Technical Manual

Hydrogen Peroxide (H_2O_2) Fluorometric Assay Kit

- Catalogue Code: MAES0001
- Size: 96T
- Research Use Only

1. Key features and Sample Types

Detection method:
Fluorimetric method

Specification:
96T

Range:
0.02-10 µmol/L

Sensitivity:
0.02 µmol/L

Storage:
2-8°C and -20°C for 6 months

Expiry:
See Kit Label

Experiment Notes:

This kit is for **research use only.**

Instructions should be strictly followed. Changes of operation may result in unreliable results.

The validity of kit is 6 months.

Do not use components from different batches of kit.

2. Background

Hydrogen peroxide (H_2O_2) is a metabolic by-product of reactive oxygen species, which is not only a signal molecule in cells, but also a source of oxidative stress. H_2O_2 is an important regulatory factor of eukaryotic signal transduction involved in cell proliferation, differentiation and migration. However, abnormal H_2O_2 can lead to oxidative cell damage and disease, such as cancer, atherosclerosis, osteoporosis and neurodegenerative diseases.

3. Intended Use

This kit can be used to measure the H_2O_2 content in serum, plasma, tissue and cells samples.

4. Detection Principle

In the presence of peroxidase, hydrogen peroxide reacts with the fluorescent probe, and the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 587 nm is proportional to the hydrogen peroxide concentration.

5. Kit components & storage

Item	Specification	Storage
Buffer Solution	60 mL × 1 vial	-20˚C, 6 months
Substrate	0.12 mL × 1 vial	-20˚C, 6 months, away from direct sunlight
Enzyme Reagent	1 vial Lyophilized	-20˚C, 6 months, away from direct sunlight
H_2O_2 Standard Solution (1 mol/L)	0.1 mL × 1 vial	-20˚C, 6 months
Protein Precipitator	20 mL × 1 vial	2-8˚C, 6 months
Alkali Reagent	6 mL × 1 vial	2-8˚C, 6 months
pH test strips	1 bag	
Black Microplate	96 wells	No requirement
Plate Sealer	2 pieces	

Materials required but not supplied

- Micropipettor
- Incubator
- Centrifuge
- Fluorescence microplate reader (Ex/Em=535 nm/587 nm)
- Tips (10 µL, 200 µL, 1000 µL)
- EP tubes (1.5 mL, 2 mL)
- Double distilled water

6. Assay Notes:

1. Avoid repeated freezing and thawing of substrate, it is recommended to aliquot the substrate into smaller quantities and store at -20℃.

2. Because H_2O_2 is very unstable, preparae the H_2O_2 standard solution freshly.

3. The prepared working solution must be stored with shading light.

4. The pH of pretreated sample should be 6.5-8.

5. Avoid the formulation of bubbles when the supernatant is transferred into the microplate.

7. Reagent preparation:

1. Preparation of **enzyme application solution:** Dissolve a vial of enzyme reagent with 120 µL of buffer solution and mix fully. The prepared enzyme application solution can be stored at -20℃ for 1 month with shading light.

2. Preparation of **working solution:** Mix the buffer solution, substrate and enzyme application solution at a ratio of 48:1:1. Prepare the needed amount fresh solution before use.

3. Preparation of **10 mmol/L H_2O_2 solution:** Dilute the 1 mol/L standard with buffer solution 1:100.

4. Preparation of **100 µmol/L H_2O_2 solution:** Dilute 10 mmol/L H_2O_2 solution with buffer solution 1:100.

5. Preparation of **10 µmol/L H_2O_2 solution:** Dilute 100 µmol/L H_2O_2 solution with buffer solution 1:100.

   Note: The purpose of gradual dilution is to reduce errors. Because H_2O_2 is very unstable, prepare fresh H_2O_2 standard solution.

8. Sample Preparation

1. **Serum sample:**
   Fresh blood should be incubated at 25°C for 30 min to clot the blood. Centrifuge the sample at 2000 g for 15 min at 4°C. Take the serum (which is the upper light yellow clarified liquid layer) to preserve it on ice for detection. If not detected on the same day, the serum can be stored at -80°C for a month.
2. **Plasma sample:**
   Place the fresh blood sample into a tube of anticoagulant and centrifuge at 700-1000g for 10 min at 4°C. Take the plasma (which is the upper light yellow clarified liquid layer, don't take white blood cells and platelets in the middle layer) to preserve it on ice for detection. If not detected on the same day, the plasma can be stored at -80°C for a month.

3. **Cell sample:**
   Collect the cells and wash the cells with PBS (0.01 M, pH 7.4) for 1~2 times. Centrifuge at 1000 g for 10 min and then discard the supernatant and keep the cell sediment. Add homogenization medium at a ratio of cell number (10^6): homogenization medium (μL) = 1: 300-500. Sonicate the sample on an ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant and preserve it on ice for detection. If not detected on the same day, the cells sample (without homogenization) can be stored at -80°C for a month.

4. **Tissue sample:**
   Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2-8°C. Absorb the water with filter paper and weigh. Homogenize at the ratio of the volume of buffer solution (2-8°C) (mL): the weight of the tissue (g) =9:1, then centrifuge the tissue homogenate for 10 min at 10000 g at 4°C. Take the supernatant to preserve it on ice for detection. If not detected on the same day, the tissue sample (without homogenization) can be stored at -80°C for a month.

Homogenized Method:
Hand-operated: Weigh the tissue and mince to small pieces (1 mm^3), put the tissue pieces into a glass homogenized tube. Add homogenized medium into the homogenized tube and place the tube into an ice bath. Using a glass tamping rod, grind up and down for 6-8 min. Alternatively, place the tissue into a mortar and add liquid nitrogen to grind fully. Then, add the homogenized medium.
Mechanical Homogenate: Weigh the tissue in an EP tube. Add the homogenized medium to homogenize the tissue with an homogenizer instrument (60 Hz, 90s) in an ice bath. (For skin, muscle and plant tissue samples, prolong homogenization time accordingly).
Ultrasonication: Treat the cells with an ultrasonic cell disruptor (200 W, 2 s/time, interval for 3 s, total time is 5 min).

Sample Notes:

The concentration should be determined before preforming the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.

If a lysis buffer is used to prepare tissue or cell homogenates, there is a possibility of causing a deviation due to the introduced chemical substance.

Dilution of Samples:

Large variances in results may be seen when performing pre-experiments. Dilute the sample according to the result of the pre-experiment and the detection range (0.02-10 µmol/L).

The recommended dilution factor for different samples is as follows (for reference only).

Sample Type:	Dilution Factor:
Human serum	1-3
Mouse serum	1-4
Mouse plasma	1-2
Porcine serum	1-3
Rat serum	1-4
HepG2 culture supernatant	1
10% Mouse liver tissue homogenate	1-3
10% Mouse brain tissue homogenate	1-3
10% Rat lung tissue homogenate	1-3
HepG2 cells (6.44 gprot/L)	1-2

Note: The diluent of serum (plasma) is buffer solution.

9. Assay Protocol

Ambient Temperature: 25-30°C

Optimum detection wavelength: Ex/Em=535 nm/587 nm

Plate Set Up:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73
B	B	B	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74
C	C	C	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75
D	D	D	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76
E	E	E	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77
F	F	F	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78
G	G	G	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79
H	H	H	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80

Note: A-H, standard wells; S1-S80, sample wells.

10. Operation Steps

The pretreatment of sample

Mix the protein precipitator and the sample according to the ratio of sample (volume): protein precipitator (volume) =1:1, centrifuge at 13000 g for 10 min, then take V_1 (eg, V_1=0.2 mL) of the supernatant, add V_2 (eg, V_2=0.04 mL) of reagent 6 to adjust the pH to 6.5-8, which is the sample to be tested.

The preparation of standard curve

Dilute 10 μmol/L H_2O_2 solution with buffer solution to a serial concentration. The recommended dilution gradient is as follows: 0, 0.5, 1, 2, 4, 6, 8, 10 μmol/L.

The measurement of samples

1. **Standard well:** Take 50 μL of standard solution with different concentrations to the wells.
   Sample well: Take 50 μL of pretreated sample to the wells.

2. Add 50 μL of working solution to each well.

3. Mix fully with microplate reader for 10 s and incubate the plate at room temperature for 10 min with shading light.

4. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 587 nm.

Operation Table

	Standard well	Sample well
Standard solution of different concentrations (μL)	50	
Pretreated sample (μL)		50
Working solution (μL)	50	50
Mix fully with microplate reader for 10 s and incubate the plate at room temperature for 10 min with shading light. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 587 nm.		

11. Calculations

Plot the standard curve by using fluorescence value (F) of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve with graph software (or EXCEL). The concentration of the sample can be calculated according to the formula based on the F value of sample. The standard curve is: y= ax + b.

1. Serum (plasma) and other liquid sample:

$$\underset{(\mu mol/L)}{H_2O_2}=(\Delta F\text{-}b)\div a\times 2\times(\frac{V_1+V_2}{V_1})\times f$$

2. Tissue and cell sample:

$$\underset{(\mu mol/gprot)}{H_2O_2}=(\Delta F\text{-}b)\div a\times 2\times(\frac{V_1+V_2}{V_1})\times f\div C_{pr}$$

y: $F_{Standard} - F_{Blank}$.
x: The concentration of standard.
a: The slope of standard curve.
b: The intercept of standard curve.
ΔF: Absolute fluorescence intensity of sample ($F_{Sample} - F_{Blank}$)
f: Dilution factor of sample before tested.
2: Dilution factor of sample in pretreatment step (V_{sample}:$V_{reagent\ 5}$=1:1).
V_1: The volume of supernatant in pretreatment step (mL).
V_2: The volume of reagent 6 in pretreatment step (mL).
C_{pr}: Concentration of protein in sample (gprot/L).

12. Performance Characteristics

Detection Range	0.02-10 μmol/L
Sensitivity	0.02 μmol/L
Average recovery rate (%)	100
Average inter-assay CV (%)	3.6
Average intra-assay CV (%)	1.1

Analysis

Dilute 10% mouse brain tissue homogenate with buffer solution for 2 times, take 0.2 mL of diluted sample, add 0.2 mL of reagent 5, mix fully, centrifuge at 13000 g for 10 min at 4˚C, add 0.04 mL of reagent 6 to adjust the pH, carry the assay according to the operation table.

The results are as follows:

Standard curve: y = 556.7 x + 11.559, the average fluorescence value of the sample is 2452, the average fluorescence value of the blank is 80, the concentration of protein in sample is 5.4 gprot/L, and the calculation result is:

$$\mathbf{H2O2}\ (\boldsymbol{\mu mol/gprot}) = (\mathbf{2452} - \mathbf{80} - \mathbf{11.559}) \div \mathbf{556.7} \times \mathbf{2} \times (\mathbf{0.2} + \mathbf{0.04}/\mathbf{0.2}) \times \mathbf{2} \div \mathbf{5.40} = \mathbf{3.77}\ \boldsymbol{\mu mol/gprot}$$

Safety Notes

Some of the reagents in the kit contain dangerous substances. Avoid touching skin and clothing.

Wash immediately with plenty of water if touching it carelessly.

All the samples and waste material should be treated according to the relevant rules of laboratory's biosafety.

Before the experiment, read the instructions carefully, and wear gloves and work clothes.

Notes:

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