Hydrogen Peroxide Assay Kit

Item No. 600050

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 well Quantity/Size	480 well Quantity/Size	Storage
10009322	Assay Buffer Tablet	1 tablet	1 tablet	RT
600051	Hydrogen Peroxide Standard	1 vial/100 µl	1 vial/100 µl	4°C
600052	Hydrogen Peroxide Detector ADHP	1 vial/100 µl	1 vial/500 µl	-20°C
600053	Horseradish Peroxidase	1 vial/100 µl	1 vial/500 µl	-20°C
600054	Catalase	1 vial/2 mg	1 vial/10 mg	-20°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure fluorescence with an excitation wavelength between 530-560 nm and an emission wavelength of 590 nm or absorbance at 570 nm.
2. Serum-free cell culture media appropriate for the cells used.
3. A source of pure water; glass distilled water or deionized water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. A plate shaker.
5. Microtiter plates for fluorometric or colorimetric assay.

INTRODUCTION

Background

Hydrogen peroxide (H_2O_2) is a major reactive oxygen species (ROS) with roles in redox signaling and oxidative stress.[1,2] It is produced during numerous cellular processes including respiration, protein folding, peroxisome activity, and oxidase catalysis. Localized and controlled generation of H_2O_2 occurs in response to various cell stimuli such as cytokines, neurotransmitters, and growth factors with membrane-bound NADPH oxidases regulating H_2O_2 flux.[1] H_2O_2 acts as a signaling molecule or induces oxidative damage and stress depending on its local concentration and the kinetics of its production and elimination, the latter of which is catalyzed primarily by catalases and peroxidases. H_2O_2 is also produced during neutrophil-mediated microorganism phagocytosis, whereby NADPH oxidase produces intraphagosomal superoxide, which is further dismutated by superoxide dismutase (SOD) into H_2O_2.[3] This extracellular H_2O_2 induces bacterial oxidative damage and is also converted to hypochlorous acid, which is more highly reactive, by myeloperoxidase (MPO), which induces microorganism death.

About This Assay

Cayman's Hydrogen Peroxide Assay Kit provides a simple method for the sensitive quantitation of extracellular H_2O_2 produced by cultured cells. H_2O_2 is detected using ADHP (10-acetyl-3,7-dihydroxyphenoxazine), a highly sensitive and stable probe for H_2O_2.[4] In a horseradish peroxidase catalyzed reaction, ADHP reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin.[5] Resorufin fluorescence can be read using an excitation wavelength between 530-560 nm and an emission wavelength of 590 nm. Alternatively, the absorbance of resorufin can be measured at 570 nm. Catalase, an H_2O_2 scavenger, is included in the kit to check specificity of the assay. Reactive oxygen species (ROS), such as H_2O_2 and superoxide are generated by phagocytes and participate in damaging invading miccroorganisms or other biologic targets.[6] This kit provides a valuable method for immunologists to assess the capacity of immune cell killing using ROS.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer Preparation**

   Dissolve the Assay Buffer Tablet (Item No. 10009322) in 100 ml of pure water. This assay buffer should be stable for approximately one year at room temperature.

2. **H_2O_2 Standard/Positive Control Preparation**

   Due to the instability of H_2O_2, it is recommended that the concentration of the H_2O_2 Standard (Item No. 600051) be assessed prior to use. To measure the H_2O_2 Standard concentration, dilute the H_2O_2 Standard 1:1,000 in pure water and read the absorbance at 240 nm in a quartz cuvette.

   The concentration (M) is equivalent to the absorbance multiplied by the dilution factor and divided by 43.6. This stock can then be diluted to 1 mM using the assay buffer to provide a working stock to begin your standard curve.

   If a quantitative measurement is not required, assume the standard is approximately 8.8 M. For standard curve preparation, follow instructions on page 9 for fluorimetric assay and page 10 for colorimetric assay.

3. **Catalase Solution Preparation**

   Reconstitute the Catalase (Item No. 600054) in assay buffer as follows: 2 mg vial (96-well kit) in 500 µl, or 10 mg vial (480-well kit) in 2.5 ml and keep on ice. If all of the reconstituted catalase will not be used at one time, aliquot and store at -20°C where it should be stable for at least one month.

4. **Enzyme Reaction Solution Preparation**

   To make 1 ml of enzyme reaction solution, sufficient for use on one 96-well plate, add 100 µl of Hydrogen Peroxide Detector ADHP (Item No. 600052) and 100 µl Horseradish Peroxidase (Item No. 600053) to 800 µl of assay buffer. This enzyme reaction solution is stable for up to one hour when kept on ice. Aliquot the 480-well vial into smaller aliquots and store at -20°C, limiting freeze-thaw cycles.

Sample Preparation

1. Seed cells in a 96-well plate at a density of 10^4-10^5 cells/well in 100 µl of serum-free culture medium. If desired, compounds or vehicle controls can be added to the 100 µl of culture medium. Control wells should be included which contain medium and experimental compounds but no cells. Include extra wells for catalase controls to determine assay specificity. We recommend that each treatment be performed in triplicate.
2. Culture the cells at 37°C for 24-48 hours, or for a period of time according to a user determined experimental protocol. Centrifuge the 96-well tissue culture plate at 400 x g for five minutes and collect the supernatant.

ASSAY PROTOCOL

Standard Curve Preparation - Fluorometric

Label eight test tubes A through H. Aliquot 990 µl of assay buffer into tube A and 500 µl into tubes B-H. Transfer 10 µl of the 1 mM stock H_2O_2 into tube A and mix throughly. Serially dilute the standard by removing 500 µl from tube A and place into tube B; mix throughly. Repeat this procedure for tube tube B to tube G. Do not add any standard to tube H. The final concentration of the standard tubes are 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0 µM, respectively.

Standard Curve Preparation - Colorimetric

Label eight test tubes A-H. Add the amount of 1 mM stock H_2O_2 and assay buffer to each tube as described in Table 1.

Tube	1mM H_2O_2 (µl)	Assay Buffer (µl)	Final Concentration (µM)
A	80	920	80
B	60	940	60
C	40	960	40
D	20	980	20
E	10	990	10
F	5	995	5
G	2.5	997.5	2.5
H	0	1,000	0

Table 1. Preparation of the H_2O_2 standards - colorimetric

Performing the Assay

1. Transfer 80 µl of each standard from tubes A-H prepared earlier to the standard wells on the 96-well assay plate.
2. Transfer 80 µl of supernatant from each well of cultured cells to the corresponding wells on the 96-well assay plate.
3. Add 10 µl of assay buffer to the sample activity wells or 10 µl of catalase solution to the assay specificity wells.
4. Add 10 µl of the enzyme reaction solution to each well and incubate plate on a shaker for 5 minutes.
5. Read the fluorescence using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm. If the colorimetric method is used, read the absorbance at 570 nm.

Calculations

Determination of the Reaction Rate

1. Calculate the average fluorescence or absorbance of each standard and sample.
2. Subtract the average fluorescence or absorbance of the blank (Standard H) from itself and from all other standards and samples including the catalase containing samples.
3. Plot the corrected fluorescence or absorbance of each standard (from step 2 above) as a function of the final H_2O_2 concentration (µM). See Figures 1 (below) and 2, on page 13, for a typical standard curve.

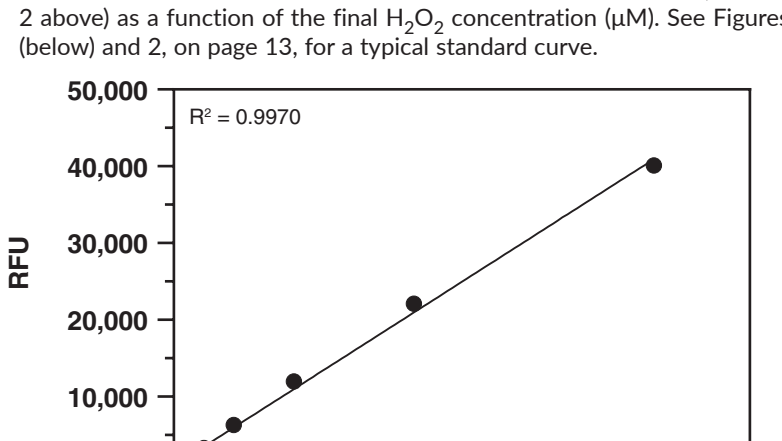

Figure 1. H_2O_2 standard curve - fluorometric

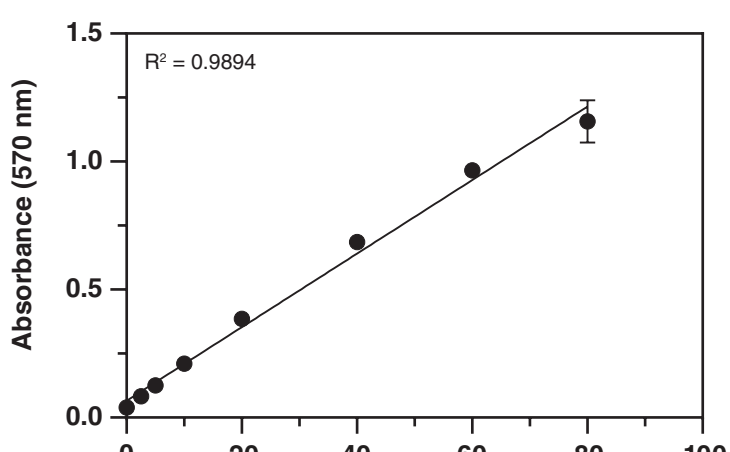

Figure 2. H_2O_2 standard curve - colorimetric

4. Subtract the catalase sample fluorescence or absorbance from the non-catalase sample fluorescence or absorbance to yield the corrected sample signal (CS).
5. Calculate the H_2O_2 concentration of the samples using the equation obtained from the linear regression of the standard curve:

$$H_2O_2 \text{ Concentration } (\mu M) = \left[\frac{CS - (y\text{-intercept})}{Slope} \right] \times \text{Sample Dilution}$$

If a high production of H_2O_2 in the samples is expected, serial dilution may be required to obtain values that fall on the standard curve.

Performance Characteristics

Sensitivity:

The lower limit of quantification (LLOQ) for the fluorometric assay is 0.16 µM and the lower limit of detection (LLOD) is 0.02 µM. The LLOQ for the colorimetric assay is 2.5 µM and the LLOD is 0.15 µM.

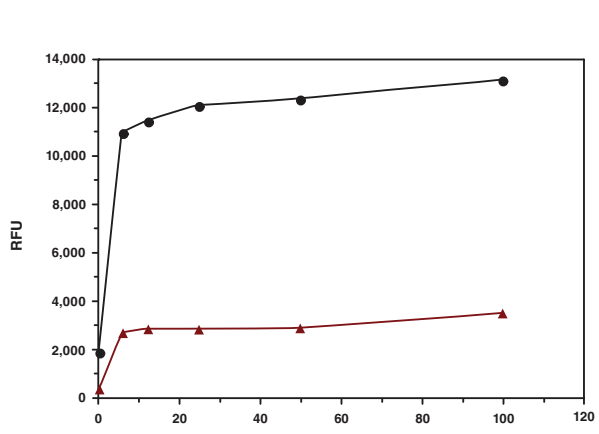

Figure 3. IFNγ and LPS together increase H_2O_2 production in RAW 264.7 cells.

RESOURCES

Troubleshooting

Problem	Possible Causes
Erratic values; dispersion of duplicates/ triplicates	A. Poor pipetting/technique B. Bubble in the well(s)
No H_2O_2 was detected in the sample and standard wells	Enzyme mixture was not prepared correctly
Erratic response curve of compound treatments	Unequal number of cells in each well

References

1. Jia, S. and Chang, C.J. A microtubule-localizing activity-based sensing fluorescent probe for imaging hydrogen peroxide in living cells. *Bioorg. Med. Chem. Lett.* **48**, 128252 (2021).
2. Lismont, C., Revenco, I., and Fransen, M. Peroxisomal hydrogen peroxide metabolism and signaling in health and disease. *Int. J. Mol. Sci.* **20(15)**, 3673 (2019).
3. Rada, B. and Leto, T.L. Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contrib. Microbiol.* **15**, 164-187 (2008).
4. Zhou, M., Diwu, Z., Panchuk-Voloshina, N., *et al.* A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal. Biochem.* **253**, 162-168 (1997).
5. Amundson, D.M. and Zhou, M. Fluorometric method for the enzymatic determination of cholesterol. *J. Biochem. Biophys. Meth.* **38**, 43-52 (1999).
6. Rosen, G.M., Pou,S., Ramos, C.L. *et al.* Free radicals and phagocytic cells. *FASEB J.* **9**, 200-209 (1995).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.