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ProductInformation

Hydrogen Peroxide Colorimetric Assay

Product Number **CS0270**
Storage Temperature –20 °C

Technical Bulletin

Product Description

Colorimetric Hydrogen Peroxide (H_2O_2) kit is a complete kit for the quantitative determination of hydrogen peroxide in biological fluids and tissue cultures. Please read the complete kit insert before performing this assay. The kit is designed to measure low concentrations of H_2O_2 in biological matrices. The kit employs a color reagent that contains a dye, xylenol orange, in an acidic solution with sorbitol and ammonium iron sulfate that reacts to produce a purple color proportional to the concentration of H_2O_2 in the sample. The exact mechanism of the color reaction is not known, but probably involves coordinated iron reacting with H_2O_2 and the dye molecule.

Hydrogen peroxide is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress-related states.[1, 2] Functioning through NF kappa-B and other factors, hydroperoxide-mediated pathways have been linked to asthma, inflammatory arthritis, arteriosclerosis, diabetic vasculopathy, osteoporosis, a number of neurodegenerative diseases and Down's Syndrome.[3-11] An interesting aspect of H_2O_2 biology is the recent report that antibodies have the capacity to convert molecular oxygen into hydrogen peroxide to contribute to the normal recognition and destruction processes of the immune system.[12, 13] Measurement of this reactive species will help to determine how oxidative stress modulates varied intracellular pathways.

Reagents

- **Hydrogen Peroxide Standard, 1 vial, Product No. H 2163** – 0.5 mL solution in water, 100,000 ng/mL, contains stabilizer, light sensitive.
- **Multiwell Plate, 1 plate, 96 wells, Prod. No. M 1443,** Ready to use.
- **Hydrogen Peroxide Color Reagent, 11 mL, Product No. H 2288** – contains xylenol orange dye in an acidic solution with sorbitol and ammonium iron sulfate.
- **Plate sealer, Product No. P 1496,** 2 each, adhesive strips.

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings between 540 and 570 nm
- Recommended Sample Diluent, 50 mM phosphate, pH 6.0
- Calibrated adjustable precision pipettes for volumes between 25μL and 1,000 μL.
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- The kit is used for cell culture supernatants, serum, urine, plasma (EDTA, heparin, sodium citrate) and other biological fluids.
- High levels of interfering substances may cause variations in assay results. Run samples and standard curve in the same matrix.
- Separate samples from cells and particulate material and assay immediately or store at –20 °C until use.
- Cell culture supernatants could be assayed undiluted, if the standards and zero blank are diluted in the same media.
- Media containing ferrous salts should be avoided, as they will interfere with sensitive detection.
- Serum, plasma, and urine samples require a 64-fold dilution in 50 mM phosphate. A suggested 64-fold dilution is 5 μL of sample + 315 μL of Sample Diluent.

Reagent Preparation

Hydrogen Peroxide Standard

1. The standard is light sensitive and should be

2. protected from direct light for prolonged periods of time.
3. Equilibrate standard solution to room temperature.
4. Pre-rinse pipette tip with the diluent before removing standard from 100,000-ng/mL tube.
5. Prepare serial standard dilutions as follows:

Tube #	Sample Diluent µL	Standard from tube #: -µL	Final Standard Concentration ng/mL
0	Standard vial 100,000 ng/mL		
1	966 µL	34 µL (0)	3,400
2	500 µL	500 µL	1,700
3	500 µL	500 µL	850
4	500 µL	500 µL	425
5	500 µL	500 µL	212
6	500 µL	500 µL	106

5. When running cell culture supernatants changes in binding may occur with running the standards and samples in media.

Storage/Stability

The kit is shipped on dry ice and should be stored at –20 °C until use.
After opening:

- Hydrogen Peroxide Color Reagent must be frozen at –20 °C. Avoid freeze/thaw cycles.
- The standard may be stored at 2-8 °C.
- The multiwell plate must be tightly sealed and stored at 2-8 °C.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate all reagents with the exception of Hydrogen Peroxide Color Reagent to room temperature (15-30 ºC).
- Hydrogen Peroxide Color Reagent must be kept at 4 °C during use.
- Warm up all samples to room temperature for 30 minutes.
- Multiwell plate: equilibrate to room temperature. Cover all unused wells tightly with plate sealer.
- When not in use all kit components should be stored as described in **Storage/Stability.**
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents past the kit shelf life.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.

Assay Procedure

1. Determine the number of wells to be used. Cover any unused wells tightly with a plate sealer.
2. Do not reuse wells.
3. Add 50 µL of Sample Diluent or cell culture media (CCM) into duplicate Blank (zero standard) wells.
4. Add 50 µL of each Standard into duplicate wells.
5. Add 50 µL of sample into duplicate wells.
6. Add 100 µL of Hydrogen Peroxide Color Reagent to all the wells.
7. Mix well by tapping the side of the plate gently for 10 seconds.
8. Incubate for 30 minutes at room temperature.
9. Read the optical density (OD) of each well using a multiwell plate reader set to 550 nm.

Add 50 µL standards, samples (1:64) and zero blank (sample diluent)

Add 100 µL of H_2O_2 Color Reagent to all wells

Mix by tapping; incubate 30 minutes at room temperature

Blank against the blank wells and read absorbance at 550 nm

H_2O_2 Assay Protocol

Results

Calculation of Results

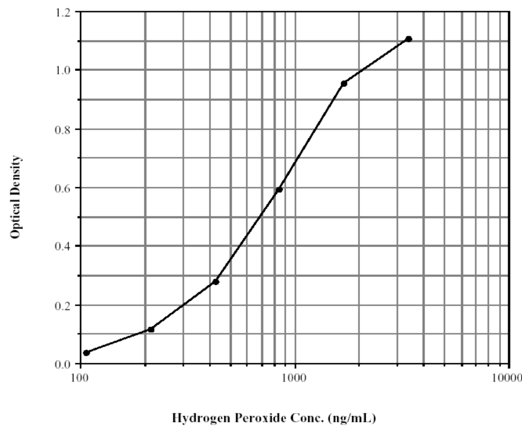
1. Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.
2. Create a standard curve by reducing the data using computer software capable of generating a linear curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a y-axis against the

concentration on the x-axis and draw the best-fit curve through the points on the graph.

3. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical Results

H_2O_2 ng/mL	OD_{550}	Average OD_{550}	Net OD_{550}
0	0.306		
	0.306	0.306	
106	0.337		
	0.346	0.341	0.035
212	0.421		
	0.421	0.421	0.115
425	0.586		
	0.584	0.585	0.279
850	0.894		
	0.904	0.899	0.593
1700	1.257		
	1.266	1.262	0.956
3400	1.405		
	1.419	1.412	1.106



This standard curve is provided for demonstration only. A standard curve should be generated for each assay run.

Precision

Intra-assay Precision (Precision within an assay) Three samples of known concentration were tested sixteen times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays) Three samples of known concentration were tested in eight separate assays to assess inter-assay precision.

	H_2O_2 ng/mL	Inter-assay %	Intra assay %
Low	314.2		10.0
Medium	772.1		3.0
High	1,606.2		4.0
Low	326.46	2.2	
Medium	768.21	1.7	
High	1,732.93	5.7	

Sensitivity

The sensitivity of the H_2O_2 assay is typically less than 51.3 ng/mL. Sensitivity was determined by subtracting two standard deviations from the mean absorbance value of sixteen zero standard replicates and calculating the corresponding concentration.

Linearity

To assess the linearity of the assay, Sample Diluent spiked with H_2O_2, was assayed using serial 2-fold dilutions.

Dilution	Observed ng/mL	Expected ng/mL	Observed: %Expected
Neat	2,880		
1:2	1,340	1,440	93%
1:4	598	720	83%
1:8	296	360	82%
1:16	163	180	90%
1:32	70	90	78%

Recovery

The recovery of H_2O_2 spiked into in different matrices was evaluated.

	Average % Recovery	Range
Cell culture media (n=4)	106%	92.5 - 116%
Human serum (n=1)	95	-
Equine heparin plasma (n=4)	94	88.7 - 99.2%
Human urine (n=1)	91	-

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