

## 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), Dessicated, Avoid Light	1 vial
Component B: H <sub>2</sub> O <sub>2</sub>	Freeze (<-15 °C), Avoid Light	1 vial (3% stabilized solution, 200 µL)
Component C: Assay Buffer	Freeze (<-15 °C), Avoid Light	1 bottle (100 mL)
Component D: Horseradish Peroxidase	Freeze (<-15 °C), Dessicated, Avoid Light	1 vial (20 units)
Component E: DMSO	Freeze (<-15 °C), Avoid Light	1 vial (1 mL)

### OVERVIEW

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> working solution (50 µL)
2. Add H<sub>2</sub>O<sub>2</sub> 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 µM (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<sub>2</sub>O<sub>2</sub> standard solution (20 mM):

Add 22.7 µL of 3% H<sub>2</sub>O<sub>2</sub> (0.88 M, Component B) into 977 µL of Assay Buffer (Component C).

**Note** Diluted H<sub>2</sub>O<sub>2</sub> solution is not stable. Any unused portions should be discarded.

### PREPARATION OF STANDARD SOLUTION

#### H<sub>2</sub>O<sub>2</sub> standard

For convenience, use the Serial Dilution Planner:

<https://www.aatbio.com/tools/serial-dilution/11501>

Add 1 µL of 20 mM H<sub>2</sub>O<sub>2</sub> stock solution into 1999 µL of Assay Buffer (Component C) to get a 10 µM H<sub>2</sub>O<sub>2</sub> standard (HS7). Take 10 µM H<sub>2</sub>O<sub>2</sub> standard and perform 1:3 serial dilutions to get serial dilutions of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> standards and test samples in a solid black 96-well microplate. HS = H<sub>2</sub>O<sub>2</sub> 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		

**Table 2.** Reagent composition for each well. Note that high concentrations of H<sub>2</sub>O<sub>2</sub> (e.g., >100 μM, final concentration) may cause reduced fluorescence signals due to the overoxidation of Amplitude™ 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<sub>2</sub>O<sub>2</sub> assay in supernatants

1. Prepare H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> working solution into each well of H<sub>2</sub>O<sub>2</sub> standard, blank control, and test samples to make the total H<sub>2</sub>O<sub>2</sub> assay volume of 100 μL/well. For a 384-well plate, add 25 μL of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> assay for cells

The Amplitude™ Fluorimetric Hydrogen Peroxide Assay Kit can be used to measure the release of H<sub>2</sub>O<sub>2</sub> from cells. The following is a suggested protocol that can be modified to meet the specific research needs.

1. The H<sub>2</sub>O<sub>2</sub> 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.

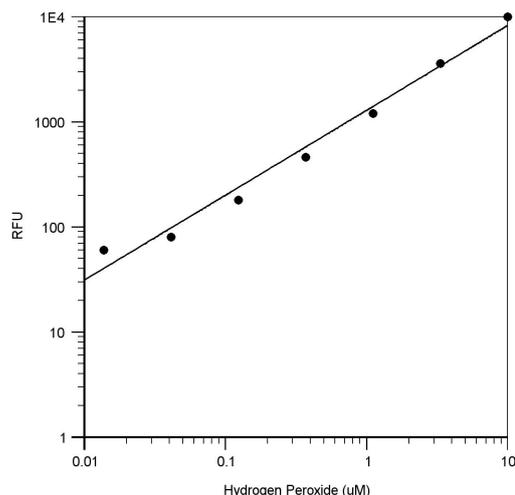
**Note** The negative controls (media alone and non-activated cells) are included for measuring background fluorescence. For a 384-well plate, use 25 μL/well of cell media instead.

3. Add 50 μL of H<sub>2</sub>O<sub>2</sub> cell working solution into each well of cells and H<sub>2</sub>O<sub>2</sub> standards. For a 384-well plate, add 25 μL of H<sub>2</sub>O<sub>2</sub> 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 baseline 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 Amplitude™ Fluorimetric Hydrogen Peroxide Assay Kit using a Gemini fluorescence microplate reader (Molecular Devices).

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