KAMIYA BIOMEDICAL COMPANY

Hydrogen Peroxide Colorimetric Detection Kit

For the quantitative determination of H_2O_2 in fresh urine, buffers and TCM

Cat. No. KT-732

For Research Use Only.

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 O_2 during respiration produces superoxide anion (O_2-·), which is spontaneously or enzymatically dismutated by superoxide dismutase to H_2O_2. Many cells produce low levels of O_2-· and H_2O_2 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 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 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.

A substantial portion of H_2O_2 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 Hydrogen Peroxide Colorimetric Detection Kit is designed to quantitatively measure H_2O_2 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 Colorimetric 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 colored product. The pink product is read at 560 nm. Increasing levels of H_2O_2 cause a linear increase in color.

COMPONENTS

Clear 96 well Half Area Plates 2 Plates
Corning Costar Plate 3695.

Hydrogen Peroxide Calibrator 220 µL
Hydrogen Peroxide at 1,000 µM in a special stabilizing solution.

Assay Buffer Concentrate 25 mL
A 5X buffer concentrate containing detergents and stabilizers.

Colorimetric Substrate 5 mL
A solution of the substrate in a special stabilizing buffer.

Horseradish Peroxidase Concentrate 120 µL
A 50X concentrated solution of HRP in a special stabilizing solution.

STORAGE

All components of this kit should be stored at 4℃ 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 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.

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 H_2O_2.

SAMPLE TYPES AND PREPARATION

Samples that need to be stored after collection should be stored at -70℃ 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 Preparation

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℃ for 3 months.

Horseradish Peroxidase (HRP) Preparation

Dilute the HRP Stock solution 1:50 with Assay Buffer using the table below:

HRP Dilution Table

	1/2 Plate	One Plate	Two Plates
HRP Stock	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

Calibrator Preparation

Hydrogen Peroxide Calibrators are prepared by labeling six tubes as #1 through #6. Briefly vortex to mix the vial of H_2O_2 calibrator. Pipet 450 µL of Assay Buffer into tube #1 and 200 µL into tubes #2 to #6. Carefully add 50 µL of the H_2O_2 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 #6. The concentration of H_2O_2 in tubes 1 through 6 will be 100, 50, 25, 12.5, 6.25, and 3.125 µM.

Use all Calibrators within 2 hours of 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

ASSAY PROTOCOL

Use the plate layout sheet on the back page to aid in proper sample and calibrator identification. Set plate parameters for a 96-well Corning Costar 3695 plate.

1. Pipet 50 µL of samples or appropriate calibrators into duplicate wells in the plate.
2. Pipet 50 µL of Assay Buffer into duplicate wells as the Zero calibrator.
3. Add 25 µL of Colorimetric Substrate to each well using a repeater pipet.
4. Initiate the reaction by adding 25 µL of the HRP Preparation to each well using a repeater pipet.
5. Incubate at room temperature for 15 minutes.
6. Read the plate at 560 nm (Acceptable Range 540-580 nm.).

CALCULATION OF RESULTS

Average the duplicate OD 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 ODs for the Zero wells. The sample concentrations obtained should be multiplied by the dilution factor to obtain neat sample values.

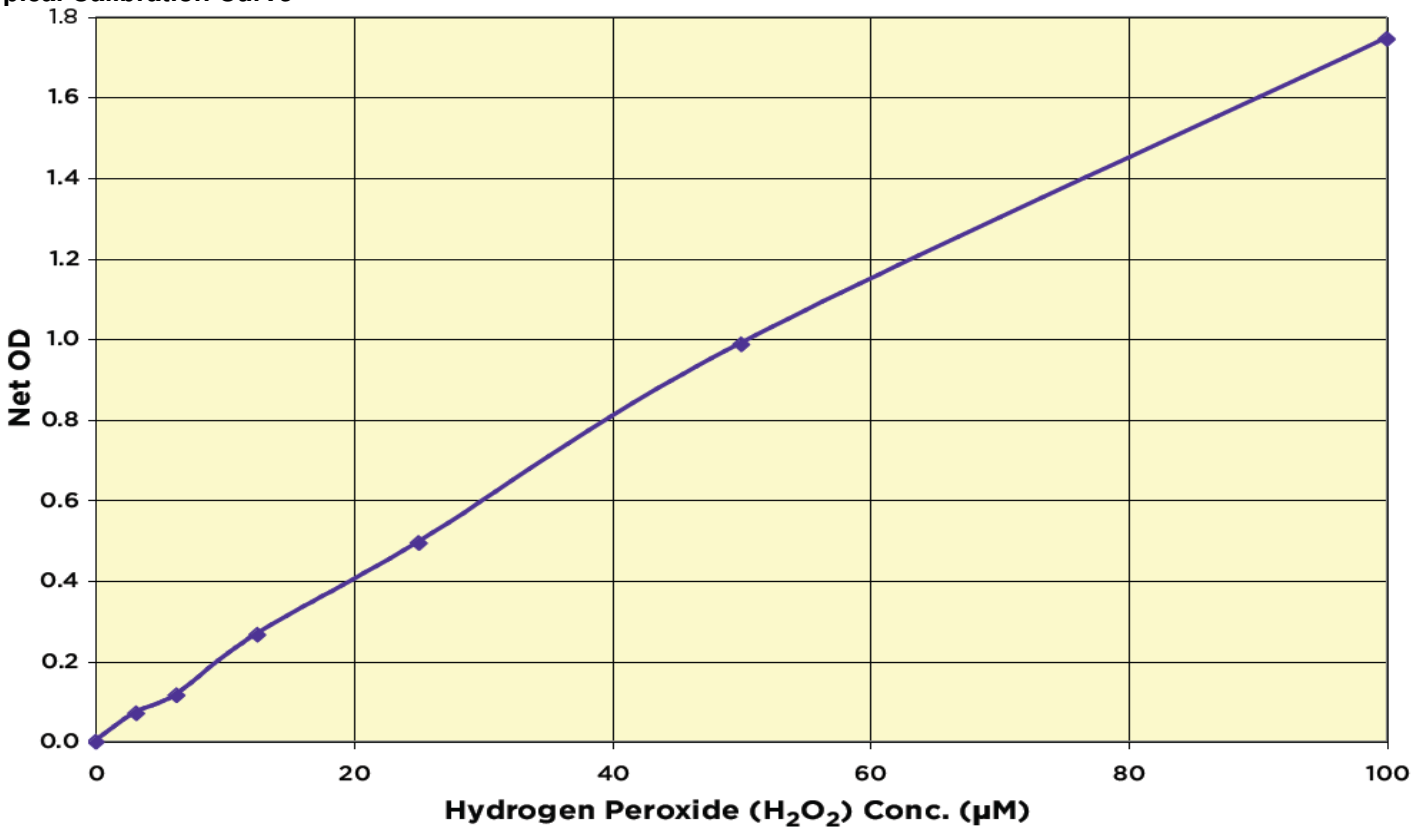
TYPICAL DATA

Sample	Mean OD	Net OD	H_2O_2 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

Always run your own calibration curves for calculation of results. Do not use these data.

Conversion Factor: 100 µM of Hydrogen Peroxide is equivalent to 3.4 µg/mL.

Typical Calibration Curve



Always run your own calibration curves for calculation of results. Do not use these data.

VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the ODs for twenty wells run for each of the zero and calibrator #6. The detection limit was determined at two (2) standard deviations from the zero along the calibration curve.

Sensitivity was determined as 1.83 µM. This is equivalent to 91.3 pmol (3.10 ng) H_2O_2 per well.

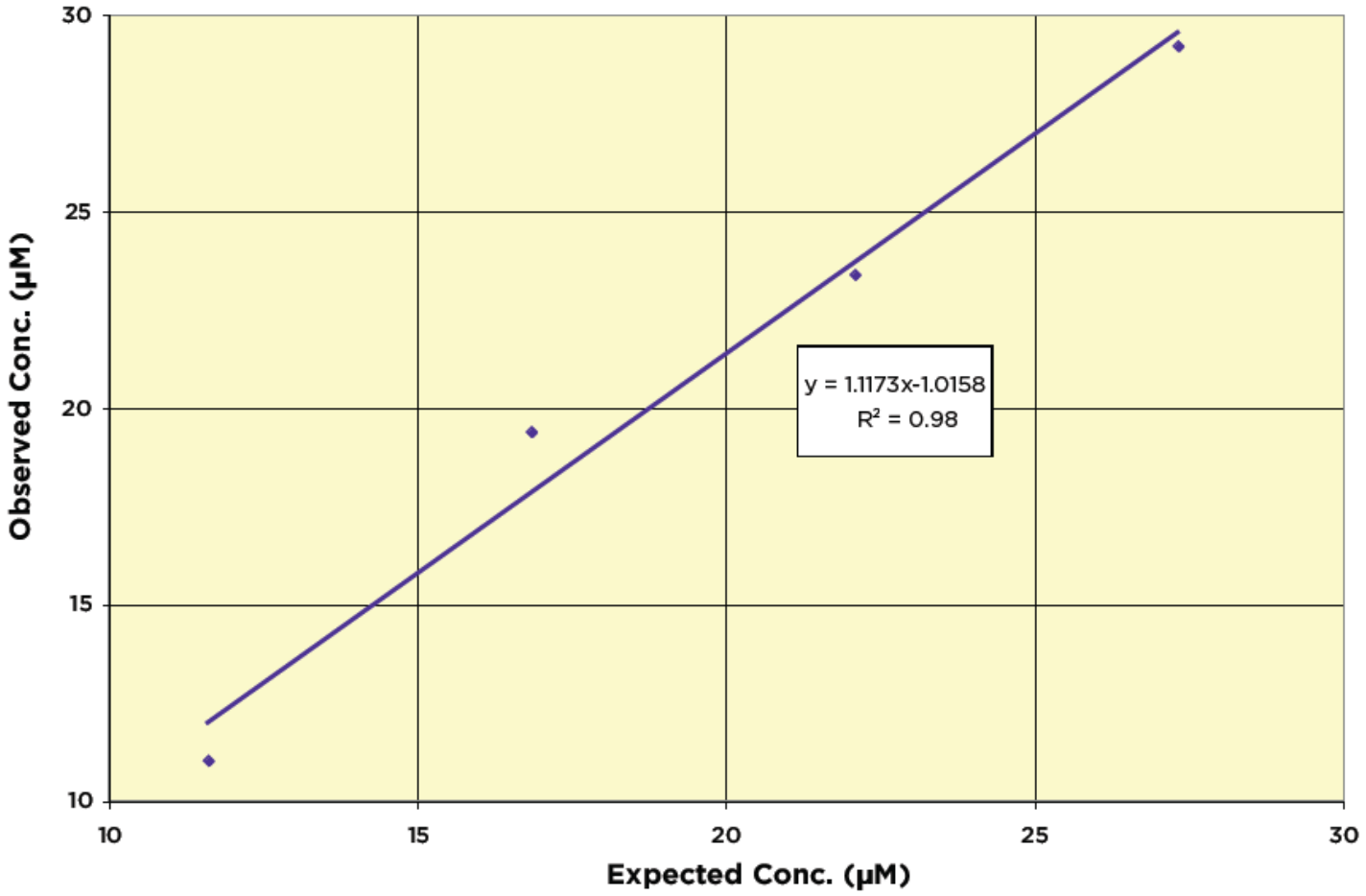
The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the zero and a low concentration human sample.

The Limit of Detection was determined as 1.96 µM. This is equivalent to 98.0 pmol (3.33 ng) H_2O_2 per well.

Linearity

Linearity was determined by taking two diluted human urine samples with known H_2O_2 concentrations and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

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%**



Intra Assay Precision

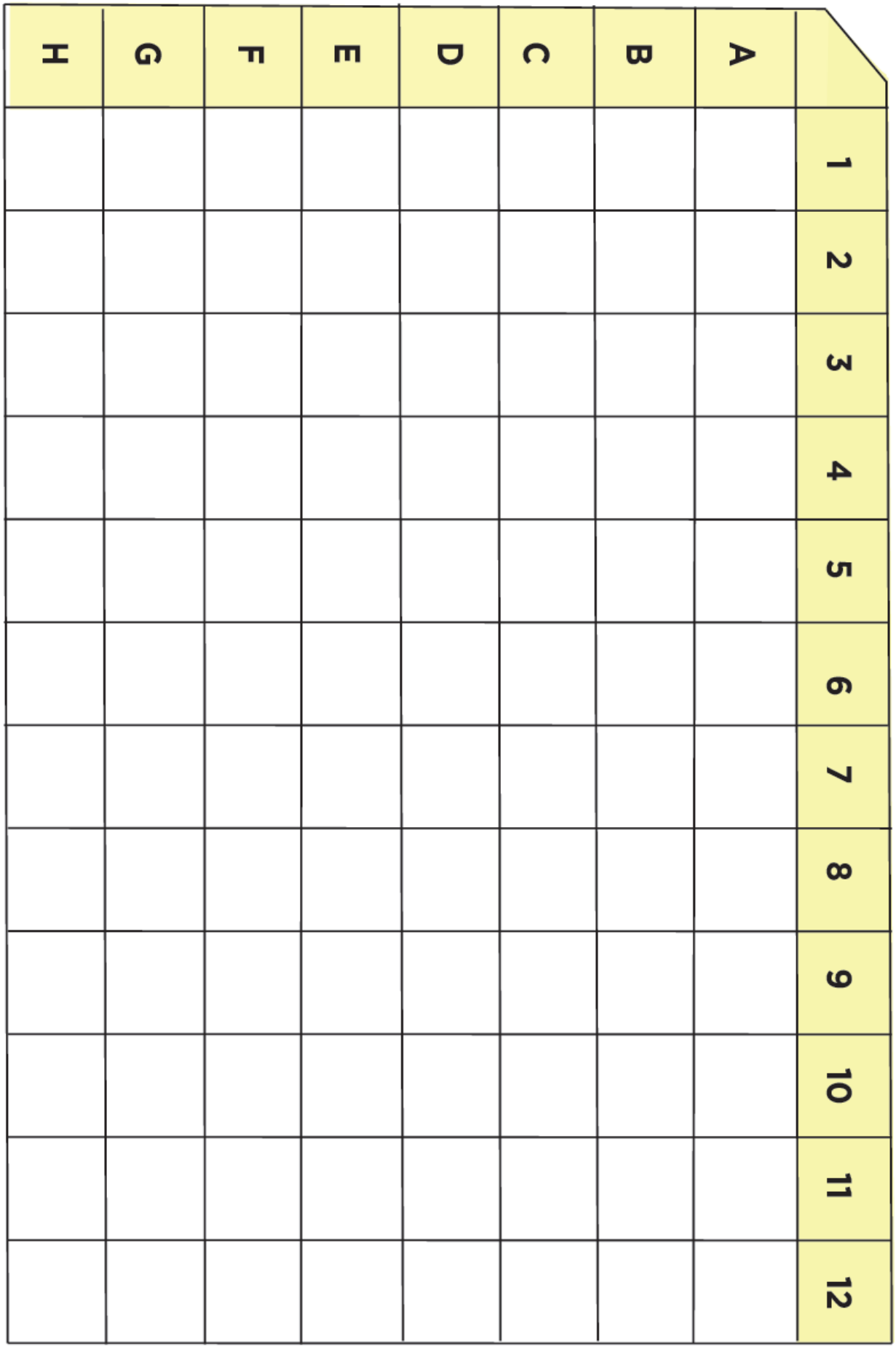
Three buffer samples were run in replicates of 20 in an assay. The mean and precision of the calculated concentrations were:

Sample	H_2O_2 Conc. (µM)	%CV
1	82.2	2.1
2	53.1	2.4
3	19.4	5.9

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	H_2O_2 Conc. (µM)	%CV
1	79.9	3.7
2	49.5	4.5
3	18.4	4.3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FOR RESEARCH USE ONLY

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