Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit

Red Fluorescence

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: #11501 (500 assays)	Keep in freezer and avoid light	Fluorescence microplate readers

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. The Amplite™ Hydrogen Peroxide Assay Kit uses our non-fluorescent 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.

The Amplite™ Fluorimetric 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 = 540 ±10/590 ±10 nm (maximum Ex/Em = 540/590 nm) or absorbance microplate reader at 576 ±5 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.
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

Components	**Amount**
Component A: Amplite™ Red 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 One 96-well 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/Em = 540/590 nm

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

1. Prepare stock solutions:

1.1 ~~Amplite™ Red peroxidase substrate stock solution (100X):~~ Add 250 ∝L of DMSO (Component E) into the vial of Amplite™ Red 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 and protect from light.

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 ∝L of 3% H_2O_2 (0.88 M, Component B) into 977∝L 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™ Red Peroxidase Substrate stock solution (100X, from step 1.1)	50 ∝L
20 U/ml Peroxidase (from step 1.2)	200 ∝L
Assay Buffer (Component C)	4.75 mL
Total volume	5 mL

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

Warning: *1) 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 ∝M would significantly decrease the assay dynamic range.*
2) 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. High concentration of H_2O_2 (e.g., >100 µM, final concentration) may cause reduced fluorescence signal due to the overoxidation of Amplite™ Red (to a non-fluorescent product).*

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, and 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 Ex/Em = 540±10 /590±10 (optimal Ex/Em = 540/590) nm 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 of 576±5 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 µL/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 3.3).
Note: For a 384-well plate, add 25 µL cells, and 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 Ex/Em= 540±10/590±10 (optimal Ex/Em = 540/590) nm using a fluorescence plate reader.

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 is 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.

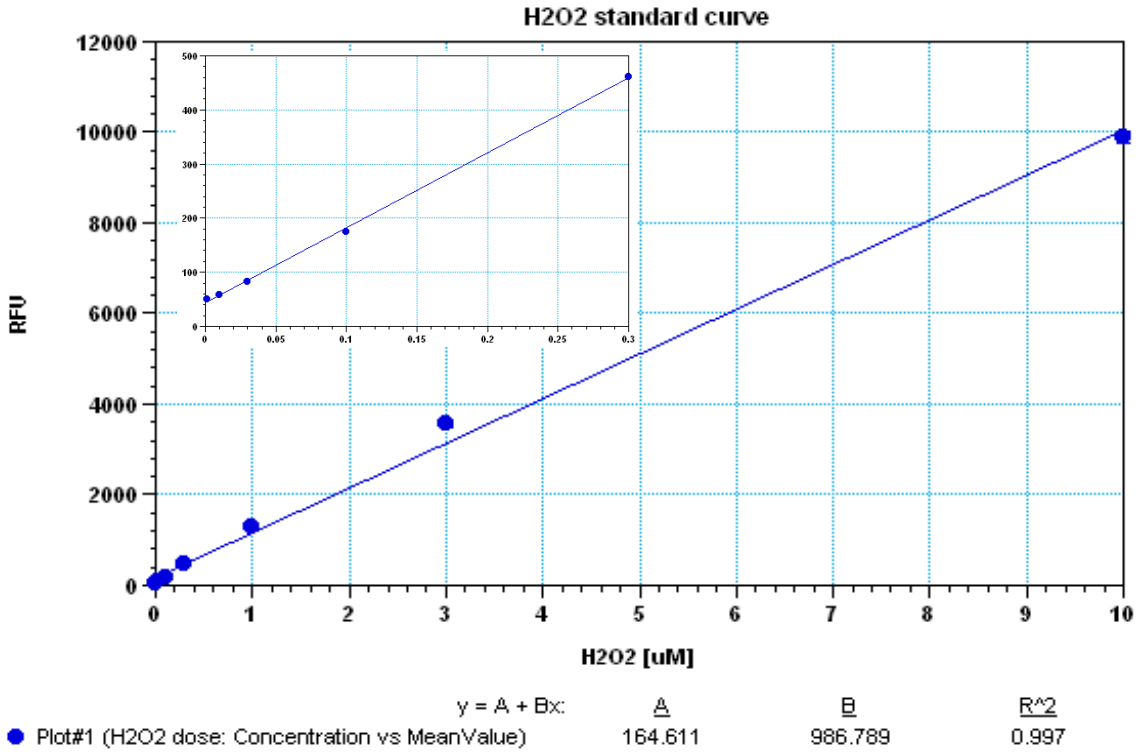

Figure 1. H_2O_2 dose response on 384-well black plate using a Gemini fluorescence microplate reader (Molecular Devices) measured with the Amplite™ Hydrogen Peroxide Assay Kit. As low as 0.03 µM H_2O_2 can be detected with 30 minutes incubation time (n=3). The insert shows the low levels of H_2O_2 detection.

References:

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