Amplite™ IR

Catalog number: 11009
Unit size: 1 mg

Component	Storage	Amount
Amplite™ IR	Freeze (<-15 °C), Dessicated, Minimize light exposure	1 mg

OVERVIEW

Our Amplite™ IR is a fluorogenic peroxidase substrate that generates near infrared fluorescence upon reaction with peroxidase and H2O2. It can be used to detect both H2O2 and peroxidase. Amplite™ IR generates a substance 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 autoabsorption and/or autofluorescence of biological samples that rarely absorb light beyond 600 nm. Unlike other HRP substrates such as dihydrofluoresceins and dihydrorhodamines, the air-oxidation of Amplite™ IR is minimal. Compared to Amplex Red™, Amplite™ IR generates the fluorescence that is pH-independent from pH 4 to 10. In addition, it has excellent water solubility. It is a superior alternative to Amplex Red™ for the detections that require low pH where Amplex Red™ has significantly reduced fluorescence. We have used Amplite™ IR to detect HRP in quite a few immunoassays. Amplite™ IR can also be used to detect trace amount of H2O2. Because H2O2 is produced in many enzymatic redox reactions, Amplite™ IR can be used in coupled enzymatic reactions to detect the activity of many oxidases and/or related enzymes/substrates or cofactors such as glucose, acetylcholine and cholesterol, L-glutamate, amino acids etc.

AT A GLANCE

Protocol summary

1. Prepare 100 µM Amplite™ IR with 0.8 U/mL peroxidase in phosphate buffer and add 50 µL in a well
2. Add H_2O_2 standards or test samples (50 µL)
3. Incubate at RT for 0-30 minutes
4. Monitor fluorescence intensity at Ex/Em = 640/680 nm

Important The following is the recommended protocol for H_2O_2 assay in solution and live cells. The protocol only provides a guideline, should be modified according to the specific needs.

KEY PARAMETERS

Instrument:	Fluorescence microplate reader
Excitation:	640 nm
Emission:	680 nm
Cutoff:	650 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.

Amplite™ IR stock solution:
Add appropriate amount of anhydrous DMSO to make 10 to 25 mM Amplite™ IR stock solution.

PREPARATION OF WORKING SOLUTION

Amplite™ IR working solution(2X):
In order to achieve final concentration per well of 50 to 100 µM in 50 mM phosphate buffer or buffer of your choice, make 100 to 200 µM concentration solution in a tube. 50 µL is required per well.

Note

Amplite™ IR is unstable in the presence of thiols such as DTT and b-mercaptoethanol. Thiols higher than 10 µM (final concentration) could significantly decrease the assay dynamic range. NADH and glutathione (reduced from: GSH) may interfere with the assay.

Note We recommend using fresh stock solution every time you perform experiments.

SAMPLE EXPERIMENTAL PROTOCOL

Run H_2O_2 assay in supernatants

1. Add 50 µL of 2X Amplite™ IR working solution (from Step 1.2) into each well of the H_2O_2 standard, blank control, and test samples 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 2X Amplite™ IR working solution into each well.

2. Incubate the reaction at room temperature for 0 to 30 minutes, protected from light.

3. Monitor the fluorescence increase at Ex/Em = 640/680 nm with a fluorescence plate reader.

   Note Amplite™ IR peroxidase substrate is easy to be self-oxidized, so read the fluorescence as soon as the H_2O_2 reaction mixture is added to increase the signal to noise ratio.

4. 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

Run H_2O_2 assay for cells:

1. Amplite™ IR 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.The Amplite™ IR working solution should be prepared as Step 1.2 except that the phosphate buffer should be replaced with the media that is used in the cell culture system. Suggested media including (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.

3. Add 50 µL of H_2O_2 reaction mixture to each well of the cells, and those of H_2O_2.

   Note For a 384-well plate, add 25 µL of cells and 25 µL of H_2O_2 reaction mixture into each well.

4. Incubate the reaction for 0 to 30 minutes at room temperature, protected from light.

5. Monitor the fluorescence increase at Ex/Em = 640/ 680 nm with a fluorescence plate reader.

   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

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of 670 nm. The absorption detection has lower sensitivity compared to fluorescence reading.

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.

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 H2O2 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

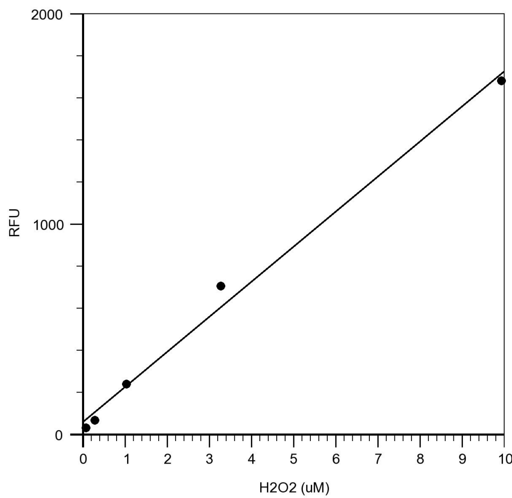

Figure 1. H2O2 dose response was measured in a solid black 96-well plate with Amplite™ Fluorimetric Hydrogen Peroxide Assay Kit.

DISCLAIMER

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