PRODUCT INFORMATION & MANUAL

Hydrogen Peroxide Assay Kit (Fluorometric)

NBP3-24507

For research use only.
Not for diagnostic or therapeutic procedures.

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Hydrogen Peroxide (H_2O_2) Fluorometric Assay Kit

Catalog No: NBP3-24507

Method: Fluorimetric method

Specification: 96T (Can detect 80 samples without duplication)

Measuring instrument: Fluorescence Microplate Reader

Sensitivity: 0.02 μmol/L

Detection range: 0.02-10 μmol/L

Average intra-assay CV (%): 1.1

Average inter-assay CV (%): 3.6

Average recovery rate (%): 100

- ▲ This kit is for research use only.
- ▲ Instructions should be followed strictly, changes of operation may result in unreliable results.
- ▲ Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

General information

▲Intended use

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

▲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.

▲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.

▲Kit components & storage

Item	Component	Specification	Storage
Reagent 1	Buffer Solution	60 mL × 1 vial	-20℃ , 12 months
Reagent 2	Substrate	0.12 mL × 1 vial	-20℃ , 12 months, shading light
Reagent 3	Enzyme Reagent	Powder × 1 vial	-20℃ , 12 months, shading light
Reagent 4	1 mol/L H_2O_2 Standard Solution	0.1 mL × 1 vial	-20℃ , 12 months
Reagent 5	Protein Precipitator	20 mL × 1 vial	2-8℃ , 12 months
Reagent 6	Alkali Reagent	6 mL × 1 vial	2-8℃ , 12 months
	pH test strips	1 bag	
	Black Microplate	96 wells	No requirement
	Plate Sealer	2 pieces	

Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other.

▲Materials prepared by users

Instruments

Fluorescence microplate reader (Ex/Em=535 nm/587 nm), Micropipettor, Incubator, Vortex mixer, Centrifuge

⚠Safety data

Some of the reagents in the kit contain dangerous substances. It should be avoided to touch the 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.

⚠Precautions

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

▲The key points of the assay

1. Avoid repeated freezing and thawing of reagent 2, it is recommended to aliquot the reagent 2 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. Prevent the formulation of bubbles when the supernatant is transferred into the microplate.

Pre-assay preparation

▲Reagent preparation

1. Preparation of reagent 3 application solution:
   Dissolve a vial of reagent 3 with 120 μL of reagent 1 and mix fully. The prepared reagent 3 application solution can be stored at -20℃ for 1 month with shading light.
2. Preparation of working solution:
   Mix the reagent 1, reagent 2 and reagent 3 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 reagent 4 with reagent 1 for 100 times.
4. Preparation of 100 μmol/L H_2O_2 solution:
   Dilute 10 mmol/L H_2O_2 solution with reagent 1 for 100 times.
5. Preparation of 10 μmol/L H_2O_2 solution:
   Dilute 100 μmol/L H_2O_2 solution with reagent 1 for 10 times.
   [Note]: The purpose of gradual dilution is to reduce errors. Because H_2O_2 is very unstable, preparae the H_2O_2 standard solution freshly.

▲Sample preparation

The samples should be prepared as conventional methods. Also please refer to appendix II.

▲Dilution of sample

It is recommended to take 2~3 samples with expected large difference to do pre-experiment before formal experiment and 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 homogenate (the concentration of protein is 6.44 gprot/L)	1-2

Note: The diluent is reagent 1.

Assay protocol

▲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.

▲Detailed operation steps

1.The pretreatment of sample

Mix the reagent 5 and the sample according to the ratio of sample (volume): reagent 5 (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.

2.The preparation of standard curve

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

Number	Standard concentrations (μmol/L)	10 μmol/L Standard (μL)	Reagent 1 (μL)
A	0	0	200
B	0.5	10	190
C	1	20	180
D	2	40	160
E	4	80	120
F	6	120	80
G	8	160	40
H	10	200	0

3.The measurement of samples

1) Standard tube: Take 50 μL of standard solution with different concentrations to the wells.
   Sample tube: Take 50 μL of pretreated sample in Step 1 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.

▲Summary operation table

	Standard tube	Sample tube
Standard solution of different concentrations (μL)	50	
Pretreated sample in Step 1 (μL)		50
Working solution (μL)	50	50
Mix fully and incubate at room temperature for 10 min with shading light. Measure the fluorescence intensity .		

▲Calculation

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.

Serum, plasma and other liquid sample

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

Tissue and Cells

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

Note:

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).

Appendix I Data

▲Example analysis

Dilute 10% mouse brain tissue homogenate with reagent 1 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℃ , add 0.04 mL of reagent 6 to adjust the pH, then 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:

$$\underset{(\mu mol/gprot)}{H_2O_2} = (2452 - 80 - 11.559) \div 556.7 \times 2 \times \frac{0.2+0.04}{0.2} \times 2 \div 5.40 = 3.77\ \mu mol/gprot$$

Appendix II Sample preparation

The following sample pretreatment methods are for reference only.

▲ Serum

Collect fresh blood and stand at 25℃ for 30 min to clot the blood. Then centrifuge at 2000 g for 15 min at 4℃ . 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℃ for a month.

▲ Plasma

Take fresh blood into the tube which has anticoagulant (heparin is used as anticoagulant), centrifuge at 700-1000 g for 10 min at 4℃ . 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℃ for a month.

▲ Tissue sample

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

▲ Cells

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 or grind with hand-operated in ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant and preserve it on ice for detection. Meanwhile, determine the protein concentration of supernatant. If not detected on the same day, the cells sample (without homogenization) can be stored at -80℃ for a month.

Note:

1. Homogenized medium: Reagent 1.

2. Homogenized method:

(1) Hand-operated: Weigh the tissue and mince to small pieces (1 mm^3), then put the tissues pieces to glass homogenized tube. Add homogenized medium into homogenized tube, place the tube into the ice bath with left hand, and insert the glass tamping rod vertically into the homogenized tube with the right hand to grind up and down for 6-8 min.

Or put the tissue into the mortar, and add liquid nitrogen to grind fully. Then add the homogenized medium to homogenize.

(2) Mechanical homogenate: Weigh the tissue to EP tube, add the homogenized medium to homogenize the tissue with homogenizer instrument (60 Hz, 90s) in the ice bath. (For samples of skin, muscle and plant tissue, the time of homogenization can be properly prolonged.)

(3) Ultrasonication: Treat the cells with ultrasonic cell disruptor (200 W, 2 s/ time, interval for 3 s, the total time is 5 min).

Appendix III References

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5. Moloney J N, Cotter T G. ROS signalling in the biology of cancer. Seminars in Cell & Developmental Biology, 2018, 80: 50-64.