Signosis, Inc.
Innovative Plate Assay Solutions

Ferroptosis and Lipid Peroxidation Combo Kit

Catalog # EA-7102 **(For Research Use Only)**

Introduction

Signosis' Ferroptosis and Lipid Peroxidation Combo Kit provides a comprehensive solution for measuring key ferroptotic markers in cells, tissues, or biological fluids in a single, streamlined workflow. This kit can perform 100 assays each for intracellular ferrous iron (Fe^{2+}), lipid peroxidation (MDA), reduced glutathione (GSH), and glutathione peroxidase (GPx) activity by integrating multiple assays into a single kit.

Principle

Ferrozine Assay

The Ferrozine Iron Assay Kit utilizes ferrozine to detect ferrous iron (Fe^{2+}) in biological samples. In this assay, ferrous iron reacts with ferrozine to form a purple-colored complex that can be quantified spectrophotometrically at an absorbance of 562 nm. Ferric iron (Fe^{3+}) can also be measured following reduction to Fe^{2+}, allowing determination of total iron content.

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.

GPx Assay

The Glutathione Peroxidase (GPx) Activity Assay determines the activity of GPx by measuring its ability to convert reduced glutathione (GSH) to oxidized glutathione (GSSG) in the presence of H2O2. The GSSG that is produced by GPx is quantified using the enzyme glutathione reductase (GR) which interacts with GSSG. GR reduces GSSG to GSH with NADPH as a cofactor, which converts the NADPH to its oxidized form, NADP+. Because NADPH can be measured spectrophotometrically at an absorbance of 340 nm, the depletion of NADPH levels in samples can be used to determine GPx activity. Since GPx activity causes NADPH to be consumed, elevated GPx activity is observed as a decrease in absorbance at 340 nm.

Materials Required but Not Provided

- PBS
- 96-well clear microplate for absorbance reading
- Microplate reader capable of measuring absorbance at 562 nm, 532 nm, 412 nm, and 340 nm.

Materials Provided

- Iron Assay Buffer (4°C)
- Iron Standard (-20°C)
- Reduction Reagent (-20°C)
- Detection Reagent (-20°C)
- 1 mM MDA Stock Solution (-20°C)
- TBA Solution (RT)
- 10 mM GSH Stock Solution (-20°C)
- DTNB Detection Reagent (-20°C)
- 1mM Peroxide Reagent (4°C)
- Serum Reagent (4°C)
- 10mM FAD (-20°C)
- 10mM GSH (-20°C)
- 10mM NADPH (-20°C)
- 1x GR Enzyme Stock (-80°C)

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

Plasma Sample Preparation

1. Centrifuge citrated or EDTA-collected blood at 4°C (1,000 x *g* for 10 minutes) to separate plasma from erythrocytes. Alternatively, blood collected without anticoagulant can be centrifuged to collect serum.
2. Transfer the plasma layer to a new tube without disturbing the buffy layer.
3. The plasma may be assayed directly or stored away at -80°C.

Cell Sample Preparation

1. Wash the cells once with PBS before lysing the cells.
2. For a 96-well culture plate, add 40 µL of lysis buffer to each well and incubate at room temperature for 10 minutes.
3. Pipette the lysis buffer up and down to detach the cells and transfer the cell lysates into a new tube.
4. If necessary, homogenize the cell lysates with a sonicator.
5. The cell lysates may be assayed directly or stored at -80°C.
6. Use PBS to dilute the cell sample to the appropriate concentration for each assay, if necessary.

Tissue Sample Preparation

1. Weigh tissue sample and add 1 mL of lysis buffer per 100mg of tissue.
2. Homogenize the tissue samples with a tissue grinder.
3. If necessary, further homogenize the tissue samples with a sonicator.
4. Centrifuge the sample at 10,000 RPM for 5 minutes to pellet the tissue debris.
5. Collect the supernatant and measure the protein concentration of the supernatant. The tissue sample can be assayed directly or stored at -80°C.
6. Use PBS to dilute the tissue sample to the appropriate concentration for each assay, if necessary.

Iron Measurement

1. Using the provided 1 mM iron standard, prepare a standard curve dilution in a 96-well clear plate as described in the table below:

Standard #	1	2	3	4	5	6
Iron Standard Volume (µL)	5	4	3	2	1	0
Assay Buffer (µL)	45	46	47	48	49	50
Iron Final Concentration (µM)	100	80	60	40	20	0
Standard Final Volume (µL)	50	50	50	50	50	50

Iron Dilution Table

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

2. Prepare samples in each well of the plate by diluting 10 µL of the sample in 40 µL of Assay Buffer.
3. For total iron measurement (Fe^{2+} and Fe^{3+}), add 5 µL of the reduction reagent to each sample or standard in the 96-well plate.
4. Mix and incubate the standards and samples at 37°C for 30 minutes.
5. Add 50 µL of detection reagent to each well containing standards or samples and incubate at 37°C for 60 minutes away from light.
6. Measure the absorbance of the plate at 562 nm using a plate reader.

MDA Measurement

1. Using the provided 1 mM MDA stock solution, prepare a standard curve dilution in a 96-well clear plate as described in the table below:

MDA Dilution Table

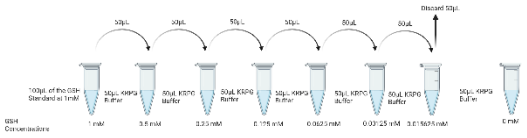
Standard #	1	2	3	4	5	6
MDA Stock Solution Volume (µL)	5	4	3	2	1	0
ddH_2O (µL)	45	46	47	48	49	50
MDA Final Concentration (µM)	100	80	60	40	20	0
Standard Final Volume (µL)	50	50	50	50	50	50

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

2. Prepare samples in each well of the plate 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 96-well plate. (TBA reactions can be heated in PCR tubes in a thermocycler if desired.)
4. Incubate the plate at 95°C for 1 hour.
5. After incubation, cool the plate on ice or a 4°C fridge for 10 minutes.
6. 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.

GPx Activity Measurement

1. Sample preparation: A baseline GSSG measurement should be performed additionally for each sample. To obtain a baseline sample, take an aliquot from each sample and heat at 98°C to deactivate all enzymes in the sample. The heated sample contains only the endogenous GSSG from the sample, which will be used as the reference for the baseline GSSG level. The unheated sample will include additional GSSG produced by GPx activity and will have higher overall GSSG levels compared to the baseline.
2. Reaction mix preparation: calculate the amount of each reagent needed to make the reaction mix according to the table below.

Component	Reaction Mix (per well/sample)
1mM Peroxide Reagent	0.9 µL
Serum Reagent	0.9 µL
10mM FAD	0.09 µL
10mM GSH	0.9 µL
10mM NADPH	0.9 µL
1x GR Enzyme	0.09 µL
PBS	86.22 µL
Total	90 µL

3. Any unused enzyme stock can be stored at -80°C for future use.
4. Add 90 µL of reaction mix to each well of the plate.
5. Add 10 µL of sample to each well with reaction mix and mix thoroughly.
6. Cover the plate and incubate at room temperature for 10-20 minutes.
7. Measure the absorbance of the plate at 340 nm using a plate reader. Multiple readings of the plate can be done at 5 minute intervals to observe potential kinetic changes in the measurements.