Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit *Red Fluorescence*

Catalog number: 11501
Unit size: 500 Tests

Component	Storage	Amount
Component A: Amplite™ Red 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 (1 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. Amplite™ Hydrogen Peroxide Assay Kit uses our Amplite™ Red peroxidase substrate to quantify hydrogen peroxide in solutions and cell extracts. 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 10 - 30 minutes
4. Monitor fluorescence intensity at Ex/Em = 540/590 nm

Important Thaw all the kit components at room temperature before starting the experiment.

Important The component A is unstable in the presence of thiols such as DTT and β-mercaptoethanol. Thiols higher than 10 uM (final concentration) would significantly decrease the assay dynamic range.

Important NADH and glutathione (reduced form: GSH) may interfere with the assay.

KEY PARAMETERS

Instrument:	Fluorescence microplate reader
Excitation:	540 nm
Emission:	590 nm
Cutoff:	570 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™ Red Peroxidase Substrate stock solution (100X):
Add 250 µL of DMSO (Component E) into the vial of Amplite™ Red Substrate (Component A). This stock solution should be used promptly.

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

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).

Note Diluted H_2O_2 solution is not stable. Any unused portions should be discarded.

PREPARATION OF STANDARD SOLUTION

H2O2 standard

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/11501

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

PREPARATION OF WORKING SOLUTION

Add 50 µL of Amplite™ Red Peroxidase Substrate stock solution (100X) and 200 µL of Peroxidase stock solution (20 U/mL) into 4.75 mL of Assay Buffer (Component C) to make a total volume of 5 mL.

Note Keep from light.

PREPARATION OF CELL SAMPLES

For guidelines on cell sample preparation, please visit
https://www.aatbio.com/resources/guides/cell-sample-preparation.html

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 standard (HS1-HS7, 0.01 to 10 µM); BL = blank control; TS = test sample.

BL	BL	TS	TS
HS1	HS1	...	...
HS2	HS2	...	...
HS3	HS3		
HS4	HS4		
HS5	HS5		
HS6	HS6		
HS7	HS7		

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Table 2. Reagent composition for each well. Note that high concentrations of H_2O_2 (e.g., >100 µM, final concentration) may cause reduced fluorescence signals due to the overoxidation of Amplite™ Red (to non-fluorescent products).

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

H_2O_2 assay in supernatants

1. Prepare H_2O_2 standards (HS), blank controls (BL), and test samples (TS) into a solid black 96-well microplate according to the layout provided in Table 1 and Table 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 into 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 instead, for a total volume of 50 µL/well.
3. Incubate the reaction at room temperature for 15 to 30 minutes, protected from light.
4. Monitor the fluorescence increase with a fluorescence plate reader at Excitation = 540 ± 10 nm, Emission = 590 ± 10 nm (optimal Ex/Em = 540/590 nm).

Note The contents of the plate can also be transferred to a white clear bottom microplate and read by an absorbance microplate reader at the wavelength of 576 ± 5 nm. However, the absorption detection has a lower sensitivity compared to that of a fluorescence reading.

H_2O_2 assay for cells

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.

1. The H_2O_2 cell working solution should be prepared as above, except that the Assay Buffer (Component C) should be replaced with the media that is used in your cell culture system. Suggested medias include (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 background fluorescence. For a 384-well plate, use 25 µL/well of cell media instead.

3. Add 50 µL of H_2O_2 cell working solution into each well of cells and H_2O_2 standards. For a 384-well plate, add 25 µL of H_2O_2 cell working solution into each well instead.
4. Incubate the reaction at room temperature for 15 to 30 minutes, protected from light.
5. Monitor the fluorescence increase with a fluorescence plate reader at Excitation = 540 ± 10 nm, Emission = 590 ± 10 nm (optimal Ex/Em = 540/590 nm).

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

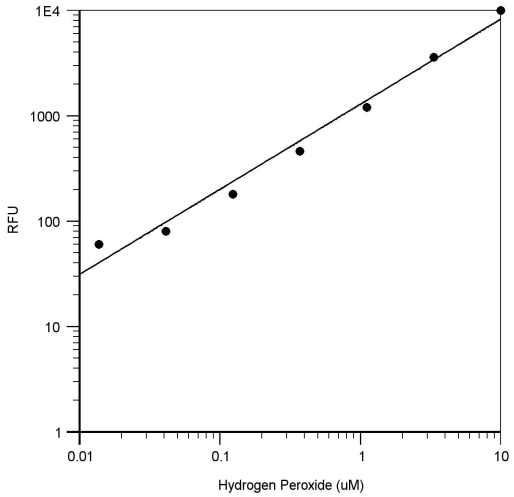

Figure 1. Hydrogen Peroxide dose response was measured in a solid black 384-well plate with the Amplite™ Flourimetric Hydrogen Peroxide Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices).

DISCLAIMER