AAT Bioquest®

Amplite™ Colorimetric Hydrogen Peroxide Assay Kit

Catalog number: 11500
Unit size: 500 Tests

Component	Storage	Amount
Component A: Amplite™ IR Peroxidase Substrate	Freeze (<-15 °C), Minimize light exposure	1 vial
Component B: H2O2	Refrigerate (2-8 °C), Minimize light exposure	1 vial (3% stabilized solution, 200 µL)
Component C: Assay Buffer	Freeze (<-15 °C)	1 bottle (100 mL)
Component D: Horseradish Peroxidase	Freeze (<-15 °C), Minimize light exposure	1 vial (20 units)
Component E: DMSO	Freeze (<-15 °C)	1 vial (0.5 mL)

OVERVIEW

Hydrogen peroxide (H2O2) 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 H2O2 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™ Colorimetric Hydrogen Peroxide Assay Kit uses our unique Amplite™ IR peroxidase substrate to quantify hydrogen peroxide in solutions and cell extracts. Upon hydrogen peroxide oxidation the colorless Amplite™ IR generates an intense blue color product that is pH-independent from pH 4 to 10. The existing colorimetric hydrogen peroxide assays (from other vendors) often have severe sample interferences caused by the inherent absorption of biological samples. The near infrared absorption of Amplite™ IR product minimizes the assay background that since the biological samples 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.

AT A GLANCE

Protocol summary

1. Prepare H_2O_2 working solution (50 µL)
2. Add H_2O_2 standards or test samples (50 µL)
3. Incubate at room temperature for 10 - 60 minutes
4. Monitor absorbance at 650 nm

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

Important Amplite™ IR Peroxidase Substrate (Component A) is unstable in the presence of thiols such as DTT and β-mercaptoethanol. If the final concentration of the thiols is higher than 10 uM, it would significantly decrease the assay dynamic range.

Important NADH and glutathione (reduced form of GSH) may interfere with the assay.

KEY PARAMETERS

Instrument:	Absorbance microplate reader
Absorbance:	650 nm
Recommended plate:	Clear bottom

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™ IR Peroxidase Substrate stock solution (100X):
Add 250 µL of DMSO (Component E) into the vial of Amplite™ IR Peroxidase Substrate (Component A).

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_2O_2 standard solution (20 mM):
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 stock solution is not stable. The unused portion should be discarded.

PREPARATION OF STANDARD SOLUTION

H_2O_2 standard

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/11500

Add 5 µL of 20 mM H_2O_2 standard solution into 995 µL of Assay Buffer (Component C) to get 100 µM H_2O_2 standard (HS7). Take 200 µL of 100 µM H_2O_2 standard to perform 1:2 serial dilutions to get serial dilutions of H_2O_2 standard (HS6 - HS1).

PREPARATION OF WORKING SOLUTION

Add 50 µL of Amplite™ IR 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_2O_2 standards and test samples in a white wall/clear bottom 96-well microplate. HS= H_2O_2 Standards (HS1 - HS7, 1.563 to 100 µM); BL=Blank Control; TS=Test Samples.

BL	BL	TS	TS

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HS1	HS1	...	...
HS2	HS2	...	...
HS3	HS3		
HS4	HS4		
HS5	HS5		
HS6	HS6		
HS7	HS7		

Table 2. Reagent composition for each well.

Well	Volume	Reagent
HS1 - HS7	50 µL	Serial Dilution (1.563 to 100 µM)
BL	50 µL	Assay Buffer (Component B)
TS	50 µL	test sample

H2O2 assay in supernatants reaction

1. Prepare H_2O_2 standards (HS), blank controls (BL), and test samples (TS) according to the layout provided in Tables 1 and 2. For a 384-well plate, use 25 µL of reagent per well instead of 50 µL.

2. Add 50 µL of H_2O_2 working solution to each well of H_2O_2 standard, blank control, and test samples to make the total H_2O_2 assay volume of 100 µL/well. For a 384-well plate, add 25 µL of H_2O_2 working solution into each well instead, for a total volume of 50 µL/well.

3. Incubate the reaction at room temperature for 10 to 30 minutes, protected from light.

4. Monitor the absorbance with an absorbance plate reader at 650 nm.

H2O2 assay for cells

The Amplite™ Colorimetric 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.

1. The H_2O_2 cell working solution should be prepared as given except that the Assay Buffer (Component C) should be replaced with the media 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.

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 the background fluorescence.

3. Add 50 µL of H_2O_2 cell working solution to each well of cells and H_2O_2 standards to make the total H_2O_2 assay volume of 100 µL/well. For a 384-well plate, add 25 µL of H_2O_2 cell working solution into each well instead, for a total volume of 50 µL/well.

4. Incubate the reaction at room temperature for 10 to 60 minutes, protected from light.

5. Monitor the absorbance with an absorbance plate reader at 650 nm.

EXAMPLE DATA ANALYSIS AND FIGURES

The reading (Absorbance) 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 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

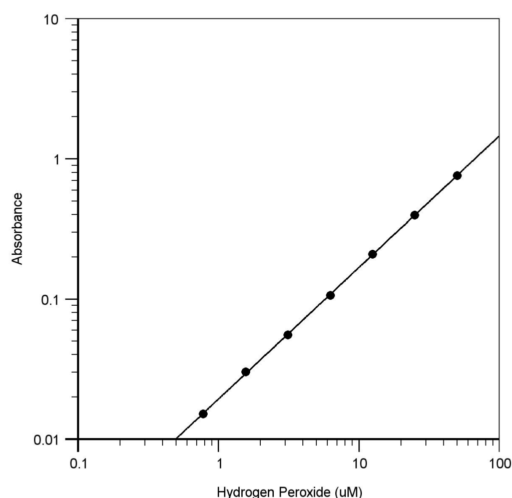

Figure 1. Hydrogen Peroxide dose response was measured in a a white wall/clear bottom 96-well plate with the Amplite™ Colorimetric Hydrogen Peroxide Assay Kit using a Spectramax absorbance microplate reader (Molecular Devices).

DISCLAIMER

AAT Bioquest provides high-quality reagents and materials for research use only. For proper handling of potentially hazardous chemicals, please consult the Safety Data Sheet (SDS) provided for the product. Chemical analysis and/or reverse engineering of any kit or its components is strictly prohibited without written permission from AAT Bioquest. Please call 408-733-1055 or email info@aatbio.com if you have any questions.

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