Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit

Near Infrared Fluorescence

Ordering Information:
Product Number: #11502 (500 assays)

Instrument Platform:
Fluorescence microplate readers

Storage Conditions:
Keep in freezer and avoid light

Introduction

Hydrogen peroxide (H_2O_2) 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 H_2O_2 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, in cell extracts and in live cells. 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 (also called 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.

The Amplite™ Hydrogen Peroxide Assay Kit provides a sensitive, one-step fluorometric assay to detect as little as 10 picomoles of H_2O_2 in a 100 µL assay volume (30 nM; Figure1). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation with no separation steps required. Its signal can be easily read by either fluorescence microplate reader with Ex/Em = 645/670 or absorbance microplate reader at 650 nm.

Kit Key Features

Broad Application: Can be used for quantifying hydrogen peroxide in solutions, in cell extracts and in live cells; and for detecting a variety of oxidase activities through enzyme-coupled reactions.
Sensitive: The kit detect as low as 10 picomoles of H_2O_2 in solution, pH insensitive.
Continuous: Easily adapted to automation with no separation required.
Convenient: Formulated to have minimal hands-on time. No wash is required.
Non-Radioactive: No special requirements for waste treatment.

Kit Components

Component	Amount
Component A: Amplite™ IR peroxidase substrate	1 vial
Component B: H_2O_2	1 vial (3% stabilized solution, 200 µL)
Component C: Assay Buffer	1 bottle (100 mL)
Component D: Horseradish Peroxidase	1 vial (20 units)
Component E: DMSO	1 vial (1 mL)

Assay Protocol (for 1 plate)

Brief Summary

Prepare H_2O_2 reaction mixture (50 µL) → Add H_2O_2 standards or test samples (50 µL) → Incubate at room temperature for 10-30 min → Read fluorescence at Ex 645 nm/Em 670 nm

Note: Thaw all the kit components to room temperature before starting the experiment.

1. Prepare stock solutions:

1.1 Amplite™ IR peroxidase substrate stock solution (100X): Add 250 µL of DMSO (Component E) into the vial of Amplite™ IR substrate (component A). The stock solution should be used promptly; any remaining solution need be aliquoted and refrozen at -20°C.
Note: Avoid repeated freeze-thaw cycles.

1.2 20 U/ml Peroxidase stock solution: Add 1 mL of assay buffer (Component C) into the vial of horseradish peroxidase (Component D).
Note: The unused HRP solution should be divided as single use aliquots and stored at -20°C.

1.3 20 mM H_2O_2 stock solution: Add 22.7 µL of 3% H_2O_2 (0.88 M, Component B) into 977µL of assay buffer (Component C).
Note: The diluted H_2O_2 solution is not stable. The unused portion should be discarded.

2. Prepare H_2O_2 reaction mixture:

2.1 Prepare the H_2O_2 reaction mixture according to the following table and kept from light:

Table 1. H_2O_2 Reaction mixture for one 96-well plate (2X)

Components	Volume
Amplite™ IR peroxidase substrate stock solution (100X, from step 1.1)	100 ul
10 U/ml Peroxidase (from step 1.2)	200 ul
Assay Buffer (Component C)	4.7 ml
Total volume	5 ml

3. Prepare Serial H_2O_2 (0 to 10 µM) solutions

Warning: *The component A is unstable in the presence of thiols such as DTT and β-mercaptoethanol. The final concentration of the thiols higher than 10 uM would significantly decrease the assay dynamic range. NADH and glutathione (reduced form: GSH) may interfere with the assay*

3.1 Add 1 µL of 20 mM H_2O_2 solution (from step 1.3) in 1999 µL of assay buffer (Component C) to get 10 µM H_2O_2 solution.
3.2 Take 200 µL of 10 µM solution to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 standard H_2O_2 solutions.
3.3 Add H_2O_2 standards and H_2O_2-containing test samples into a 96-well solid black microplate as described in Tables 2 and 3

Table 2. Layout of H_2O_2 standards and test samples in a solid black 96-well microplate:

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

Note: HS= H_2O_2 standards, BL=Blank control, TS=test samples.

Table 3. Reagent composition for each well:

H_2O_2 Standard	**Blank Control**	**Test Sample**
Serial dilutions* (50 µL)	Assay buffer (Component C): 50 µL	50 µL

**Note: Add the serially diluted H_2O_2 standards from 0.01 µM to10 µM into wells from HS1 to HS7 in duplicate.*

4. Run H_2O_2 Assay in supernatants Reaction

4.1 Add 50 µL of H_2O_2 reaction mixture (from step 2.1) to each well of the H_2O_2 standard, blank control, and test samples (see step 3.3) so that the total H_2O_2 assay volume is 100 µL/well
Note: For a 384-well plate, add 25 µL sample, 25 µL of H_2O_2 reaction mixture *per well.*
4.2 Incubate the reaction for 15 to 30 minutes at room temperature, protected from light.
4.3 Monitor the fluorescence increase with 645nm excitation and 670 nm emission by using a fluorescence plate reader.
Note: The contents of the plate can also be transferred to a white clear bottom plate and read by absorbance microplate reader at the wavelength 670 nm. The absorption detection has lower sensitivity compared to fluorescence reading.

5. Run H_2O_2 Assay for Cells

The Amplite™ 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 for your specific research needs.

5.1 The H_2O_2 reaction mixture should be prepared as step 2.1 except that the assay buffer (Component C) should be replaced with the media that is 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.
5.2 Prepare cells in 96-well plate (50-100 uL/well), and activate the cells as desired.
Note: The negative controls (media alone and non-activated cells) are included for measuring background fluorescence.
5.3 Add 50 µL of H_2O_2 reaction mixture (from step 5.1) to each well of the cells, and those of H_2O_2 standards (from step 3).
Note: For a 384-well plate, add 25 µL cells, 25 µL of H_2O_2 reaction mixture *per well.*
5.4 Incubate the reaction for 15 to 30 minutes at room temperature, protected from light.
5.5 Monitor the fluorescence increase with 645nm excitation and 670 nm emission by using a fluorescence plate reader.

6. Run Data Analysis

The fluorescence in blank wells (with the assay buffer only) is used as a control, and is subtracted from the values for those wells with the H_2O_2 reactions. The typical data are shown in Figure 1 (H_2O_2 standard curve).
Note: The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.

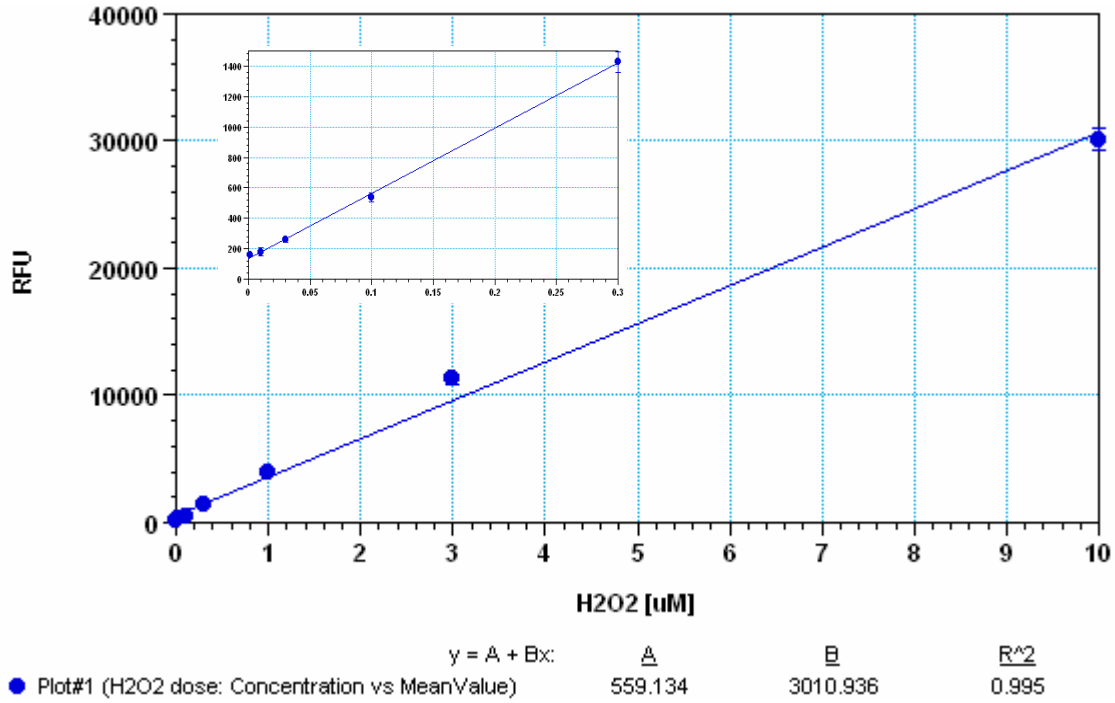

Figure 1. H_2O_2 dose response on 384-well black plate using a fluorescence microplate reader (NovoStar Labtech) measured with the Amplite™ Hydrogen Peroxide Assay Kit. As low as 0.01 µM H_2O_2 can be detected with 30 minutes incubation time (n=3). The insert shows the low levels of H_2O_2 detection.

References:

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