Hydrogen Peroxide Colorimetric Detection Kit

Cat#: orb511100 (User Manual)

1. Introduction

Hydrogen peroxide was first described in 1818 by Louis Jacques Thénard. Today, industrially, hydrogen peroxide is manufactured almost exclusively by the autoxidation of a 2-alkyl-9,10-dihydroxyanthracene to the corresponding 2-alkyl anthraquinone in the Riedl-Pfleiderer or anthraquinone process.

In biological systems incomplete reduction of O2 during respiration produces superoxide anion (O2-·), which is spontaneously or enzymatically dismutated by superoxide dismutase to H2O2. Many cells produce low levels of O2-· and H2O2 in response to a variety of extracellular stimuli, such as cytokines (TGF-ß1, TNF-a, and various interleukins), peptide growth factors (PDGF, EGF, VEGF, bFGF, and insulin), the agonists of heterotrimeric G protein–coupled receptors (GPCR) such as angiotensin II, thrombin, lysophosphatidic acid, sphingosine 1-phosphate, histamine, and bradykinin, and by shear stress1. The addition of exogenous H2O2 or the intracellular production in response to receptor stimulation affects the function of various proteins, including protein kinases, protein phosphatases, transcription factors, phospholipases, ion channels, and G proteins. In 1894, Fenton2 described the oxidation of tartaric acid by Fe2+ and H2O2. H2O2 and O2 may participate in the production of singlet oxygen and peroxynitrite and the generation of these species may be concurrent with reactions involving iron, and under some circumstances they might be important contributors to H2O2 toxicity3,4.

A substantial portion of H2O2 lethality involves DNA damage by oxidants generated from iron-mediated Fenton reactions5,6. Damage by Fenton oxidants occurs at the DNA bases or at the sugar residues. Sugar damage is initiated by hydrogen abstraction from one of the deoxyribose carbons, and the predominant consequence is eventual strand breakage and base release7,8.

ICT's Hydrogen Peroxide Colorimetric Detection Kit is designed to quantitatively measure H2O2 in a variety of samples. This kit is validated for use in fresh urine, buffers, and tissue culture media (TCM). It is species independent. Please read the complete kit insert before performing this assay. A hydrogen peroxide standard is provided to generate a standard curve for the assay. All samples should be read off the standard curve. Samples are mixed with the Colorimetric H2O2 Detection Substrate and the reaction is initiated by addition of horseradish peroxidase (HRP). The reaction is incubated at room temperature for 15 minutes. The HRP reacts with the substrate in the presence of hydrogen peroxide to convert the colorless substrate into a colored product. The pink product is read at 560 nm. Increasing levels of H2O2 cause a linear increase in color.

This kit is for research use only and is not for use in diagnostic procedures.

2. Kit Contents

- 2 Clear Half-Area 96-well Microwell Plates #267

• 1 vial Hydrogen Peroxide Standard (220 µL) #6604: Hydrogen Peroxide at 1,000 µM in a special stabilizing solution (Section 12).
• 1 bottle Assay Buffer Concentrate, 5X (25 mL) #6605: A 5X buffer concentrate containing detergents and stabilizers (Section 10).
• 1 vial Colorimetric H2O2 Detection Substrate (5 mL) #6608: A solution of the substrate in a special stabilizing buffer.
• 1 vial Horseradish Peroxidase Concentrate, 50X (120 µL) #6609:
A 50X concentrated solution of HRP in a special stabilizing solution (Section 11).

3. Required Materials

• Repeater pipet with disposable tips capable of dispensing 25 µL.

4. Storage

All components of this kit should be stored at 4°C until the expiration date.

5. Safety Data Sheets (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

6. Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product. The supplied hydrogen peroxide standard consists of a very dilute H2O2 solution (< 0.001%).

7. Detection Equipment

• 96-well plate reader capable of reading absorbance at 560 nm
(Acceptable Range 540-580 nm.). Set plate parameters for a 96-well Corning Costar 3695 plate.
• Software for converting colorimetric intensity readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

8. Sample Types and Preparation

Samples that need to be stored after collection should be stored at -70°C or lower, preferably after being frozen in liquid nitrogen. Urine samples can be used after being diluted ≥ 1:10. This assay has been validated for buffer and media samples.

Table 1: HRP Dilution			
1/2 Plate		1 Plate	2 Plates
HRP Stock Concentrate, 50X	30 µL	60 µL	110 µL
Assay Buffer	1.47 mL	2.94 mL	5.39 mL
Total Volume	1.5 mL	3 mL	5.5 mL

Table 2: Standard Preparation						
Std 1		Std 2	Std 3	Std 4	Std 5	Std 6
Assay Buffer (µL)	450	200	200	200	200	200
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Vol of Addition (µL)	50	200	200	200	200	200
Final Conc (µM)	100	50	25	12.5	6.25	3.125

9. Sample Preparation

Dilute samples ≥ 1:10 with Assay Buffer prior to running in the assay.

10. Assay Buffer Preparation

Dilute Assay Buffer Concentrate, 5X (#6605) 1:5 by adding one part of the concentrate to four parts of deionized water. Diluted Assay Buffer is stable at 4°C for 3 months.

11. Horseradish Peroxidase (HRP) Preparation

Dilute the Horseradish Peroxidase Concentrate, 50X (#6609) 1:50 with Assay Buffer. See Table 1: HRP Dilution; for example if using 1 plate:

1. Measure 2.94 mL Assay Buffer.
2. Add 60 µL Horseradish Peroxidase Concentrate, 50X.
3. Mix. The total volume is 3 mL for 1 plate.

12. Standard Preparation

1. Hydrogen Peroxide Standards are prepared by labeling six tubes as #1 through #6.
2. Briefly vortex to mix the vial of Hydrogen Peroxide Standard (#6604).
3. Pipet 450 µL of Assay Buffer (Section 10) into tube #1 and 200 µL into tubes #2 to #6.
4. Carefully add 50 µL of the Hydrogen Peroxide Standard to tube #1 and vortex completely.
5. Take 200 µL of the solution in tube #1 and add it to tube #2 and vortex completely.
6. Repeat this for tubes #3 through #6.
7. The concentration of H2O2 in tubes 1 through 6 will be 100, 50, 25, 12.5, 6.25, and 3.125 µM (See Table 2: Standard Preparation).

• Use all Standards within 2 hours of preparation.

Table 3: Typical Data

Always run your own standard curves for calculation of results. Do not use these data. Conversion Factor: 100 µM of Hydrogen Peroxide is equivalent to 3.4 µg/mL.

Sample	Mean OD	Net OD	H2O2 Conc. (µM)
Zero	0.075	0	0
Standard 1	1.820	1.745	100
Standard 2	1.062	0.987	50
Standard 3	0.569	0.494	25
Standard 4	0.341	0.266	12.5
Standard 5	0.190	0.115	6.25
Standard 6	0.146	0.071	3.125
Sample 1	1.453	1.378	76.7
Sample 2	0.434	0.359	18.3

Figure 1: Typical Standard Curve

Always run your own standard curve for calculation of results.
Do not use this data.

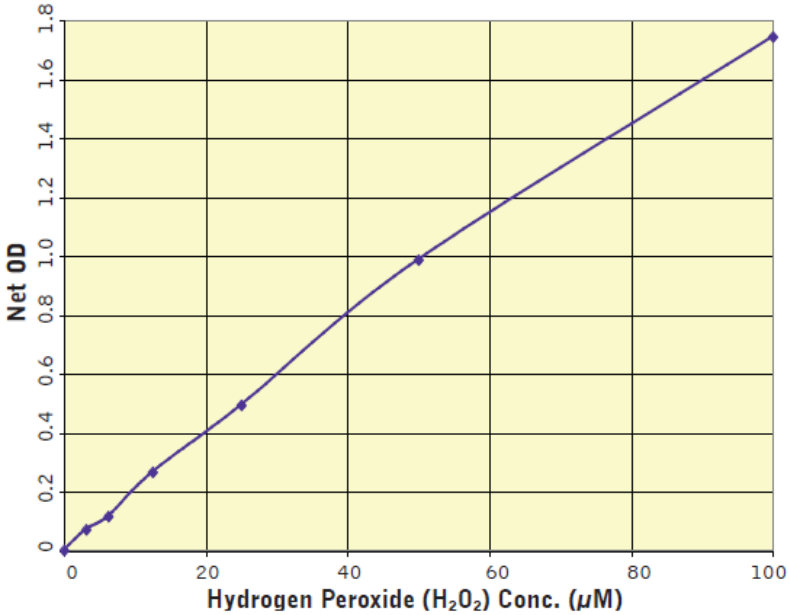
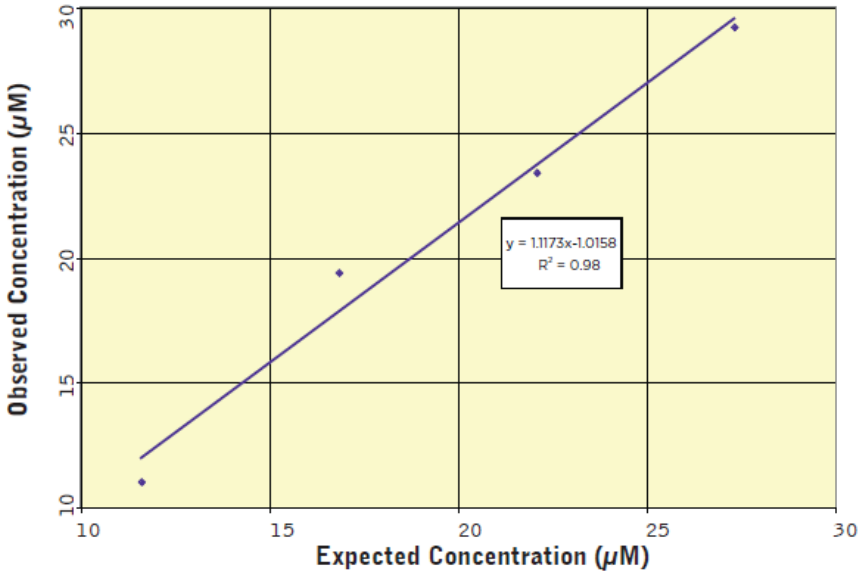


Table 4: Sample Linearity Data

High Urine	Low Urine	Observed Conc. (µM)	Expected Conc. (µM)	% Recovery
80%	20%	29.2	27.3	106.8
60%	40%	23.4	22.1	105.8
40%	60%	19.4	16.9	114.9
20%	80%	11.0	11.6	94.9
			Mean Recovery	105.6%

Figure 2: Sample Linearity Curve



13. Assay Protocol

1. Use the plate layout sheet on the back page (Figure 3) to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3695 plate.
2. Pipet 50 µL of samples or appropriate standards into duplicate wells in the plate.
3. Pipet 50 µL of Assay Buffer into duplicate wells as the zero standard.
4. Add 25 µL of Colorimetric H2O2 Detection Substrate (#6608) to each well using a repeater pipet.
5. Initiate the reaction by adding 25 µL of the HRP Preparation (Section 11) to each well using a repeater pipet.
6. Incubate at room temperature for 15 minutes.
7. Read the plate at 560 nm (Acceptable Range 540-580 nm).

14. Calculation of Results

1. Set up 96-well visible absorbance plate reader software to automatically subtract the mean absorbance of the zero 0-well standard wells from all standard and sample OD readings. This 0-well OD subtraction can be performed after the plate reader has completed the OD scan of the plate.
2. Manually or via plate reader software, calculate the average OD reading for each of the duplicate standards and samples.
3. Create a standard curve using software-derived linear regression analysis. Select the four-parameter logistic curve (4PLC) fitting option for this step (see Figure 1: Typical Standard Curve).

4. Multiply curve derived sample concentration values by initial sample dilution factor to obtain the H2O2 concentration present in neat samples.

15. Validation Data: Sensitivity

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the zero and standard #6 (low standard). The theoretical detection limit in Assay Buffer was determined at two (2) standard deviations from the zero along the standard curve.
Sensitivity was determined as 1.83 µM. This is equivalent to 91.3 pmol (3.10 ng) H2O2 per well.

16. Validation Data: Limit of Detection

The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the zero and a low concentration human sample.
The Limit of Detection was determined as 1.96 µM. This is equivalent to 98.0 pmol (3.33 ng) H2O2 per well.

17. Linearity

Linearity was determined by taking two diluted human urine samples with known H2O2 concentrations and mixing them in the ratios given; see Table 4: Sample Linearity Data. The measured concentrations were compared to the expected values based on the ratios used. Figure 2 illustrates a linear plot of observed versus expected concentrations in a human sample.

Table 5: Intra Assay Precision
Three buffer samples were run in replicates of 20 in an assay. The mean and precision of the calculated concentrations were:

Sample	H2O2 Conc. (µM)	%CV
1	82.2	2.1
2	53.1	2.4
3	19.4	5.9

Table 6: Inter Assay Precision
Three buffer samples were run in duplicate in twelve assays run over multiple days by three operators. The mean and precision of the calculated concentrations were:

Sample	H2O2 Conc. (µM)	%CV
1	79.9	3.7
2	49.5	4.5
3	18.4	4.3

18. References

1. Rhee, S. G., Bae, Y. S., Lee, S. R. & Kwon, J. Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. Sci STKE 2000, pe1, doi:10.1126/stke.2000.53.pe1 (2000).
2. Fenton, H. J. H. LXXIII. - Oxidation of Tartaric Acid in the presence of Iron. J. Chem. Soc., Trans., 65, 899-910, doi:10.1039/CT8946500899 (1894)
3. Sies, H. Damage to plasmid DNA by singlet oxygen and its protection. Mutat Res 299, 183-191 (1993).

4. Squadrito, G. L. & Pryor, W. A. The formation of peroxynitrite in vivo from nitric oxide and superoxide. Chem Biol Interact 96, 203-206 (1995).
5. Imlay, J. A. & Linn, S. DNA damage and oxygen radical toxicity. Science 240, 1302-1309 (1988).
6. Mello-Filho, A. C. & Meneghini, R. Iron is the intracellular metal involved in the production of DNA damage by oxygen radicals. Mutat Res 251, 109-113 (1991).
7. von Sonntag, C. The chemical basic of radiation biology. 238-249 (Taylor & Francis, 1987).
8. Henle, E. S., Roots, R., Holley, W. R. & Chatterjee, A. DNA strand breakage is correlated with unaltered base release after gamma irradiation. Radiat Res 143, 144-150 (1995).

Figure 3: Plate Layout Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												