MANUAL

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ISO 13485:2016

RayBio® Hydrogen Peroxide (H_2O_2) Colorimetric Assay Kit

Catalog #: MA-H2O2

Introduction

Hydrogen Peroxide (H_2O_2) is a significant reactive oxygen species (ROS) playing essential roles in redox signaling and oxidative stress. It's generated during various cellular processes, including respiration, protein folding, peroxisome activity, and oxidase catalysis. High doses of hydrogen peroxide can be toxic to eukaryotic cells, leading to the oxidation of DNA, lipids, and proteins, potentially causing mutagenesis and cell death. Peroxide-induced cellular damage is associated with the development of numerous pathological conditions, such as aging, asthma, arthritis, diabetes, cardiovascular disease, atherosclerosis, Down's Syndrome, and neurodegenerative diseases.

The RayBio® Hydrogen Peroxide (H_2O_2) Colorimetric Assay Kit offers a straightforward, reproducible, and sensitive method for measuring hydrogen peroxide concentrations in plasma, serum, cell lysates, urine, and other biological fluid samples, without the need for prior treatment. This colorimetric probe reacts with H_2O_2 and the horseradish peroxidase enzyme (HRP), producing a pink-colored product with less background interference and enhanced stability compared to the commonly used Xylenol Orange (FOX) colorimetric assay for H_2O_2. The intensity of the pink color, measured at 520nm, corresponds to the amount of hydrogen peroxide present in the sample.

Storage

The entire kit may be stored at < -20 °C for up to 6 months from the date of shipment. For prepared reagent storage, see table below.

Component	Size / Description	Storage After Preparation
Microplate (Item A)	A 96-well (12 strips x 8 wells) plate	RT*
Sample Buffer	10 ml	2–8 °C, 4 weeks
Assay Buffer	25 ml	2–8 °C, 4 weeks
Hydrogen Peroxide Standard	1 amber vial (50 µl of 8.82M)	Do not store and reuse
Horseradish Peroxidase Concentrate	2 amber vials (Lyophilized)	Do not store and reuse
Colorimetric Probe	2 amber vials (Lyophilized)	Do not store and reuse

RT = room temperature

**Return unused wells to the pouch containing desiccant pack, reseal along entire edge.*

Additional Materials Required

1. Microplate reader capable of measuring absorbance at 520 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Multi-channel pipettes to deliver 20 µl to 200 µl volumes
4. Tubes to prepare sample dilutions
5. 50 ml conical tubes

Sample Tips and General Considerations

NOTE: Optimal methods of sample preparation will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.

1. Cell lysates can be prepared as follows:

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1,000 x g for 10 min) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at $2x10^7$ cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 minutes.
It is recommended that sample protein concentrations should be determined using a total protein assay.
Lysates should be used immediately or aliquot and stored at -70 °C. Thawed lysates should be kept on ice prior to use.
General tips for preparing lysate samples can be viewed on the online Resources page of the website: https://www.raybiotech.com/tips-on-sample-preparation/

2. Plasma samples:

Collect blood with an anticoagulant such as citrate, EDTA or oxalate and mix by inversion. Centrifuge the blood at 1000 x g at 4°C for 10 minutes. Collect plasma supernatant without disturbing the white buffy layer. Sample should be tested immediately or frozen at -80°C for storage. Samples can be assayed directly.

3. Serum samples:

Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Samples should be tested immediately or frozen at -80°C for storage. Samples can be assayed directly.

4. Urine samples:

To remove insoluble particles, spin at 10,000 x g for 5 min. The supernatant can be assayed directly.

Note: If the calculated hydrogen peroxide concentration of the sample is higher than 100 µM, dilute the sample in sample buffer and repeat the assay.

Reagent Preparation

REAGENT	PREPARATION
Microplate (Item A)	No preparation
Sample Buffer	
Assay Buffer	
Hydrogen Peroxide Standard	See “Standard Preparation” section.
Horseradish Peroxidase Concentrate	For each vial, reconstitute with 1 ml Assay Buffer.
Colorimetric Probe	For each vial, reconstitute with 1 ml Assay Buffer.
Chromogenic Working Solution	Add 1 mL reconstituted Horseradish Peroxidase and 1 mL reconstituted Colorimetric Probe into 6 mL Assay Buffer to make Chromogenic Working Solution. This is enough to assay 55 wells. Prepare the additional vial as needed. The solution is stable for 1 day at 2-8°C in the dark.

Standard Preparation

To prepare a dilution series of standard in the concentration range of 0 μM – 100 μM (see Table below),
Note: Diluted hydrogen peroxide is unstable and should be kept on ice while being used. Do not store diluted hydrogen peroxide standard solutions.

1. Label 8 microtubes #1 through #8 for the following concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0 μM.
2. Pipette 988.5 μL Sample Buffer into labeled tube #1, and 500 μL Sample Buffer into labeled tubes #2 – tube #8.
3. To make 8.8mM Hydrogen Peroxide standard stock: Pipette 2 μL 8.8M Hydrogen Peroxide Standard (provided) into 1998 μL Sample Buffer to get 8.8mM Hydrogen Peroxide Standard.
4. Pipette 11.5 μL 8.8mM Hydrogen Peroxide Standard into tube #1, to make 100μM Hydrogen Peroxide Standard.
5. To make the 50 μM standard, pipette 500 μL of tube #1 into the tube labeled #2. Mix thoroughly.
6. Repeat this step with successive concentration, preparing a dilution series as shown in the Table below. Each time, use 500 μL of the prior concentration until the 1.5625 μM is reached. Mix each tube thoroughly before the next transfer. Standards should be prepared freshly, mixed thoroughly and used immediately.

Labeled Tubes	Hydrogen peroxide Standard (μL)	Sample Buffer (μL)	Standard Conc. (μM)
1	11.5 μL of 8.8mM H_2O_2	988.5 μL	100
2	500 μL of Tube #1	500 μL	50
3	500 μL of Tube #2	500 μL	25
4	500 μL of Tube #3	500 μL	12.5
5	500 μL of Tube #4	500 μL	6.25
6	500 μL of Tube #5	500 μL	3.125
7	500 μL of Tube #6	500 μL	1.5625
8	0 μL	500 μL	0

Assay Procedure

Each hydrogen peroxide standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add 50 µL diluted Hydrogen Peroxide standards or unknown samples to the 96-well microtiter plate.
2. Add 150 µL Chromogenic Working Solution (See “Reagent Preparation”) to each well. Incubate the plate at room temperature for 15 minutes gently mixing.
3. Measure the absorbance at 520nm using a plate reader.

Calculation of Results

Subtract the blanks

Average the absorbance value of the blank wells (Standard 0 µM) and subtract this from the absorbance values of all the other wells. These are the corrected absorbance.

Plotting the standard curves

Make a plot of corrected absorbance at 520nm as a function of hydrogen peroxide concentration.

Determination of sample hydrogen peroxide concentration

Hydrogen peroxide (µM) = $\frac{OD_{Sample} - OD_{Blank}}{Slope} \times DF$

OD_{Sample} = Optical density (OD) reading of the Sample

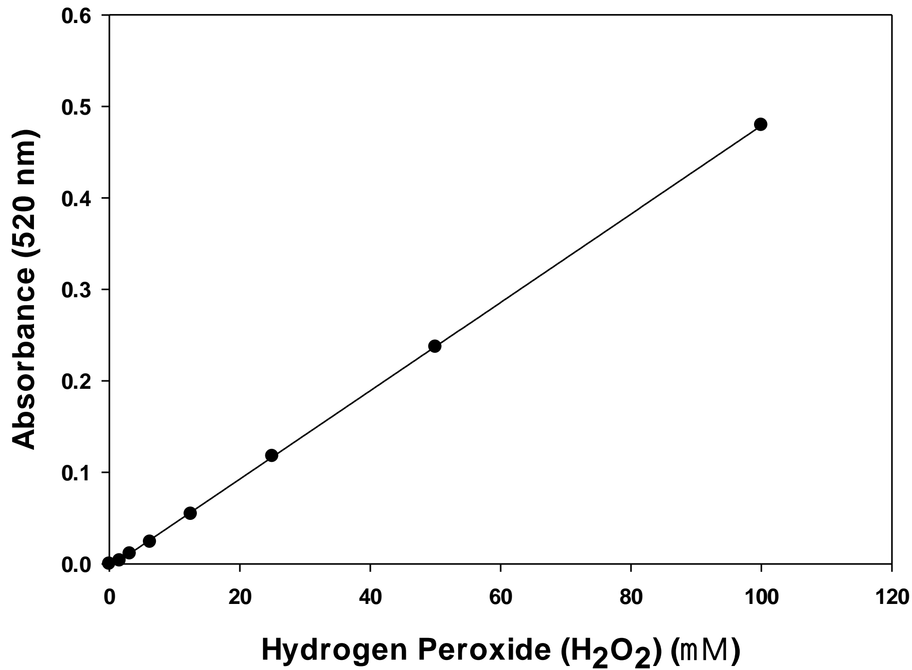
OD_{Blank} = Optical density (OD) reading of the Blank (Standard 0 µM)

Slope is from the plot of hydrogen peroxide concentration vs. Absorbance shown in Typical data below

DF = Sample Dilution factor (DF = 1 for undiluted Samples)

Note: If the calculated hydrogen peroxide concentration of the sample is higher than 100 µM, dilute the sample in sample buffer and repeat the assay.

A. Typical Data



These standard curves are for demonstration only. A standard curve must be run with each assay.

B. Reproducibility

Intra-assay Precision (Precision within an assay):
To assess intra-assay precision, 16 wells per sample (total of 4 samples) were tested on a single plate. The intra-assay coefficient of variation was found to be 1.3%.

Inter-assay Precision (Precision between assays):
To assess inter-assay precision, 4 samples were tested in separate assays (n=4). The inter-assay coefficient of variation was found to be 2.6%.

This product is for research use only.