Product Manual

OxiSelect™ Hydrogen Peroxide/Peroxidase Assay Kit (Fluorometric)

Catalog Number

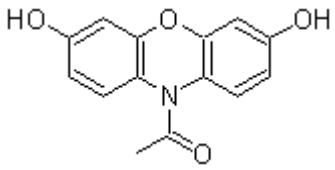
STA-344 **500 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Oxidative stress is a physiological condition where there is an imbalance between concentrations of reactive oxygen species (ROS) and antioxidants. Research has shown that excessive ROS accumulation will lead to cellular injury, such as damage to DNA, proteins, and lipid membranes. Peroxides, such as hydrogen peroxide (H_2O_2), are some of the most well documented ROS produced under oxidative stress conditions. Hydrogen peroxide is an ROS that is a toxic product of normal aerobic metabolism and pathogenic ROS production involving oxidase and superoxide dismutase reactions. Hydrogen peroxide is poisonous to eukaryotic cells and in high doses can initiate oxidation of DNA, lipids, and proteins, which can lead to mutagenesis and cell death. The cellular damage caused by peroxides have been implicated in the development of many pathological conditions, such as ageing, asthma, arthritis, diabetes, cardiovascular disease, atherosclerosis, Down's Syndrome, and neurodegenerative diseases.

Cell Biolabs' OxiSelect™ Hydrogen Peroxide/Peroxidase Assay Kit is a simple HTS-compatible assay for measuring hydrogen peroxide concentrations or peroxidase activities in biological samples without any need for pretreatment. In the presence of H_2O_2 and horseradish peroxidase (HRP), non-fluorescent ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine) is oxidized to the highly fluorescent Resorufin. The probe has less background and greater stability. The ADHP-based H_2O_2 detection is at least one order of magnitude more sensitive than the commonly used Xylenol Orange (FOX) colorimetric assay for H_2O_2. The probe can be also used as an ultrasensitive assay for peroxidase activity when H_2O_2 is in excess. The kit has a detection sensitivity limit of 50 nM (H_2O_2) or 0.1 mU/mL (Peroxidase). Each kit provides sufficient reagents to perform up to 500 assays, including standard curve and unknown samples.



ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine)

Assay Principle

The OxiSelect™ Hydrogen Peroxide/Peroxidase Assay Kit is a sensitive quantitative fluorometric assay for hydrogen peroxide or peroxidase. In the presence of HRP, ADHP reacts with H_2O_2 in a 1:1 stoichiometry to produce highly fluorescent Resorufin. The Resorufin product can be easily read by a fluorescence microplate reader with an excitation of 530-560 nm and an emission of 590 nm. Fluorescence values are proportional to the H_2O_2 or peroxidase levels within the samples. The H_2O_2 or peroxidase content in unknown samples is determined by comparison with its respective standard curve.

Related Products

1. STA-320: OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)
2. STA-330: OxiSelect™ TBARS Assay Kit (MDA Quantitation)
3. STA-341: OxiSelect™ Catalase Activity Assay Kit
4. STA-342: OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence)
5. STA-345: OxiSelect™ ORAC Activity Assay Kit

Kit Components (shipped at room temperature)

1. ADHP Probe (Part No. 234401): One 250 µL amber tube of a 10 mM solution in DMSO.
2. HRP (Part No. 234402): One 100 µL tube of a 100 U/mL solution in glycerol*.
3. Hydrogen Peroxide (Part No. 234102): One 100 µL amber tube of an 8.8 M solution.
4. 10X Assay Buffer (Part No. 234403): One 25 mL bottle.

**Note: One unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20ºC.*

Materials Not Supplied

1. Distilled or deionized water
2. 1X PBS for sample dilutions
3. Standard 96-well fluorescence black microtiter plate and/or black cell culture microplate

Storage

Upon receipt, aliquot and store the ADHP probe and HRP at -20ºC. Avoid multiple freeze/thaw cycles. Store the remaining kit components at 4ºC. ADHP is light sensitive, must be stored accordingly.

Preparation of Reagents

Note: All reagents must be brought to room temperature prior to use.

- 1X Assay Buffer: Dilute the stock 10X Assay Buffer 1:10 with deionized water for a 1X solution. Stir or vortex to homogeneity.
- ADHP/HRP Working Solution (Hydrogen Peroxide Assay): If measuring hydrogen peroxide, prepare an ADHP/HRP Working Solution by adding ADHP to a final concentration of 100 µM and HRP to a final concentration of 0.2 U/mL in 1X Assay Buffer (eg. Add 50 µL ADHP stock solution and 10 µL HRP stock solution to 4.940 mL 1X Assay Buffer). This volume is enough for ~100 assays. The ADHP/HRP Working Solution is stable for 1 day. Prepare only enough for immediate use.
- ADHP/H_2O_2 Working Solution (Peroxidase Assay): If measuring peroxidases, prepare the ADHP/H_2O_2 Working Solution by adding ADHP to a final concentration of 100 µM and H_2O_2 to a final concentration of 2 mM in 1X Assay Buffer. First perform a 1:1000 dilution of the stock H_2O_2 in 1X Assay Buffer. Use only enough for immediate applications (eg. Add 5 µL of H_2O_2 to 4.995 mL 1X Assay Buffer). This solution has a concentration of 8.8 mM. Use this 8.8 mM H_2O_2 solution to prepare a 2 mM H_2O_2 solution in ADHP/1X Assay Buffer (eg. Add 50 µL ADHP stock

solution and 1.14 mL of the prepared 8.8 mM H_2O_2 solution to 3.81 mL 1X Assay Buffer). This volume is enough for ~100 assays. The Working Solution is stable for 1 day. Prepare only enough for immediate use.

Preparation of Samples

- Cell culture supernatant: To remove insoluble particles, centrifuge at 10,000 rpm for 5 min. The supernatant can be assayed directly or diluted as necessary. Prepare the H_2O_2 standard curve in the same non-conditioned media. Serum should be avoided, as it interferes with the assay.

Note: Maintain pH between 7 and 8 for optimal working conditions as the ADHP is unstable at high pH (>8.5).

- Cell lysate: Resuspend cells at 1-2 x 10^6 cells/mL in PBS or 1X Assay Buffer. Homogenize or sonicate the cells on ice. Centrifuge to remove debris. Cell lysates can be assayed undiluted or titrated as necessary.
- Plasma, Serum or Urine: To remove insoluble particles, centrifuge at 10,000 rpm for 5 min. The supernatant can be assayed directly or diluted as necessary. Undiluted serum or plasma may interfere with the assay.

Notes:

- *All samples should be assayed immediately or stored at -80°C for up to 1-2 months. Run proper controls as necessary. Optimal experimental conditions for samples must be determined by the investigator. Always run a standard curve with samples.*
- *A serial dilution will be necessary depending on the total H_2O_2 or peroxidase present. Extremely high levels of H_2O_2 (≥ 500 µM final concentration) or peroxidase (≥ 100 mU/mL) can lower the fluorescence because excess H_2O_2 or peroxidase can further oxidize the reaction product, Resorufin, to nonfluorescent product Resazurin.*
- *Samples with NADH concentrations above 10 µM and glutathione concentrations above 50 µM will oxidize the ADHP probe and could result in erroneous readings. To minimize this interference, it is recommended that superoxide dismutase (SOD) be added to the reaction at a final concentration of 40 U/mL (Votyakova and Reynolds, Ref. 2).*
- *Avoid samples containing DTT or β-mercaptoethanol since Resorufin is not stable in the presence of thiols (above 10 µM).*

Preparation of Standard Curves

1. **H_2O_2 Standard:** To prepare the H_2O_2 standards, first perform a 1:1000 dilution of the stock H_2O_2 in 1X Assay Buffer. Prepare only enough for immediate use (e.g., Add 5 µL of H_2O_2 to 4.995 mL 1X Assay Buffer). This solution has a concentration of 8.8 mM.
2. Use this 8.8 mM H_2O_2 solution to prepare standards in the concentration range of 0µM – 100µM by further diluting in 1X Assay Buffer; see Table 1 below. H_2O_2 diluted solutions and standards should be prepared fresh.

Standard Tubes	8.8 mM H_2O_2 Standard (µL)	1X Assay Buffer (µL)	H_2O_2 (µM)
1	11.5	988.5	100
2	500 of Tube #1	500	50
3	500 of Tube #2	500	25
4	500 of Tube #3	500	12.5
5	500 of Tube #4	500	6.25
6	500 of Tube #5	500	3.125
7	500 of Tube #6	500	1.56
8	500 of Tube #7	500	0.78
9	500 of Tube #8	500	0.39
10	500 of Tube #9	500	0.195
11	500 of Tube #10	500	0.098
12	0	500	0

Table 1. Preparation of H_2O_2 Standards

- **Peroxidase Standard:** To prepare the peroxidase standards, first perform a 1:1000 dilution of the stock HRP in 1X Assay Buffer (e.g., Add 5 µL of HRP stock to 4.995 mL 1X Assay Buffer). Prepare only enough for immediate use. This solution has a concentration of 100 mU/mL. Use this 100 mU/mL solution to prepare standards in the concentration range of 0 mU/mL – 10 mU/mL by further diluting in 1X Assay Buffer (see Table 2 below). HRP diluted solutions and standards should be prepared fresh.

Standard Tubes	100 mU/mL HRP Standard (µL)	1X Assay Buffer (µL)	HRP (mU/mL)
1	100	900	10
2	500 of Tube #1	500	5
3	500 of Tube #2	500	2.5
4	500 of Tube #3	500	1.25
5	500 of Tube #4	500	0.625
6	500 of Tube #5	500	0.3125
7	500 of Tube #6	500	0.1563
8	0	500	0

Table 2. Preparation of HRP Standards

Assay Protocol

I. Hydrogen Peroxide

1. Prepare and mix all reagents thoroughly before use. Each sample, including unknowns and standards, should be assayed in duplicate or triplicate.
2. Add 50 µL of each sample (H_2O_2 standard, control or unknown) into an individual microtiter plate well.
3. Add 50 µL of ADHP/HRP Working Solution to each well. Mix the well contents thoroughly and incubate for 30 minutes at room temperature protected from light.

Note: This assay is continuous (not terminated) and therefore may be measured at multiple time points to follow the kinetics of the reactions.

4. Read the plate with a fluorescence microplate reader equipped for excitation in the 530-570 nm range and for emission in the 590-600 nm range.

5. Calculate the concentration of peroxide within samples by comparing the sample RFU to the standard curve. Subtract the value from the zero H_2O_2 control.

II. Peroxidase

1. Prepare and mix all reagents thoroughly before use. Each sample, including unknowns and standards, should be assayed in duplicate or triplicate.
2. Add 50 µL of each sample (HRP standard, control or unknown) into an individual microtiter plate well.
3. Add 50 µL of ADHP/ H_2O_2 Working Solution to each well. Mix the well contents thoroughly and incubate for 30 minutes at room temperature protected from light.
   Note: This assay is continuous (not terminated) and therefore may be measured at multiple time points to follow the kinetics of the reactions.
4. Read the plate with a fluorescence microplate reader equipped for excitation in the 530-570 nm range and for emission in the 590-600 nm range.
5. Calculate the concentration of peroxidase within samples by comparing the sample RFU to the standard curve. Subtract the value from the zero HRP control.

Example of Results

The following figures demonstrate typical Hydrogen Peroxide/Peroxidase Assay results. One should use the data below for reference only. This data should not be used to interpret actual results.

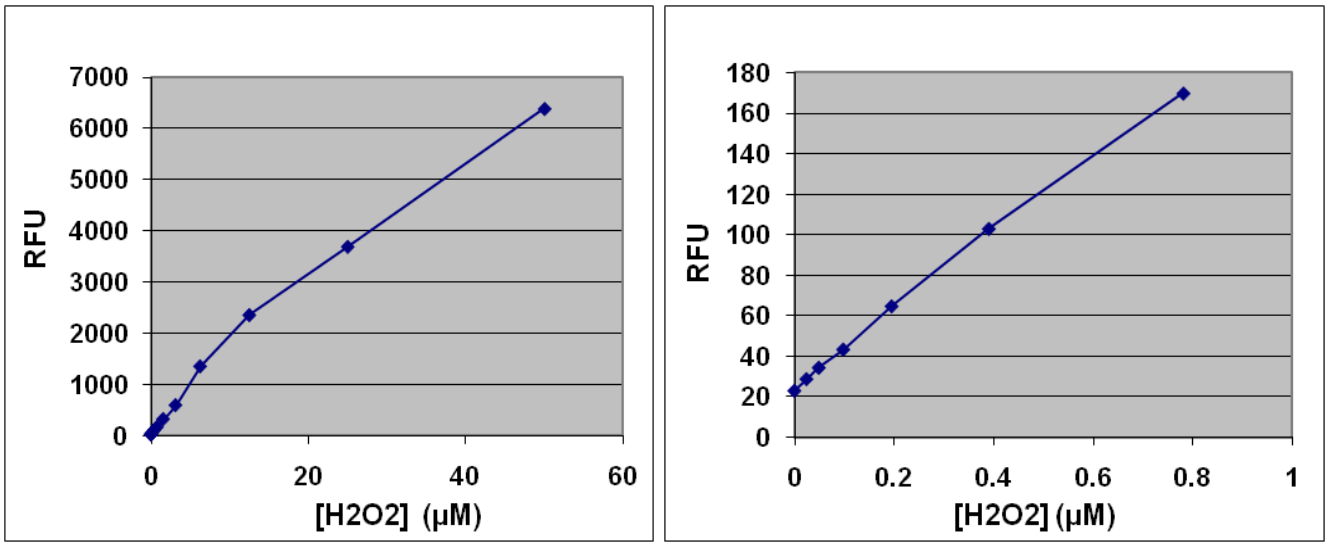


Figure 1. H_2O_2 Standard Curve.

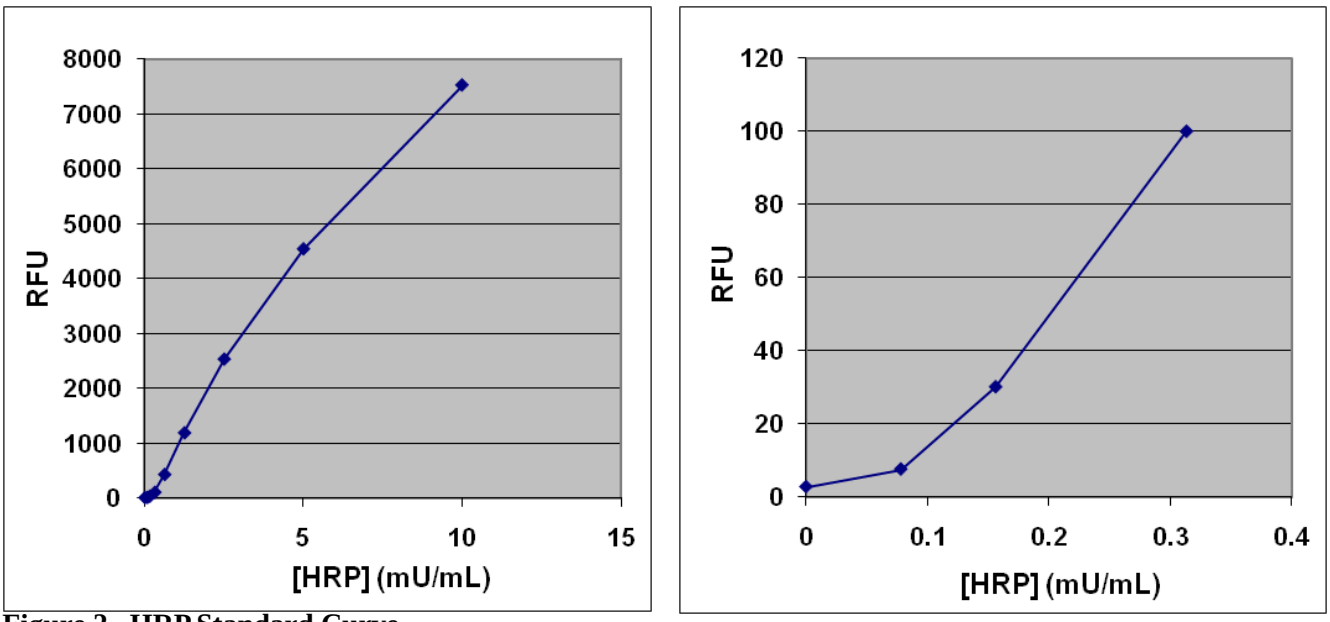


Figure 2. HRP Standard Curve.

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Recent Product Citations

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