Hydrogen Peroxide (urinary) Assay Kit

Item No. 706011

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
706016	Hydrogen Peroxide	1 vial/100 µl
706012	Reagent 1	1 vial/400 µl
706014	Reagent 2	1 vial/30 ml
10005395	Catalase	2 vials
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

E-Mail: 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 at 0-4°C 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 absorbance at 595 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

The detection of reactive oxygen species (ROS) is fundamental to the elucidation of the role of these short-lived oxygen-derived products in normal cell function and signal transduction. Hydrogen peroxide (H_2O_2) is a ubiquitous, toxic metabolic by-product of aerobic respiration, oxidative stress, and oxidative injury. Left unquenched, H_2O_2 can react with ferric ions *via* the Fenton reaction to produce the hydroxyl radical, one of the most reactive and damaging free radical species known. H_2O_2 is produced both non-enzymatically and enzymatically by the superoxide dismutase enzymes, and is reduced to water by catalase and by the glutathione peroxidase/reductase system.

About This Assay

Cayman's Hydrogen Peroxide (urinary) Assay Kit is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by H_2O_2 under acidic conditions, (equation 1). The ferric ion binds to the dye xylenol orange (3,3′-*bis*[N,N-di(carboxymethyl)amino-methyl]-o-cresolsulfone-phthalein, sodium salt) to form a stable colored complex which can be measured at 595 nm, (equation 2).

(1) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + OH^-$

(2) $Fe^{3+} + XO \longrightarrow Fe^{3+}\text{-}XO$

In the presence of sorbitol, there is a substantial chain oxidation of ferrous ion, increasing the sensitivity of the assay.[1] The specificity of this reaction for H_2O_2 is demonstrated by the addition of catalase as an H_2O_2 scavenger. The sensitivity and the specificity of the Cayman assay make it well suited to measure H_2O_2 in urine, where the H_2O_2 levels in humans typically range from 1-100 µM, depending on health and dietary intake.[2-6]

PRE-ASSAY PREPARATION

Reagent Preparation

1. Working Reagent

Transfer 20 ml of Reagent 2 (Item No. 706014) to a clean glass beaker and then add 200 µl of Reagent 1 (Item No. 706012). Mix thoroughly and cover with tin foil. This is the Working Reagent to be used in the assay. This amount of reagent is sufficient for the entire 96-well plate. If not using the total plate, then adjust the amount of Reagent 1 and 2 accordingly. When stored at 4°C, the Working Reagent is stable for 12 hours.

2. Catalase

These vials (Item No. 10005395) contain a solution of bovine liver catalase in lyophilized form. Prior to use, reconstitute one vial with 250 µl of HPLC-grade water and store on ice. One reconstituted vial will be sufficient for 20 wells. Reconstitute only the number of vials needed to assay the samples. The reconstituted enzyme is stable for two hours at 4°C.

Sample Preparation

Catalase and glutathione peroxidase decrease H_2O_2 concentrations to extremely low or undetectable levels in normal tissue. The assay has not been validated in cell lysates or cell media. Plasma also contains very low H_2O_2 levels and can not be measured with this assay. However, the assay can be used to quantify H_2O_2 levels in urine. It is important to assay for H_2O_2 on fresh samples, as H_2O_2 levels will accumulate overtime upon storage at -80°C.

Urine

1. Collect urine in a clean beaker or flask and store on ice. If not assaying on the same day, freeze the sample at -80°C.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of H_2O_2 standards and samples to be measured in duplicate is given below in Figure 1. We suggest you record the contents of each well on the template sheet provided (see page 19).

	1	2	3	4	5	6	7	8	9	10	11	12
A	SA	SA	1	1	5	5	9	9	13	13	17	17
B	SB	SB	C1	C1	C5	C5	C9	C9	C13	C13	C17	C17
C	SC	SC	2	2	6	6	10	10	14	14	18	18
D	SD	SD	C2	C2	C6	C6	C10	C10	C14	C14	C18	C18
E	SE	SE	3	3	7	7	11	11	15	15	19	19
F	SF	SF	C3	C3	C7	C7	C11	C11	C15	C15	C19	C19
G	SG	SG	4	4	8	8	12	12	16	16	20	20
H	X	X	C4	C4	C8	C8	C12	C12	C16	C16	C20	C20

SA-SG - Standards A-G
1-20 - Samples
C1-20 - Samples + Catalase
X - Extra Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps to 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

- Do not expose the pipette tip to the reagent(s) already in the well.
- The final volume of the assay is 230 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time. However, a H_2O_2 standard curve must be run simultaneously with each set of samples.
- If the expected H_2O_2 concentration of the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is recommended that the samples and H_2O_2 standards be assayed at least in duplicate (triplicate recommended).
- Use the Working Reagent in the assay.
- Monitor the absorbance at 595 nm using a plate reader.

Standard Preparation

Dilute 10 µl of the H_2O_2 Standard (Item No. 706016) with 20 ml of HPLC-grade water and mix thoroughly. Remove 1 ml and dilute with 9 ml of HPLC-grade water and mix thoroughly. This is the stock H_2O_2 standard.

Take seven clean glass test tubes and mark them A-G. Add the amount of H_2O_2 stock and HPLC-grade water to each tube as described in Table 1. The diluted standards are stable for two hours at room temperature.

Tube	Stock H_2O_2 (µl)	HPLC-grade water (µl)	Final Concentration (µM)
A	0	1,000	0
B	25	975	11
C	50	950	22
D	75	925	33
E	100	900	44
F	125	875	55
G	150	850	66

Table 1. H_2O_2 standards

Performing the Assay

1. **H_2O_2 Standard Wells** - add 20 µl of standard (tubes A-G) and 10 µl of HPLC-grade water per well in the designated wells on the plate (see **Sample Plate Format**, Figure 1, page 7).
2. **Sample Wells** - Each sample should have at least two wells that will not contain catalase and two wells that will contain catalase. Add 20 µl of sample to the sample and sample + catalase wells. Then add 10 µl of catalase to the catalase wells and 10 µl of HPLC-grade water to the non-catalase wells.
3. Add 200 µl of Working Reagent to each well. Cover the plate with the plate cover and incubate on a shaker for one hour at room temperature.
4. Remove the plate cover and read the absorbance at 595 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard, sample, and sample + catalase.
2. Subtract the average absorbance of standard A from itself and from all other standards and samples including the catalase containing samples.
3. Plot the corrected absorbance of standards (from step 2 above) as a function of the final H_2O_2 concentration (µM) from Table 1. See Figure 2 (on page 13) for a typical standard curve.
4. Subtract the catalase sample absorbance from the non-catalase sample absorbance to yield the corrected sample absorbance.
5. Calculate the H_2O_2 concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

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

Performance Characteristics

Precision:

When a series of 48 urine measurements were performed on the same day, the intra-assay coefficient of variation was 5.5%. When a series of 16 urine measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.6%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 11-66 µM H_2O_2.

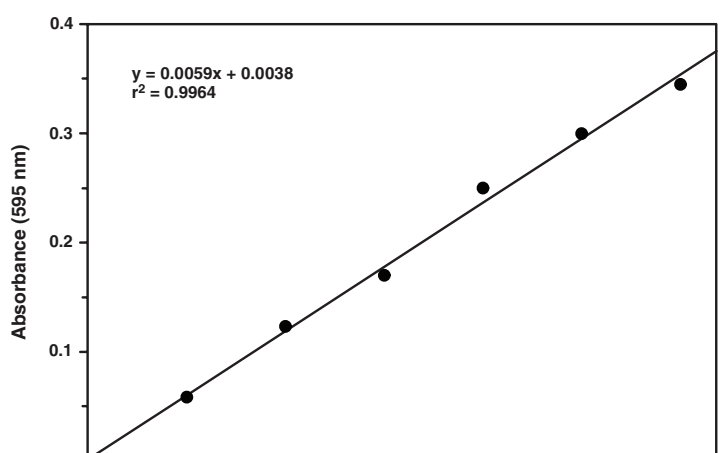


Figure 2. H_2O_2 standard curve

Interferences

The following reagents were tested in the assay for interference in the assay:

Reagent		Will Interfere (Yes or No)
Detergents	SDS (≤1%)	No
	Triton X-100 (≤0.1%)	No
	Polysorbate 20 (≤0.1%)	No
	CHAPS (100 mM)	Yes
Buffers	Tris (100 mM)	No
	HEPES (100 mM)	Yes
	MES (200 mM)	Yes
	Phosphate (100 mM)	No
Protease Inhibitors/ Chelators	Antipain (0.1 mg/ml)	Yes
	PMSF (1 mM)	Yes
	Leupeptin (≤1 mg/ml)	No
	Chymostatin (1 mg/ml)	Yes
	EGTA (≤5 mM)	No
	EDTA (1 mM)	Yes
Sugars	Mannitol (≤100 mM)	No
	Sucrose (≤100 mM)	No
	Glucose (≤100 mM)	No
Solvents	Ethanol (10 µl)	Yes
	Methanol (10 µl)	Yes
	Dimethylsulfoxide (10 µl)	Yes
Others	Glutathione (≤1 mM)	No
	Glycerol (≤5%)	No
	BSA (≤0.1%)	No
	BHT (1%)	Yes

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	A. The H_2O_2 concentration was too low B. The sample was too dilute	Re-assay at a lower dilution
Absorbance over 1.2 in the sample wells	Too much H_2O_2 was added to well(s)	Dilute samples with HPLC-grade water and re-assay
Catalase did not eliminate the sample absorbance	A. There is not any H_2O_2 present in the sample B. The catalase has deteriorated	A. Reconstitute a new catalase vial and re-assay B. Re-assay the sample at a lower dilution

References

1. Deiana, L., Carru, C., Pes, G., *et al.* Spectrophotometric measurement of hydroperoxides at increased sensitivity by oxidation of Fe^{2+} in the presence of xylenol orange. *Free Rad. Res.* **31**, 237-244 (1999).
2. Halliwell, B., Clement, M.V., and Long, L.H. Hydrogen peroxide in the human body. *FEBS Lett.* **486**, 10-13 (2000).
3. Hiramoto, K., Kida, T., and Kikugawa, K. Increased urinary hydrogen peroxide levels caused by coffee drinking. *Biol. Pharm. Bull.* **25(11)**, 1467-1471 (2002).
4. Long, L.H. and Halliwell, B. Coffee drinking increases levels of urinary hydrogen peroxide detected in healthy human volunteers. *Free Radic. Res.* **32**, 463-467 (2000).
5. Long, L.H., Evans, P.J., and Halliwell, B. Hydrogen peroxide in human urine: implications for antidoxidant defense and redox regulation. *Biochem. Biophys. Res. Commun.* **262**, 605-609 (1999).
6. Kuge, N., Kohzuki, M., and Sato, T. Relation between Natriuresis and urinary excretion of hydrogen peroxide. *Free Radic. Res.* **30**, 119-123 (1999).

Related Products

Aconitase Assay Kit - Item No. 705502
Aconitase Fluorometric Assay Kit - Item No. 700600
Antioxidant Assay Kit - Item No. 709001
Ascorbate Assay Kit - Item No. 700420
Catalase Assay Kit - Item No. 707002
Glutathione Assay Kit - Item No. 703002
Glutathione Peroxidase Assay Kit - Item No. 703102
Glutathione Reductase Assay Kit - Item No. 703202
Glutathione S-Transferase Assay Kit - Item No. 703302
8-Isoprostane EIA Kit - Item No. 516351
Lipid Hydroperoxide (LPO) Assay Kit - Item No. 705002
Lipid Hydroperoxide (LPO) Assay Kit (96 well) - Item No. 705003
Protein Carbonyl Assay Kit - Item No. 10005020
Protein Carbonyl Fluorometric Assay Kit - Item No. 700490
Superoxide Dismutase Assay Kit - Item No. 706002
TBARS Assay Kit - Item No. 10009055
L-Theanine Fluorometric Assay Kit - Item No. 700570
Thioredoxin Reductase Assay Kit - Item No. 10007892
Xanthine Oxidase Assay Kit - Item No. 10010895

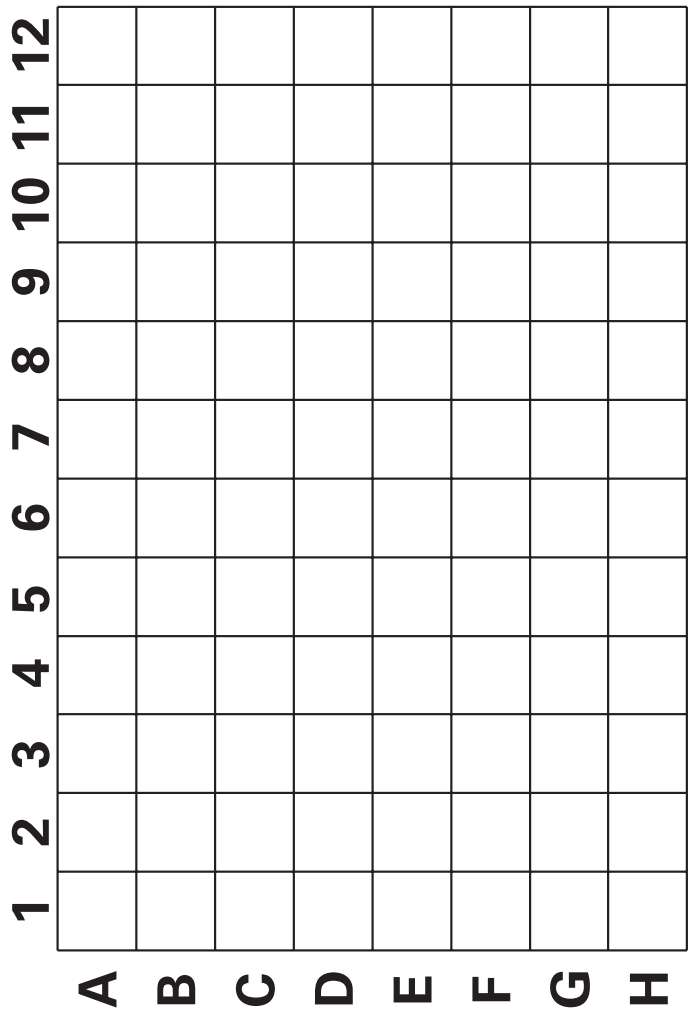
Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



NOTES

This document is copyrighted. All rights are reserved. This document may not, in whole or part, be copied, photocopied, reproduced, translated, or reduced to any electronic medium or machine-readable form without prior consent, in writing, from Cayman Chemical Company.

©10/03/2011, Cayman Chemical Company, Ann Arbor, MI, All rights reserved. Printed in U.S.A.