Hydrogen Peroxide Cell-Based Assay Kit

Item No. 600050

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GENERAL INFORMATION

Materials Supplied

For best results, upon receipt of the delivered kit, remove components and store as stated below.

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

If You Have Problems

Technical Service Contact Information

Phone:	888-526-5351 (USA and Canada only) or 734-975-3888
Fax:	734-971-3641
Email:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the capacity to measure fluorescence using an excitation wavelength between 530-560 nm and an emission wavelength of 590 nm.
2. Serum-free cell culture media appropriate for the cells used.
3. A source of pure water; glass distilled water or HPLC-grade water are acceptable.
4. A 96-well black plate.

INTRODUCTION

About This Assay

Cayman's H_2O_2 Cell-Based Assay Kit provides a simple fluorometric 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.[1] In a horseradish peroxidase catalyzed reaction, ADHP reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin.[2] Resorufin fluorescence can be read using an excitation wavelength between 530-560 nm and an emission wavelength of 590 nm. Catalase, an H_2O_2 scavenger, is included in the kit to check specificity of the assay. Reactive oxygen species, such as hydrogen peroxide (H_2O_2) and superoxide are generated by phagocytes and participate in damaging invading miccroorganisms or other biologic targets.[3] 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 each Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of UltraPure water. This buffer should be stable for approximately one year at room temperature.

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

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

   The concentration (M) is equivalent to the absorbance x the dilution factor divided by 43.6. This stock can then be diluted to 1 mM to provide a 100X working stock to begin your standard curve.

   To prepare the standard for use in the H_2O_2 assay: obtain eight clean test tubes and label them #1 through #8. Aliquot 990 µl of serum-free culture medium into tube #1 and 500 µl into tubes #2-#8. Transfer 10 µl of the 1 mM stock H_2O_2 into tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and place into tube #2; mix thoroughly. Repeat this procedure for tube #3 to tube #7. Do not add any standard to tube #8, your blank. The final concentrations of the standard tubes are 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0 µM, respectively.

   If a quantitative measurement is not required, assume the standard is approximately 8.8 M and dilute it to 10 mM for the positive control. Dilute 1:1,000 immediately prior to use. This solution is not stable and should be discarded after use.

3. **Catalase Solution Preparation**

   Reconstitute the Cell-Based Assay 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. Keep on ice and aliquot for storage at -20°C if multiple uses are anticipated. The reconstituted catalase is stable for one month at -20°C.

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 Cell-Based Assay Hydrogen Peroxide Detector ADHP (Item No. 600052) and 100 µl Cell-Based Assay Horseradish Peroxidase (Item No. 600053) to 800 µl of the 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 to avoid repeated freeze/thaw cycles.

ASSAY PROTOCOL

Performing the Assay

Procedure

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 your typical experimental protocol. Centrifuge the 96-well tissue culture plate at 400 x g for five minutes.
3. Transfer 80 µl of supernatant from each well of the cultured cells or from the standard tubes prepared earlier to a new 96 well black plate. Add 10 µl of Assay Buffer (sample activity measurement) or 10 µl of the Catalase Solution (to test assay specificity) to appropriate wells.
4. Add 10 µl of the Enzyme Reaction Solution to each well.
5. Within five minutes, read the fluorescence intensity of each well (excitation = 530 nm; emission = 590 nm).

ANALYSIS

Calculations

Determination of the Reaction Rate

1. Calculate the average fluorescence of each standard and sample.
2. Subtract the average fluorescence of the blank (Standard tube #8) from itself and from all other standards and samples including the catalase containing samples.
3. Plot the corrected fluorescence of the standards (from step 1 above) as a function of the final H_2O_2 concentration (µM). See Figure 1 (below) for a typical standard curve.

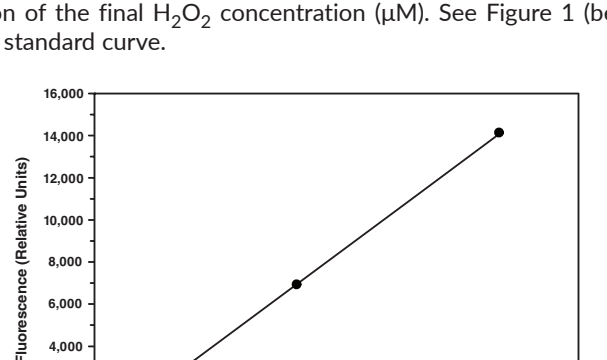

Figure 1. Example H_2O_2 standard curve

4. Subtract the catalase sample fluorescence from the non-catalase sample fluorescence to yield the corrected sample fluorescence.
5. Calculate the H_2O_2 concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected fluorescence values for each sample.

$$H_2O_2 \text{ Concentration (µM)} = \left[\frac{\text{Corrected sample fluorescence - (y-intercept)}}{\text{Slope}}\right] \text{ x Dilution}$$

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

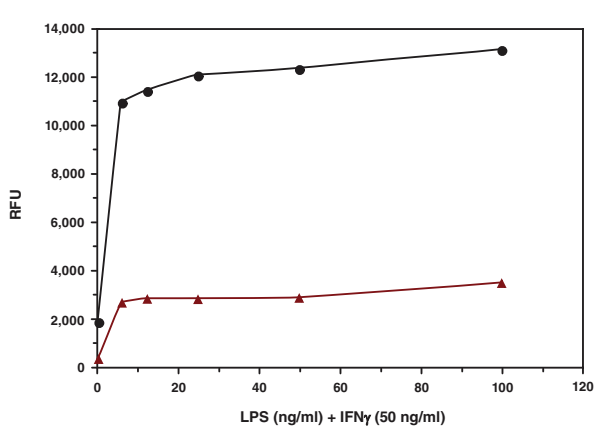

Figure 2. IFNγ and LPS together increase H_2O_2 production in RAW 264.7 cells.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No H_2O_2 was detected in the sample and standard wells	Enzyme mixture was not prepared correctly	Prepare a fresh enzyme mixture and re-assay
Erratic response curve of compound treatments	Unequal number of cells in each well	Make sure each well contains the same number of cells

References

1. 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).
2. Amundson, D.M. and Zhou, M. Fluorometric method for the enzymatic determination of cholesterol. *J. Biochem. Biophys. Meth.* **38**, 43-52 (1999).
3. 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.