Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit

Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 11501 (500 assays)	Keep in freezer Avoid exposure to 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 a variety of intracellular pathways.

This Amplite™ Fluorimetric 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. It provides a sensitive, one-step fluorometric assay to detect as little as 3 picomoles of H_2O_2 in a 100 µL assay volume (30 nM, Figure 1). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and readily adapted to automation. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = ~540/590 nm or an absorbance microplate reader at ~570 nm.

Kit Key Features

Broad Application:	Can be used for quantifying hydrogen peroxide in solutions, in cell extracts and in live cells; and can also be used for detecting a variety of oxidase activities through enzyme-coupled reactions.
Sensitive:	Detect as low as 10 picomoles of H_2O_2 in solution.
Continuous:	Easily adapted to automation without a separation step.
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 minutes → Monitor fluorescence intensity at Ex/Em = 540/590 nm

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

1. Prepare stock solutions:

1.1 100X Amplite™ Red peroxidase substrate stock solution: Add 250 µL of DMSO (Component E) into the vial of Amplite™ Red Substrate (Component A). The stock solution should be used promptly; any remaining solution should 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 into 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 977 µL 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:

Prepare the H_2O_2 reaction mixture according to the following table and keep 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 µL
20 U/ml Peroxidase Stock Solution (from Step 1.2)	200 µL
Assay Buffer (Component C)	4.75 mL
Total volume	5 mL

3. Prepare serial dilutuions of H_2O_2 standard (0 to 10 µM):

Warning 1: *The component A is unstable in the presence of thiols such as DTT and β-ercaptoethanol. Thiols higher than 10 µM (final concentration) would significantly decrease the assay dynamic range.*
Warning 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) into 1999 µL of Assay Buffer (Component C) to get a 10 µM H_2O_2 standard.

3.2 Take 200 µL of 10 µM H_2O_2 standard to perform 1:3 serial dilutions to get 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 µM serial dilutions of H_2O_2 standard.

3.3 Add serial dilutions of H_2O_2 standard and H_2O_2-containing test samples into a solid black 96-well 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) into each well of H_2O_2 standard, blank control, and test samples (see Step 3.3) to make the total H_2O_2 assay volume of 100 µL/well.
Note: For a 384-well plate, add 25 µL of sample and 25 µL of H_2O_2 reaction mixture in each well.

4.2 Incubate the reaction at room temperature for 15 to 30 minutes, protected from light.

4.3 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 540 ± 10 /590 ± 10 nm (optimal Ex/Em = 540/590 nm).
Note: 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 576 ±5 nm. The absorption detection has lower sensitivity compared to fluorescence reading.

5. Run 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.

5.1 The H_2O_2 reaction mixture should be prepared as Step 2 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 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.

5.3 Add 50 µL of H_2O_2 reaction mixture (from Step 5.1) into each well of cells and H_2O_2 standards (from Step 3.3).
Note: For a 384-well plate, add 25 µL of cells and 25 µL of H_2O_2 reaction mixture into each well.

5.4 Incubate the reaction at room temperature for 15 to 30 minutes, protected from light.

5.5 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em= 540 ± 10/590 ± 10 nm (optimal Ex/Em = 540/590 nm).

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. A H_2O_2 standard curve is shown in Figure 1.
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.

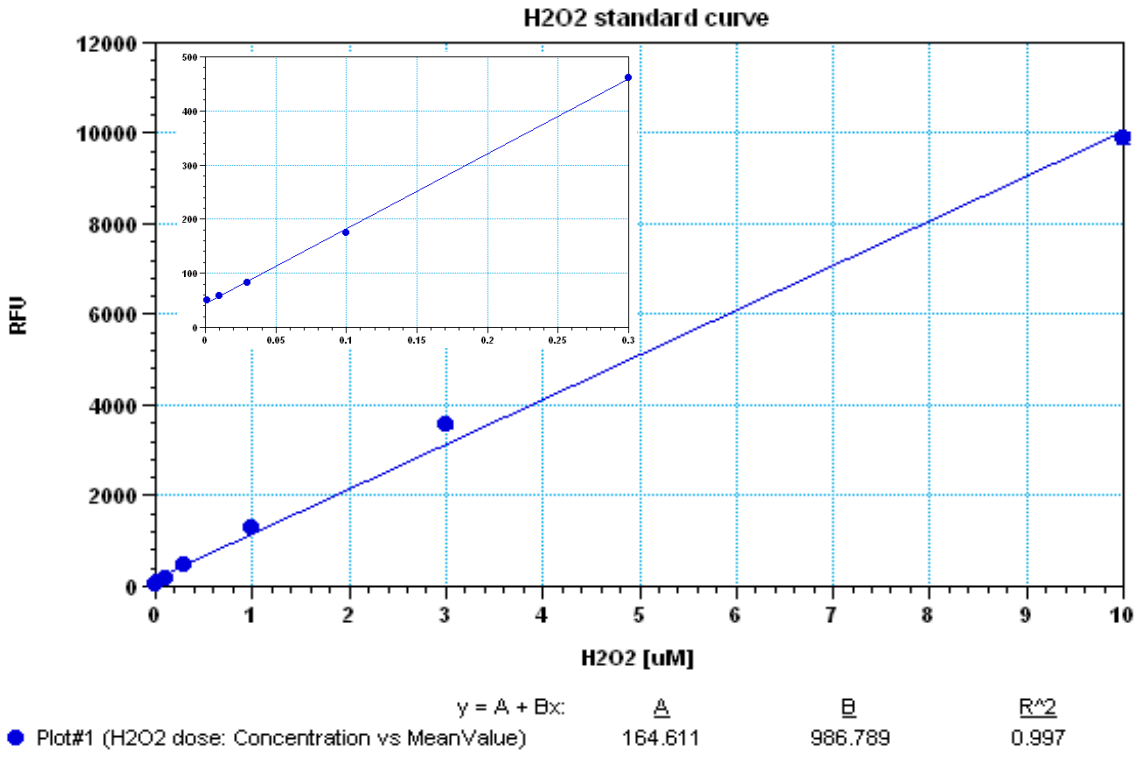

Figure 1. H_2O_2 dose response was measured in a 384-well black plate with the Amplite™ Florimetric Hydrogen Peroxide Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices). As low as 0.03 µM H_2O_2 can be detected with 30 minutes incubation (n=3). The insert shows the low levels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

References

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