Hydrogen Peroxide Assay Kit

Item No. 600050

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 well Quantity/Size	480 well Quantity/Size	Storage
10009322	Cell-Based Assay Buffer Tablet	1 tablet	1 tablet	RT
600051	Hydrogen Peroxide Standard	1 vial/110 µl	1 vial/110 µl	4°C
600052	Hydrogen Peroxide Detector ADHP	1 vial/120 µl	1 vial/550 µl	-20°C
600053	Horseradish Peroxidase	1 vial/120 µl	1 vial/550 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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Hydrogen Peroxide Assay Kit. 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 capable of measuring fluorescence with excitation and emission wavelengths of 530-540 and 590 nm, respectively, or a plate reader capable of measuring absorbance at 570 nm
2. Serum-free cell culture media appropriate for the cells used
3. A source of pure water; glass-distilled is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. An orbital microplate shaker
5. Microtiter plates for fluorometric or colorimetric assays
6. Adjustable pipettes; multichannel or repeating pipettor recommended
7. Materials used for Sample Preparation (see page 10)

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 fluorometric or colorimetric methods for the quantification of extracellular H_2O_2 produced by cultured cells. In the presence of horseradish peroxidase (HRP), H_2O_2 reacts stoichiometrically with an HRP substrate, ADHP, to produce the compound resorufin.[4,5] Resorufin fluorescence can be easily quantified at excitation and emission wavelengths of 530-540 and 585-595 nm, respectively. Alternatively, the absorbance of resorufin can be measured at 570 nm. Catalase, an H_2O_2 scavenger, is included in the kit for verifying the specificity of the assay. 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.

This assay offers the option to measure absorbance or fluorescence. It is at the user's discretion to choose the mode of measurement (and corresponding standard curve preparation) that best fits their needs. When read fluorometrically, this assay has a range of 0-10 µM and a lower limit of detection (LLOD) of 0.02 µM. When read colorimetrically, the range is 0-80 µM with an LLOD of 0.15 µM.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Cell-Based Assay Buffer Preparation**

   Dissolve the Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of pure water. The Cell-Based Assay Buffer will be stable for at least one year when stored at room temperature.

2. **Hydrogen Peroxide Standard/Positive Control Preparation**

   One vial of Hydrogen Peroxide Standard (Item No. 600051) contains 110 µl of 8,800 mM of a hydrogen peroxide solution. Due to the instability of H_2O_2, it is recommended that the concentration of the Hydrogen Peroxide Standard be assessed prior to use. To measure the Hydrogen Peroxide Standard concentration, dilute the Hydrogen Peroxide Standard 1:1,000 in pure water and read the absorbance at 240 nm in a 1 cm quartz cuvette.

   Use the following formula to calculate the concentration:

$$[H_2O_2 \text{ Standard}] \text{ (mM)} = \left[\frac{A_{240}}{43.6\ M^{-1}cm^{-1} \times 1\ cm} \right] \times 1{,}000\ mM/1\ M \times 1{,}000$$

   If a quantitative measurement is not required, assume the standard is approximately 8,800 mM.

   Dilute Hydrogen Peroxide Standard with Cell-Based Assay Buffer to a concentration of 1 mM. This will be a bulk standard. If using 8,800 mM standard concentration, add 10 µl of Hydrogen Peroxide Standard to 990 µl of Cell-Based Assay Buffer and mix gently, then transfer 10 µl of this solution to 870 µl of Cell-Based Assay Buffer and mix gently. For standard curve preparation, follow instructions on page 13 for fluorometric assay and page 14 for colorimetric assay.

3. **Catalase Solution Preparation**

   Reconstitute the Catalase (Item No. 600054) as follows: 2 mg vial in 500 µl (96-well kit) or 10 mg vial in 2.5 ml (480-well kit) of Cell-Based Assay Buffer and keep it on ice. If not using the reconstituted catalase all at once, prepare aliquots and store at -20°C where it will be stable for at least one month.

4. **Enzyme Reaction Solution Preparation**

   To make 1 ml of Enzyme Reaction Solution, which is sufficient for use on one 96-well plate, add 100 µl of Hydrogen Peroxide Detector ADHP (Item No. 600052) and 100 µl of Horseradish Peroxidase (Item No. 600053) to 800 µl of Cell-Based Assay Buffer. This enzyme reaction solution is stable for up to one hour when kept on ice.

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 serum-free culture medium. Control wells, which contain medium and experimental compounds without cells, should be included. Include extra wells for catalase controls to determine assay specificity. It is 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 at room temperature and collect the supernatant.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in triplicate is shown in Figure 1. It is suggested that the contents of each well are recorded on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	1	1	1	9	9	9	17	17	17
B	S2	S2	S2	2	2	2	10	10	10	18	18	18
C	S3	S3	S3	3	3	3	11	11	11	19	19	19
D	S4	S4	S4	4	4	4	12	12	12	20	20	20
E	S5	S5	S5	5	5	5	13	13	13	21	21	21
F	S6	S6	S6	6	6	6	14	14	14	22	22	22
G	S7	S7	S7	7	7	7	15	15	15	23	23	23
H	S8	S8	S8	8	8	8	16	16	16	24	24	24

S1-S8 = Standard Wells
1-24 = Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all of the wells.
- All reagents should be prepared as described above. The Catalase Solution should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate (triplicate is preferred), but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with excitation and emission wavelengths of 530-540 and 585-595 nm, respectively, or monitor the absorbance at 570 nm.

Standard Curve Preparation

NOTE: This assay can be read using fluorescence or absorbance. Choose the standard curve preparation that matches the format needed. Both standard curves do not need to be prepared.

Fluorometric Standard Curve Preparation

Label eight clean glass or polystyrene test tubes A-H. Pipette 990 µl of Cell-Based Assay Buffer to tube A. Pipette 500 µl of Cell-Based Assay Buffer to tubes B-H. Transfer 10 µl of the H_2O_2 bulk standard (1 mM) to tube A. Mix gently. Serially dilute the standard by removing 500 µl from tube A and placing it into tube B. Mix gently. Next, remove 500 µl from tube B and place it into tube C. Mix gently. Repeat the process for tubes D-G. Do not add any standard to tube H.

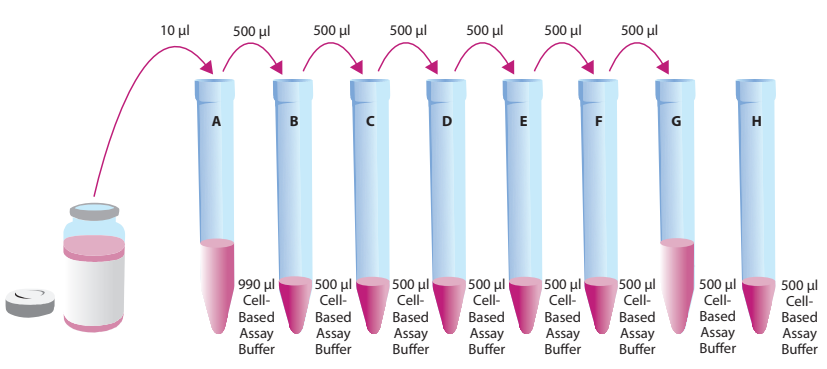


Figure 2. Preparation of the H_2O_2 standards for the fluorometric assay format

Colorimetric Standard Curve Preparation

Label eight test tubes A-H. Add the amount of H_2O_2 bulk standard (1 mM, see page 8 for preparation) and Cell-Based Assay Buffer to each tube as described in Table 1.

Tube	1 mM 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 for the colorimetric assay format

Performing the Assay

1. **Standard Wells:** Add 10 µl of Cell-Based Assay Buffer and 80 µl of standard (tubes A-H) per well in the designated wells on the plate (see **Sample plate format**, Figure 1, page 11).
2. **Sample Wells:** Add 10 µl of Cell-Based Assay Buffer and 80 µl of sample to at least two wells
3. Add 10 µl of the Catalase Solution to the assay specificity wells.
4. Initiate the reactions by adding 10 µl of the Enzyme Reaction Solution to all of the wells being used.
5. Incubate for 5 minutes at room temperature on a shaker.
6. Read the fluorescence using excitation and emission wavelengths of 530-540 and 585-595 nm, respectively. If the colorimetric method is used, read absorbance at 570 nm.

Reagent	Standard Wells (µl)	Sample Wells (µl)	Assay Specificity Wells (µl)
Standard	80	--	--
Sample	--	80	80
Assay Buffer	10	10	--
Catalase Solution	--	--	10
Initate reactions			
Enzyme Reaction Solution	10	10	10

Table 2. Pipetting summary

ANALYSIS

Calculations

1. Determine the average fluorescence or absorbance of each standard and sample.
2. Subtract the average fluorescence or absorbance value of standard H from itself and all other standards and samples, including the catalase-containing samples, to obtain corrected standard or sample measurements (CSM) for each standard and sample.
3. Plot the CSM values of each standard as a function of the final concentration of H_2O_2 from Figure 2 on page 13 or Table 1 on page 14, respectively. See Figures 3 and 4, on pages 17 and 18, for typical standard curves.
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 substituting the CSM for each sample.

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

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

Performance Characteristics

The assay range is 0-10 µM for the fluorometric assay and 0-80 µM for the colorimetric assay.

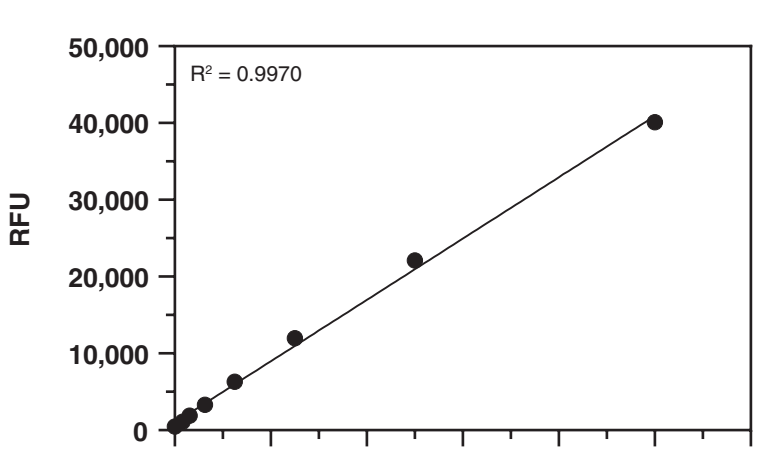


Figure 3. H_2O_2 standard curve for the fluorometric format

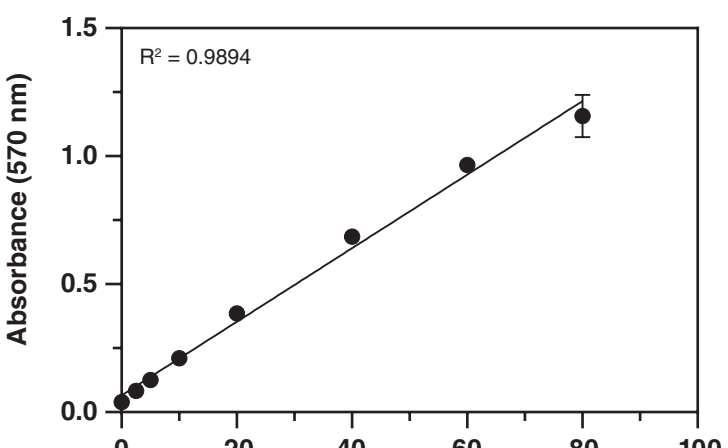


Figure 4. H_2O_2 standard curve for the colorimetric format

Sensitivity:

The LLOD is defined as a concentration two standard deviations higher than the mean zero value. The LLOD for the fluorometric assay is 0.02 µM, and the LLOD for the colorimetric assay is 0.15 µM.

The lower limit of quantification (LLOQ) is the lowest standard concentration in which absorbance (450 nm) – (1.64 x S.D.) is higher than the mean zero value of absorbance (450 nm) + (1.64 x S.D.). The LLOQ for the fluorometric assay is 0.16 µM, and the LLOQ for the colorimetric assay is 2.5 µM.

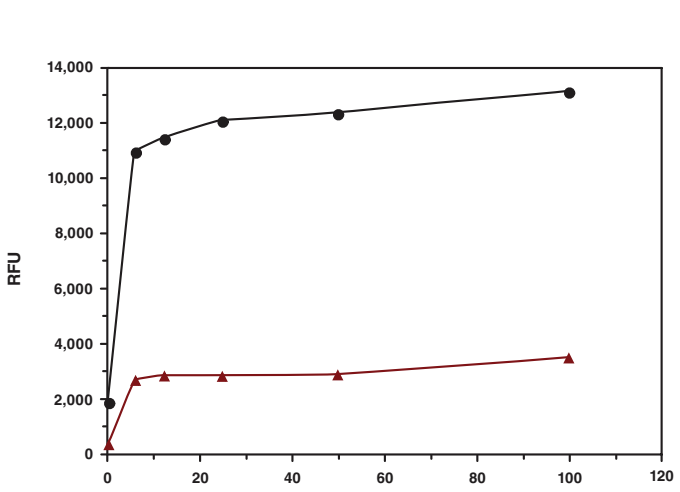


Figure 5. IFNγ- and LPS-induced H_2O_2 production in RAW 264.7 cells

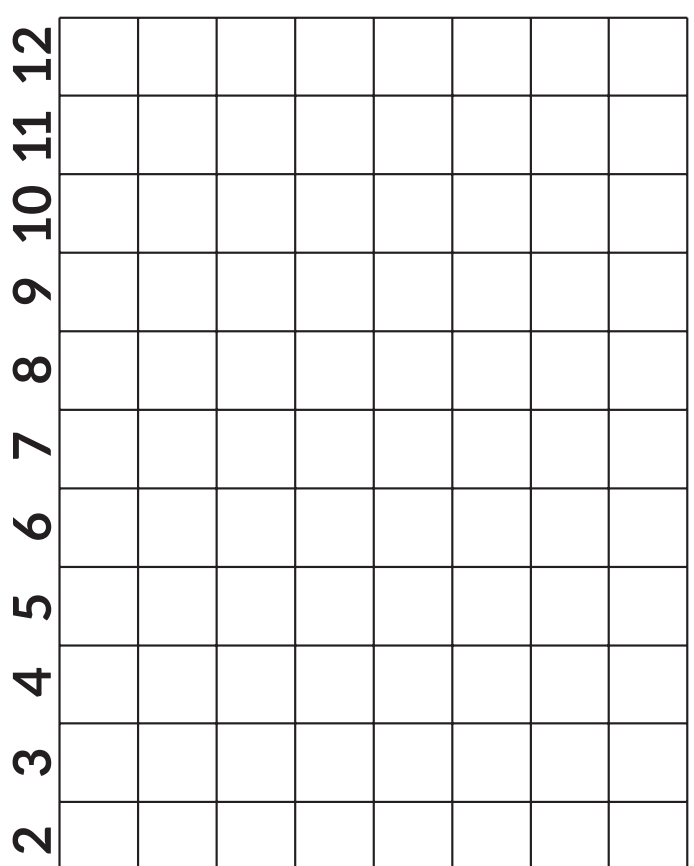
RESOURCES

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. *Bioorg. Med. Chem. Lett.* **48**, 128252 (2021).
2. Lismont, C., Revenco, I., and Fransen, M. *Int. J. Mol. Sci.* **20(15)**, 3673 (2019).
3. Rada, B. and Leto, T.L. *Contrib. Microbiol.* **15**, 164-187 (2008).
4. Zhou, M., Diwu, Z., Panchuk-Voloshina, N., *et al. Anal. Biochem.* **253**, 162-168 (1997).
5. Amundson, D.M. and Zhou, M. *J. Biochem. Biophys. Meth.* **38**, 43-52 (1999).
6. Rosen, G.M., Pou,S., Ramos, C.L. *et al. 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.