multichannel pipette if possible.**
4. For the control well, add 10 µL of PBS to one of the wells with WST working solution.
5. Incubate the plate at 37°C for 45 minutes. The plate can be incubated for an additional hour or two if a stronger signal is desired.
6. Measure the absorbance of the plate at 450 nm using a plate reader.