KAMIYA BIOMEDICAL COMPANY

Hydrogen Peroxide Fluorescent Detection Kit

For detection in fresh urine, buffers, and tissue culture media

Cat. No. KT-733

For Research Use Only. Not for Use in Diagnostic Procedures.

PRODUCT INFORMATION
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Background

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 stress. 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, Fenton 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 toxicity.

A substantial portion of H2O2 lethality involves DNA damage by oxidants generated from iron-mediated Fenton reactions. 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.

PRINCIPLE

The K-ASSAY® Hydrogen Peroxide Fluorescent Detection Kit is designed to quantitatively measure H2O2 in a variety of samples. Please read the complete kit insert before performing this assay. A hydrogen peroxide calibrator is provided to generate a calibration curve for the assay and all samples should be read off the calibration curve. Samples are mixed with the Fluorescent Substrate and the reaction initiated by addition of horseradish peroxidase. 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 fluorescent product. The fluorescent product is read at 590 nm with excitation at 570 nm. Increasing levels of H2O2 cause a linear increase in fluorescent product.

COMPONENTS

- Black 96 Well Half Area Plates — 2 Plates
- Hydrogen Peroxide Calibrator — 220 µL
- Fluorescent Detection Reagent — 5 mL
- Assay Buffer Concentrate — 25 mL
- Horseradish Peroxidase Concentrate — 60 µL

Storage

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

Other Materials Required

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

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 calibrator contains very dilute H2O2.

Sample Types
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.

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

Reagent Preparation

Assay Buffer
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable at 4°C for 3 months.

Horseradish Peroxidase (HRP) Preparation
Dilute the HRP Stock solution 1:100 with Assay Buffer using the table below:

HRP Dilution Table

	1/2 Plate	One Plate	Two Plates
HRP Stock	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

Calibrator Preparation
Hydrogen Peroxide Calibrators are prepared by labeling seven tubes as #1 through #7. Briefly vortex to mix the vial of H2O2 calibrator. Pipet 450 µL of Assay Buffer into tube #1 and 200 µL into tubes #2 to #7. Carefully add 50 µL of the H2O2 Calibrator to tube #1 and vortex completely. Take 200 µL of the solution in tube #1 and add it to tube #2 and vortex completely. Repeat this for tubes #3 through #7. The concentration of H2O2 in tubes 1 through 7 will be 10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µM.

Use all Calibrators within 2 hours of 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

ASSAY PROTOCOL

1. Use the plate layout sheet on the last page of the insert to aid in proper sample and calibrator identification. Set plate parameters for a 96-well Corning Costar 3694 plate.
2. Pipet 50 µL of samples or appropriate calibrators into duplicate wells in the plate.

3. Pipet 50 µL of Assay Buffer into duplicate wells as the Zero calibrator.

4. Add 25 µL of Fluorescent Substrate to each well using a repeater pipet.

5. Initiate the reaction by adding 25 µL of the HRP Preparation to each well using a repeater pipet.

6. Incubate at room temperature for 15 minutes.

7. Read the fluorescent emission at 585 ± 5 nm with excitation at 575 ± 5 nm. Please contact your plate reader manufacturer for suitable filter sets.

Calculation of Results

Average the duplicate FLU readings for each calibrator and sample. Create a calibration curve by reducing the data using computer software capable of generating a four-parameter logistic curve (4PLC) fit, after subtracting the mean FLUs for the Zero wells. The sample concentrations obtained should be multiplied by the dilution factor to obtain neat sample values.

Typical Data

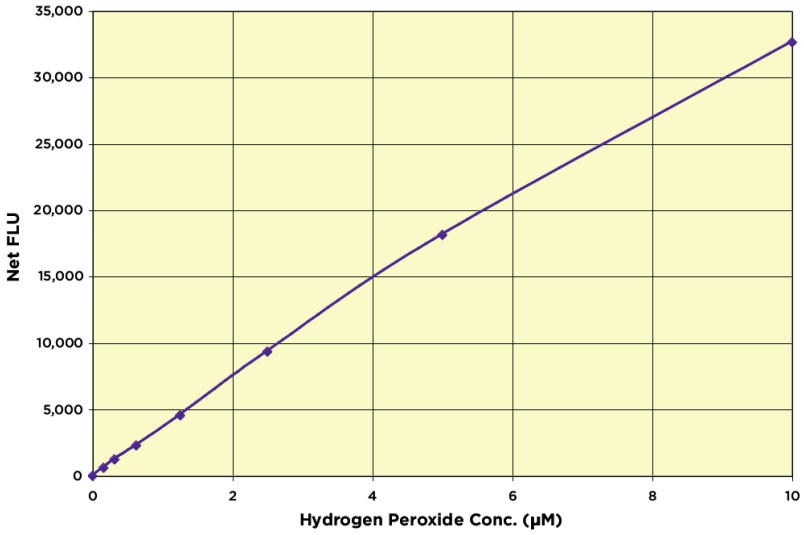
Always run your own calibration curve for calculation of results.

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

Do not use this data.

Conversion Factor: 100 nM of Hydrogen Peroxide is equivalent to 3.4 ng/mL.

Typical Calibration Curve



Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the FLUs for twenty wells run for each of the zero and calibrator #7. The detection limit was determined at two (2) standard deviations from the zero along the calibration curve.
Sensitivity was determined as 0.038 µM. This is equivalent to 1.9 pmol (64.6 pg) H2O2 per well

The Limit of Detection 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) H2O2 per well

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

Inter Assay Precision

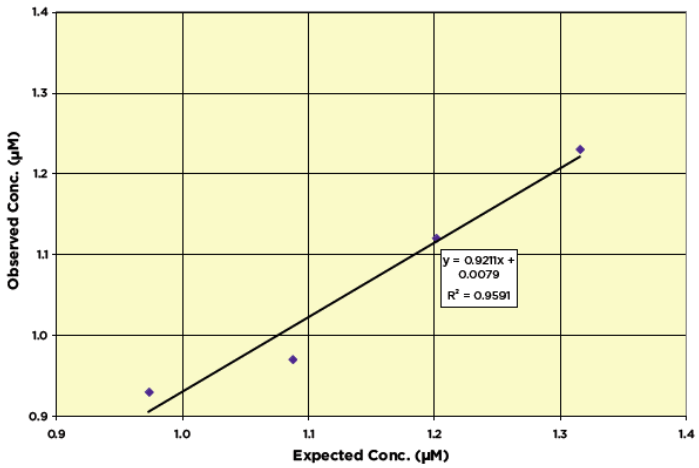
Three buffer samples were run in duplicates in fourteen assays run over multiple days by three operators. The mean and precision of the calculated concentrations were:

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

Linearity

Linearity was determined by taking two RPMI-1640 media samples with known H2O2 concentrations and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

High RPMI	Low 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%**



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FOR RESEARCH USE ONLY

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