Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit *Near Infrared Fluorescence*

Catalog number: 11502
Unit size: 500 Tests

Component	Storage	Amount
Component A: Amplite™ IR Peroxidase Substrate	Freeze (<-15 °C), Minimize light exposure	1 vial
Component B: H2O2	Refrigerate (2-8 °C), Minimize light exposure	1 vial (3% stabilized solution, 200 µL)
Component C: Assay Buffer	Freeze (<-15 °C)	1 bottle (100 mL)
Component D: Horseradish Peroxidase	Freeze (<-15 °C), Minimize light exposure	1 vial (20 units)
Component E: DMSO	Freeze (<-15 °C)	1 vial (0.5 mL)

OVERVIEW

Hydrogen peroxide (H2O2) is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress-related states. It is involved in a number of biological events that have been linked to asthma, atherosclerosis, diabetic vasculopathy, osteoporosis, a number of neurodegenerative diseases and Down's syndrome. Perhaps the most intriguing aspect of H2O2 biology is the recent report that antibodies have the capacity to convert molecular oxygen into hydrogen peroxide to contribute to the normal recognition and destruction processes of the immune system. Measurement of this reactive species will help to determine how oxidative stress modulates varied intracellular pathways. This Amplite™ Hydrogen Peroxide Assay Kit uses our unique Amplite™ IR peroxidase substrate to quantify hydrogen peroxide in solutions and cell extracts. Amplite™ IR generates the fluorescence that is pH-independent from pH 4 to 10. Thus it is superior alternative to ADHP (Amplex Red™) for the detections that require low pH where ADHP (Amplex Red™) has reduced fluorescence. In addition, Amplite™ IR generates a product that has maximum absorption of 647 nm with maximum emission at 670 nm. This near infrared absorption and fluorescence minimize the assay background that is often caused by the autofluorescence of biological samples that rarely absorb light beyond 600 nm. It can also be used to detect a variety of oxidase activities through enzyme-coupled reactions. The kit is an optimized 'mix and read' assay that is compatible with HTS liquid handling instruments.

AT A GLANCE

Protocol summary

1. Prepare H_2O_2 working solution (50 µL)
2. Add H_2O_2 standards or test samples (50 µL)
3. Incubate at room temperature for 0 - 30 minutes
4. Monitor fluorescence intensity at Ex/Em = 640/680 nm (Cutoff = 665 nm)

Important Thaw all the kit components at room temperature before starting the experiment. The Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit can be used to measure the release of H_2O_2 from cells. The following is a suggested protocol that can be modified to meet the specific research needs. NADH and glutathione (reduced form of GSH) may interfere with the assay.

KEY PARAMETERS

Instrument:	Fluorescence microplate reader
Excitation:	640 nm
Emission:	680 nm
Cutoff:	665 nm
Recommended plate:	Solid black

PREPARATION OF STOCK SOLUTIONS

Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles.

1. Amplite™ IR Peroxidase Substrate stock solution (100X):
Add 250 µL of DMSO (Component E) into the vial of Amplite™ IR Peroxidase Substrate (Component A) to make 100X Amplite™ IR Peroxidase Substrate stock solution.

Note Amplite™ IR Peroxidase Substrate (Component A) is unstable in the presence of thiols such as DTT and β mercaptoethanol. If the final concentration of the thiols is higher than 10 µM, it would significantly decrease the assay dynamic range.

2. Peroxidase stock solution (20 U/mL):
Add 1 mL of Assay Buffer (Component C) into the vial of Horseradish Peroxidase (Component D) to make 20 U/mL Peroxidase stock solution.

3. H_2O_2 standard solution (20 mM):
Add 22.7 µL of 3% H_2O_2 (0.88 M, Component B) into 977 µL of Assay Buffer (Component C) to make 20 mM H_2O_2 standard solution.

Note The diluted H_2O_2 stock solution is not stable. The unused portion should be discarded.

PREPARATION OF STANDARD SOLUTION

H_2O_2 standard

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/11502

Add 1 µL of 20 mM H_2O_2 standard solution into 1999 µL of Assay Buffer (Component C) to get 10 µM H_2O_2 standard (HS7). Take 10 µM H_2O_2 standard (HS7) and perform 1:3 serial dilutions to get serially diluted H_2O_2 standard (HS6 - HS1) with Assay Buffer (Component C).

PREPARATION OF WORKING SOLUTION

Add 50 µL of 100X Amplite™ IR Peroxidase Substrate stock solution and 200 µL of 20 U/mL Peroxidase stock solution into 4.75 mL of Assay Buffer (Component C) to make H_2O_2 working solution. Keep from light.

SAMPLE EXPERIMENTAL PROTOCOL

Table 1. Layout of H_2O_2 standards and test samples in a solid black 96-well microplate. HS= H_2O_2 Standards (HS1 - HS7, 0.01 to 10 µM); BL=Blank Control; TS=Test Samples

BL	BL	TS	TS
HS1	HS1	...	...
HS2	HS2	...	...
HS3	HS3		
HS4	HS4		
HS5	HS5		

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HS6	HS6		
HS7	HS7		

Table 2. Reagent composition for each well.

Well	Volume	Reagent
HS1 - HS7	50 µL	Serial Dilutions (0.01 to 10 µM)
BL	50 µL	Assay Buffer (Component C)
TS	50 µL	test sample

Run H_2O_2 assay in supernatants reaction:

1. Prepare H_2O_2 standards (HS), blank controls (BL), and test samples (TS) according to the layout provided in Tables 1 and 2. For a 384-well plate, use 25 µL of reagent per well instead of 50 µL.

2. Add 50 µL of H_2O_2 working solution to each well of H_2O_2 standard, blank control, and test samples to make the total H_2O_2 assay volume of 100 µL/well. For a 384-well plate, add 25 µL of H_2O_2 working solution into each well instead, for a total volume of 50 µL/well.

3. Incubate the reaction at room temperature for 0 to 30 minutes, protected from light.

4. Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 640/680 nm (Cutoff =665nm).

   Note Amplite™ IR Peroxidase Substrate is easy to be self-oxidized, so read the fluorescence as soon as the H_2O_2 working solution was added to increase the signal to noise ratio. The contents of the plate can also be transferred to a white clear bottom plate and read by an absorbance microplate reader at the wavelength of 650 nm. The absorption detection has lower sensitivity compared to the fluorescence reading.

Run H_2O_2 assay for cells:

1. The H_2O_2 working solution should be prepared as above except that the Assay Buffer (Component C) should be replaced with the media used in your cell culture system. Suggested media including (a) Krebs Ringers Phosphate Buffer (KRPB); (b) Hanks Balanced Salt Solution (HBSS); or (c) Serum-free media.

2. Prepare cells in a 96-well plate (50 - 100 µL/well), and activate the cells as desired.

   Note The negative controls (media alone and non-activated cells) are included for measuring the background fluorescence.

3. Add 50 µL of H_2O_2 working solution into each well of cells and H_2O_2 standards. For a 384-well plate, add 25 µL of cells and 25 µL of H_2O_2 working solution into each well.

4. Incubate the reaction at room temperature for 0 to 30 minutes, protected from light.

5. Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 640/680 nm (Cutoff = 665nm).

EXAMPLE DATA ANALYSIS AND FIGURES

The reading (RFU) obtained from the blank standard well is used as a negative control. Subtract this value from the other standards' readings to obtain the base-line corrected values. Then, plot the standards' readings to obtain a standard curve and equation. This equation can be used to calculate Hydrogen Peroxide samples. We recommend using the Online Linear Regression Calculator which can be found at:

https://www.aatbio.com/tools/linear-logarithmic-semi-log-regression-online-calculator

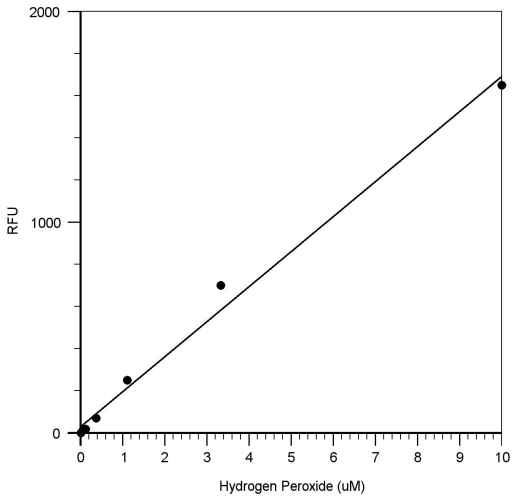

Figure 1. Hydrogen Peroxide dose response was measured in a solid black 96-well plate with Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit.

DISCLAIMER