Hydrogen Peroxide Fluorescent Detection Kit

Kit #9131, 2 plates

RESEARCH USE ONLY

1. Introduction

Hydrogen peroxide was first described in 1818 by Louis Jacques Thénard. Today, hydrogen peroxide is industrially 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 O_2 during respiration produces superoxide anion ($O_2^{-}\cdot$), which is spontaneously or enzymatically dismutated by superoxide dismutase to H_2O_2. Many cells produce low levels of $O_2^{-}\cdot$ and H_2O_2 in response to a variety of extracellular stimuli, such as cytokines (TGF-ß1, TNF-α, 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 stress[1]. The addition of exogenous H_2O_2 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, Fenton[2] described the oxidation of tartaric acid by Fe^{2+} and H_2O_2. H_2O_2 and O_2 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 H_2O_2 toxicity[3,4].

A substantial portion of H_2O_2 lethality involves DNA damage by oxidants generated from iron-mediated Fenton reactions[5,6]. 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 release[7,8].

ICT's Hydrogen Peroxide Fluorescent Detection Kit is designed to quantitatively measure H_2O_2 in a variety of samples. This kit is validated for use in fresh urine, buffers, and tissue culture media (TCM) samples. 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 Fluorescent H_2O_2 Detection Substrate and the reaction is initiated by addition of horseradish peroxidase. The reaction is incubated at room temperature for 15 minutes. HRP is oxidized by hydrogen peroxide present in the sample. Oxidized HRP then reacts with the substrate to convert the colorless substrate into the fluorescent form. The fluorescent product is read at 590 nm with excitation at 570 nm. Increasing levels of H_2O_2 cause a linear increase in fluorescent product. This kit is for research use only and is not for use in diagnostic procedures.

Learn more about all of ICT's products at www.immunochemistry.com or call 1-800-829-3194.

2. Kit Contents

- 2 black half-area polystyrene 96-well microtiter plates #268
- 1 vial of Hydrogen Peroxide Standard (220 µL) #6603: Hydrogen Peroxide at 100 µM in a special stabilizing solution (Section 12).
- 1 bottle of Assay Buffer Concentrate (25 mL) #6605: A 5X buffer concentrate containing detergents and stabilizers (Section 10).
- 1 vial of Fluorescent H_2O_2 Detection Substrate (5 mL) #6606: A solution of the substrate in a special stabilizing buffer.
- 1 vial of Horseradish Peroxidase Concentrate (60 µL) #6607: A 100X concentrated solution of HRP in a special stabilizing solution (Section 11).

3. Required Materials

- Repeater pipet with disposable tips capable of accurately dispensing 25 µL.

4. Storage

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

5. MSDS

Material 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 H_2O_2 solution.

7. Detection Equipment

The assay can be analyzed with a fluorescence plate reader:

- 96-well plate reader capable of reading fluorescence at 580-590 nm with excitation at 570-580 nm. Set plate parameters for a 96-well Corning® Costar 3694 plate.
- Software for converting fluorescent 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
Horseradish Peroxidase Concentrate	15 µL	30 µL	55 µL
Assay Buffer	1.485 mL	2.97 mL	5.445 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	Std 7
Assay Buffer (µL)	450	200	200	200	200	200	200
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Vol of Addition (µL)	50	200	200	200	200	200	200
Final Conc (µM)	10	5	2.5	1.25	0.625	0.313	0.1569

9.Sample Preparation

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

10. Assay Buffer Preparation

Dilute Assay Buffer Concentrate (#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 Horseradish Peroxidase Concentrate (#6607) 1:100 with Assay Buffer. See Table 1: HRP Dilution; for example if using 1 plate:

1. Measure 2.97 mL Assay Buffer.
2. Add 30 µL Horseradish Peroxidase Concentrate.
3. Mix. The total volume is 3 mL for 1 plate.

12. Standard Preparation

1. Hydrogen peroxide standards are prepared by labeling seven tubes as #1 through #7.
2. Briefly vortex to mix the vial of H_2O_2 Standard (#6603).
3. Pipet 450 µL of Assay Buffer into tube #1 and 200 µL into tubes #2 to #7.
4. Carefully add 50 µL of the H_2O_2 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 #7.
7. The concentration of H_2O_2 in tubes 1 through 7 will be 10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µM (See Table 2: Standard Preparation).

• Use all Standards within 2 hours of preparation.

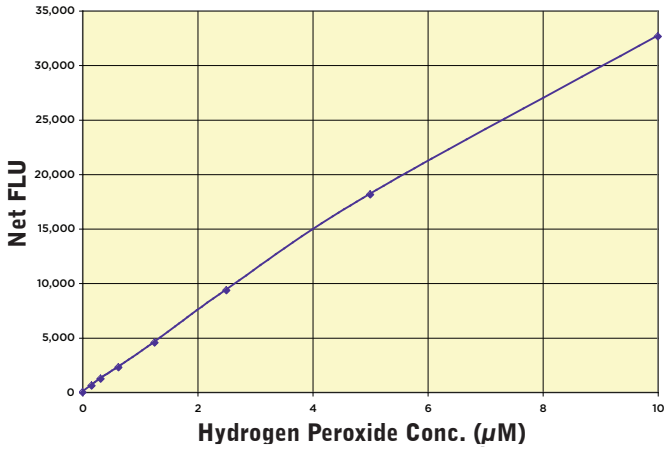
Table 3: Typical Data

Always run your own standard curves for calculation of results. Do not use this data. Conversion Factor: 100 nM of hydrogen peroxide is equivalent to 3.4 ng/mL.

Sample	Mean FLU	Net FLU	H_2O_2 Conc. (µM)
Zero	3,782	0	0
Standard 1	36,417	32,635	10
Standard 2	21,919	18,137	5
Standard 3	13,134	9,352	2.5
Standard 4	8,333	4,551	1.25
Standard 5	6,072	2,290	0.625
Standard 6	5,031	1,249	0.313
Standard 7	4,398	616	0.156
Sample 1	6,578	2,796	0.76
Sample 2	24,680	20,898	5.85

Figure 1: Typical Standard Curve

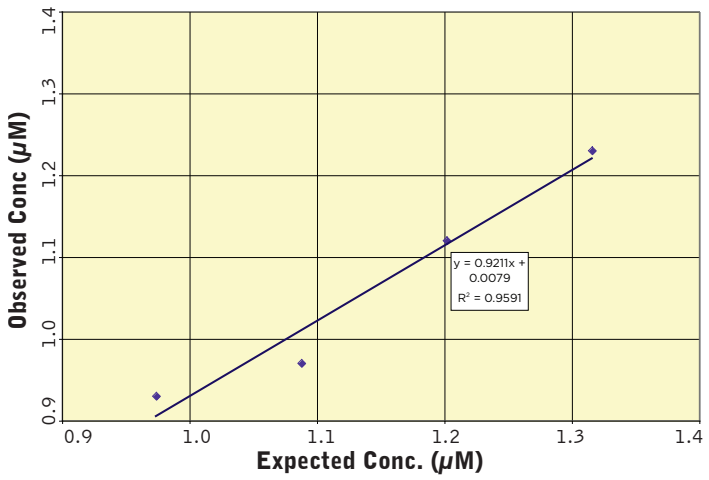
Always run your own standard curves for calculation of results. Do not use this data.



ImmunoChemistry TECHNOLOGIES LLC

Table 4: Sample Linearity Data				
High H_2O_2 RPMI	Low H_2O_2 RPMI	Observed Conc. (μM)	Expected Conc. (μM)	% Recovery
80%	20%	1.23	1.32	93.5
60%	40%	1.12	1.20	93.2
40%	60%	0.97	1.09	89.2
20%	80%	0.93	0.97	95.5
			Mean Recovery	**92.8%**

Figure 2: Sample Linearity Curve
Always run your own standard curves for calculation of results.
Do not use this data.



13. Assay Protocol

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3694 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 Fluorescent H_2O_2 Detection Substrate (#6606) 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. Replace tips each time to minimize sample carry over.
6. Incubate at room temperature for 15 minutes.
7. Read the fluorescent emission at 585 $\pm$ 5 nm with excitation at 575 $\pm$ 5 nm. Please contact your plate reader manufacturer for suitable filter sets.

14. Calculation of Results

1. Set up fluorescence plate reader software to subtract mean of the zero 0-well FLU values from all standard and sample fluorescence readings. This 0-well FLU subtraction step can be performed after the plate reader has completed the fluorescence scan of the plate.
2. Manually or via plate reader software, calculate the average FLU 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 H_2O_2 concentration present in neat samples.

15. Validation Data: Sensitivity

Sensitivity was calculated by comparing the FLUs for twenty wells run for each of the zero and standard #7 (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 0.038 μM. This is equivalent to 1.9 pmol (64.6 pg) H_2O_2 per well.

16. Validation Data: Limit of Detection

The limit of detection in a human sample was determined in a similar manner by comparing the FLUs for twenty wells run for each of the zero and a low concentration human sample.

The Limit of Detection was determined as 0.052 μM. This is equivalent to 2.6 pmol (88.4 pg) H_2O_2 per well.

17. Linearity

Linearity was determined by taking two RPMI-1640 media samples with known H_2O_2 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 concentration values.

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	H_2O_2 Conc. (μM)	%CV
1	6.27	3.6
2	3.21	3.8
3	0.98	5.7

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

Sample	H_2O_2 Conc. (μM)	%CV
1	5.86	4.3
2	3.00	7.0
3	0.88	12.1

18. References

1. Rhee, S.G., et al. Hydrogen peroxide: A key messenger that modulates protein phosphorylation through cysteine oxidation. Science's stke. (2000). Available at: http://stke.sciencemag.org/cgi/content/abstract/sigtrans;2000/53/pe1
2. Fenton, HJH. J. Chem. Soc. (Lond.) 65, 899–910. (1894).
3. Sies, H. Mutat. Res. 299, 183–191. (1993).
4. Squadrito, G.L. and 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. and Linn, S. DNA damage and oxygen radical toxicity. Science 240, 1302–1309. (1988).
6. Mello-Filho, A.C. and 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. In: The Chemical Basis of Radiation Biology. pp. 238–249, Taylor and Francis, New York. (1987).
8. Henle, E.S. et al. DNA strand breakage is correlated with unaltered base release after gamma irradiation. Radiat. Res. 143, 144–150. (1995).

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

Thank you for using this kit. If you have any questions or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788, or send an email to help@immunochemistry.com.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

ImmunoChemistry TECHNOLOGIES LLC

ImmunoChemistry Technologies, LLC
9401 James Avenue South #155
Bloomington, MN 55431 USA

toll-free: 1-800-829-3194
local: 952-888-8788
fax: 952-888-8988
www.immunochemistry.com