

EZ-Hydrogen Peroxide /Peroxidase Assay Kit

Oxidative Stress Assay Kit

Cat. No. DG-PER500

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

▪ Product Description

Oxidative stress occurs due to an imbalance between Reactive Oxygen Species (ROS) and antioxidants. The excessive accumulation of ROS leads to cellular damage, such as damage to DNA, proteins, and lipid cell membranes. Peroxides like Hydrogen Peroxide (H_2O_2), which are typical byproducts of ROS under oxidative stress conditions, are toxic in eukaryotic cells. At high concentrations, they can cause oxidation of DNA, lipids, and proteins, leading to mutations or even cell death. Cellular damage caused by peroxides is associated with the development of various diseases, including aging, asthma, arthritis, diabetes, cardiovascular diseases, and neurodegenerative disorders.

The EZ-Hydrogen Peroxide/Peroxidase Assay Kit uses Oxi-Probe to measure Hydrogen Peroxide (H_2O_2) or Peroxidase activity. It offers a simple experimental procedure and high sensitivity.

This kit can be used to detect H_2O_2 present in experimental samples (such as cells or tissues) as well as H_2O_2 generated by enzyme-related reactions, allowing for the detection of small amounts of Hydrogen Peroxide or Peroxidase activity.

▪ Kit Contents and Storage Conditions

Components	500 assay	Storage
● Oxi-Probe (MW=257)	5 vials	
● Dimethylsulfoxide (DMSO), anhydrous	700 $\mu\ell$	
● Horseradish peroxidase (HRP, 10U**)	1 vial	-20°C
● Hydrogen peroxide (H_2O_2 , 3%, MW=34)	200 $\mu\ell$	
5X Reaction Buffer (pH 7.4, 0.25 M)	28 mL	

* The kit remains stable for 6 months when stored at -20°C before opening.

* The Oxi-Probe reagent is highly sensitive to air. It is recommended to use it shortly after opening and to protect it from light exposure.

** HRP 1U (unit) = The amount of enzyme that catalyzes the formation of 1 mg of purpurogallin from pyrogallol in 20 seconds (at 20°C and pH 6.0).

▪ Preparation of Stock Solution

Solution	Preparation	Storage
10mM Oxi-Probe	Dissolve the Oxi-Probe and DMSO vial at room temperature. Add 60 μl of DMSO to one Oxi-Probe vial and mix thoroughly. ➤ <u>One vial of Oxi-Probe is sufficient for 100 assays.</u>	Use the opened vial on the same day. Store any remaining solution at -20°C and mix thoroughly before use. <u>**Protect from light.</u>
1X Reaction Buffer	Mix 4 mL of 5X Reaction Buffer with 16 mL of deionized water (dH_2O).	-
10U/mL horseradish-peroxidase (HRP)	Add 1 mL of 1X Reaction Buffer to the HRP vial and mix thoroughly.	Dispense the remaining solution into smaller volumes and store at -20°C .
20mM Hydrogen-peroxide (H_2O_2)	Mix 23 μl of 3.0% H_2O_2 with 977 μl of deionized water (dH_2O).	The mixed solution has very low stability and is difficult to store. Prepare only the required amount for each experiment.

* This protocol is optimized for measurements using a 96-well microplate (total volume 100 μl).

* If using a standard fluorometer or a microplate of a different size, adjust the volume accordingly.

▪ Interference

1. The product formed during the Oxi-Probe reaction, Resorufin, is unstable in the presence of thiol compounds such as dithiothreitol (DTT) and 2-mercaptoethanol.
2. The final concentration of DTT and 2-mercaptoethanol in the sample should not exceed 10 μM during the experiment.
3. Pay attention to the pH during the experiment (optimal pH = 7~8).
4. The absorbance or fluorescence values of the final product, Resorufin, are affected by pH. Below $\text{pK}_a = 6.0$, the absorbance or fluorescence wavelength of Resorufin changes, and sensitivity significantly decreases. Additionally, Oxi-Probe becomes unstable at pH above 8.5, making accurate measurements difficult. Therefore, ensure the experiment is conducted at pH 7~8, using the Reaction Buffer (pH 7.4) provided in the kit.

▪Preparation of Samples

1. Cell Culture Supernatant

- ① Centrifuge at 10,000 rpm for 5 minutes to remove undissolved particles.
- ② Supernatants can be analyzed directly or diluted. However, it should fall within the H_2O_2 standard range.
- ③ Serum may cause interference during analysis, so it is recommended to remove it or avoid analyzing it.

2. Cell Lysate

- ① Resuspend the cells at $1 \sim 2 \times 10^6$ cells/mL in PBS or 1X Assay Buffer. (Cell concentration can vary depending on the type of cells.)
- ② Homogenize or sonicate the resuspended solution on ice.
- ③ Centrifuge to remove debris and use the supernatant.
- ④ The supernatant should be used directly or diluted to fall within the H_2O_2 standard range.

3. Plasma or Urine

- ① Centrifuge at 10,000 rpm for 5 minutes to remove undissolved particles.
- ② Supernatants can be analyzed directly or diluted, but it must fall within the H_2O_2 standard range.

Notes:

1. All samples should be analyzed immediately. If not, they can be stored at -80°C for up to about one month. However, the optimal experimental conditions for the samples should be determined by the experimenter. Always run the standard curve solution alongside the samples during the experiment.
2. To accurately measure the total H_2O_2 and peroxidase present in the sample, perform serial dilution before measurement. Excessive H_2O_2 or peroxidase can further oxidize the reaction products, so very high concentrations of H_2O_2 (\geq approximately 500 μM , final concentration) or peroxidase (\geq approximately 100 mU/mL) may lead to lower measured values.
3. NADH concentrations greater than 10 μM or glutathione concentrations greater than 50 μM can interfere with analysis and lead to inaccurate measurements. To minimize such interference, it may be helpful to add superoxide dismutase (SOD) to achieve a final concentration of 40 U/mL .

▪ General Protocol

I . Hydrogen Peroxide (H₂O₂) Assay

1. H₂O₂ standard preparation

: Prepare the H₂O₂ standard solution by mixing the 20 mM H₂O₂ solution and 1X Reaction Buffer as follows.

- ① It is recommended that the standard be measured for each experiment.
- ② If you are not using Standard curve, prepare positive/negative control.
 - Positive control: 10 μ M H₂O₂ solution - 50 μ l
 - Negative control: 1X Reaction Buffer (without H₂O₂) - 50 μ l

Standard Tube No.	H ₂ O ₂ standard	20mM H ₂ O ₂	1X Reaction Buffer	Final H ₂ O ₂ Concentration
1	10 μ M	0.5 μ l	999.5 μ l	5 μ M
2	5 μ M	500 of Tube #1	500 μ l	2.5 μ M
3	2.5 μ M	500 of Tube #2	500 μ l	1.25 μ M
4	1.25 μ M	500 of Tube #3	500 μ l	0.625 μ M
5	0.625 μ M	500 of Tube #4	500 μ l	0.3125 μ M
6	0.3125 μ M	500 of Tube #5	500 μ l	0.15625 μ M
7	0.15625 μ M	500 of Tube #6	500 μ l	0.078125 μ M
8	0 μ M	0	500 μ l	0 μ M

Table. protocol for H₂O₂ standard solution

2. Add 50 μ l of each of the H₂O₂ standard solutions prepared in 1 to the 96well plate. For accurate measurement, it is recommended to prepare the standard and sample at least two replicates and experiment.

3. Sample preparation

Put the prepared samples into the 96well plate in 50 μ l each.

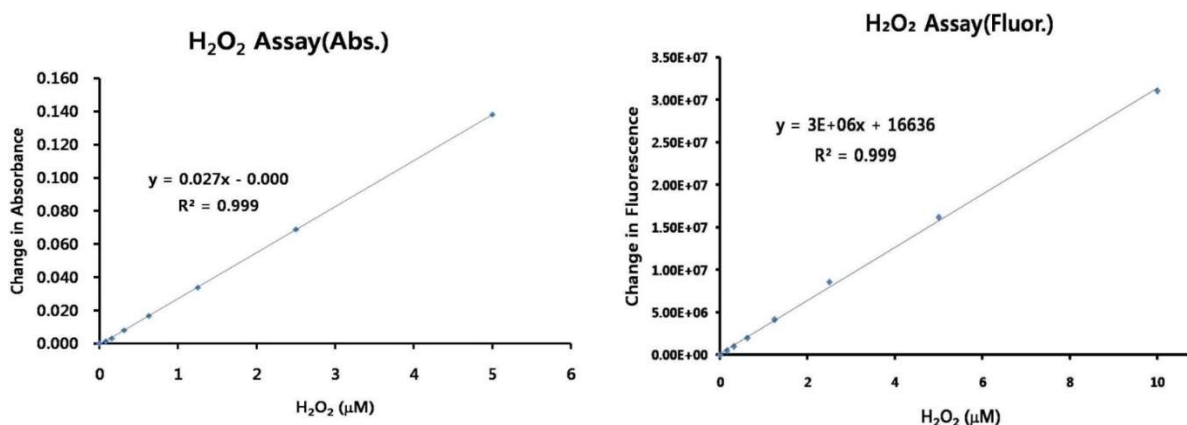
- If the concentration of H₂O₂ in the sample is too high, it may oxidize resorufin, the final product of the reaction, making accurate measurement difficult. Perform a preliminary experiment by serially diluting the sample to determine the approximate amount of the sample to be measured. (Use 1X Reaction Buffer for dilution of the sample.)

4. **Oxi-Probe/HRP Working Solution preparation:** (100assay)

Components	Volume
10mM Oxi-Probe	50 μl
10U/ml horseradish-peroxidase (HRP)	100 μl
1X Reaction Buffer	4.85 ml

Table. protocol for Oxi-Probe/HRP Working Solution

5. Add 50 μL of the Oxi-Probe/HRP Working Solution to each well of the plate containing the H_2O_2 standard solution (or control) and sample.
6. Incubate the plate in the dark at room temperature for 30 minutes.
7. After the reaction is complete, measure the reaction values using a plate reader.
 - ① Using Fluorescence plate reader – Excitation: 530 ~ 560nm
Emission: 580 ~ 590nm
(optimal Ex/Em = 540/590)
 - ② Using Absorbance plate reader – 560nm
8. Calculation
 - ① Using Standard curve: To correct the readings, subtract the No- H_2O_2 control value (standard #8) from the O.D. value of each well. This will give you the adjusted O.D. value for each sample.
 - ② Not using Standard Curve: Use the following formula to obtain the value.



$$\text{H}_2\text{O}_2 (\mu\text{M}) = \frac{A - B}{C - B} \times 5 \mu\text{M}$$

A: The measurement value for each well.

B: The measurement value of the negative control.

C: The measurement value of the positive control.

Fig. Hydrogen peroxide(H_2O_2) standard curve. Assay was performed following the kit protocol.
(Left - used the absorbance plate reader, Right - used the Fluorescence plate reader)

II. Peroxidase Assay

1. Peroxidase standard preparation

: Dilute 10U/mL horseradish-peroxidase (HRP) to 1/1000 (10mU/mL HRP).

Mix 10mU/mL horseradish-peroxidase (HRP) and 1X Reaction Buffer as follows to create an HRP standard solution.

- ① Always measure the standard with each experiment.
- ② If you are not using Standard curve, prepare positive/negative control.
 - Positive control: 2 mU/mL horseradish-peroxidase (HRP) - 50 μ l
 - Negative control: 1X Reaction Buffer (without HRP) - 50 μ l

Standard Tube No.	HRP standard	10mU/mL HRP	1X Reaction Buffer	Final HRP Concentration
1	2 mU/mL	200 μ l	800 μ l	1 mU/mL
2	1 mU/mL	500 of Tube #1	500 μ l	0.5 mU/mL
3	0.5 mU/mL	500 of Tube #2	500 μ l	0.25 mU/mL
4	0.25 mU/mL	500 of Tube #3	500 μ l	0.125 mU/mL
5	0.125 mU/mL	500 of Tube #4	500 μ l	0.0625 mU/mL
6	0.0625 mU/mL	500 of Tube #5	500 μ l	0.03125 mU/mL
7	0.03125 mU/mL	500 of Tube #6	500 μ l	0.015625 mU/mL
8	0 mU/mL	0	500 μ l	0 mU/mL

Table. protocol for HRP standard solution

2. Add 50 μ l of each of the HRP standard solutions prepared in 1 to the 96-well plate.
 - For accurate measurement, it is recommended to prepare at least two replicates for both the standard and the sample.

3. Sample preparation

Put the prepared samples into the 96well plate in 50 μ l each.

- If the concentration of HRP in the sample is too high, it may oxidize the final product, resorufin, making accurate measurement difficult. Perform a preliminary experiment by serially diluting the sample you wish to measure.
- Determine the approximate amount of the sample. (Use 1X Reaction Buffer for sample dilution.)

4. Oxi-Probe/H₂O₂ Working Solution preparation: (100assay)

Components	Volume
10mM Oxi-Probe	50 μ l
20mM Hydrogen Peroxide (H ₂ O ₂)	500 μ l
1X Reaction Buffer	4.45mL

Table. protocol for Oxi-Probe/H₂O₂ Working Solution.

5. Add 50 μl of the Oxi-Probe/H₂O₂ Working Solution to each well of the plate containing the HRP standard solution (or control) and the samples.
6. Incubate the plate in the dark at room temperature for 30 minutes.
7. After the reaction is complete, measure the reaction values using a plate reader.
 - ① Using Fluorescence plate reader – Excitation: 530 ~ 560nm
Emission: 580 ~ 590nm
(optimal Ex/Em = 540/590)
 - ② Using Absorbance plate reader – 560nm
8. Calculation
 - ① Using Standard curve: To correct the readings, subtract the No-H₂O₂ control value (standard #8) from the O.D. value of each well. This will give you the adjusted O.D. value for each sample.
 - ② Not using Standard Curve: Use the following formula to obtain the value.

$$\text{HRP } (\mu\text{M/mL}) = \frac{\text{A} - \text{B}}{\text{C} - \text{B}} \times 1 \mu\text{M/mL}$$

A: The measurement value for each well.

B: The measurement value of the negative control.

C: The measurement value of the positive control.

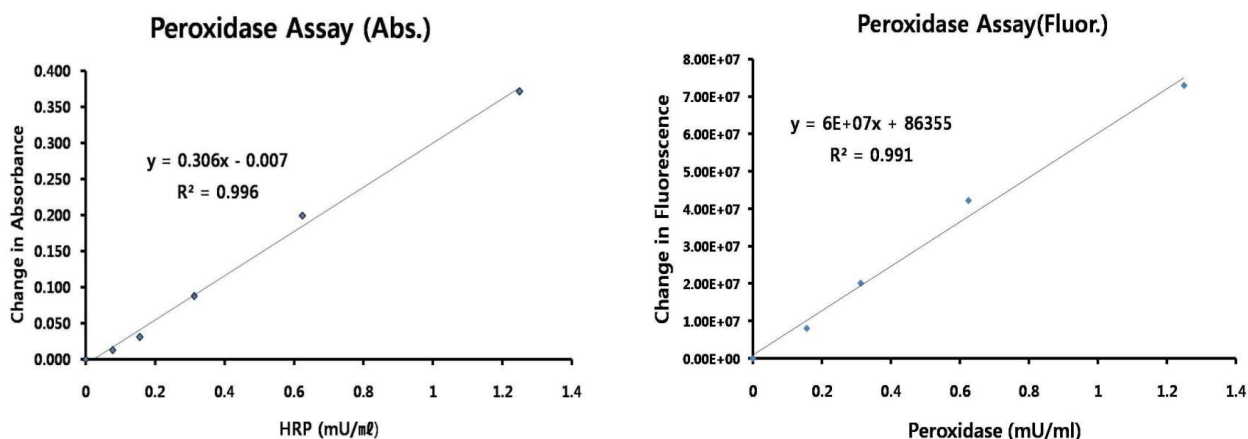


Fig. HRP standard curve. Assay was performed following the kit protocol.
(Left - used the absorbance plate reader, Right - used the Fluorescence plate reader)