PRODUCT INFORMATION & MANUAL

Reactive Oxygen Species/ ROS Assay Kit (Fluorometric)

NBP3-25793

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

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Reactive Oxygen Species (ROS) Fluorometric Assay Kit

Catalog No: NBP3-25793

Method: Fluorimetric method

Specification: 96T

Instrument: Fluorescence Microplate reader, Flow Cytometry

- ▲ 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 reactive oxygen species (ROS) in fresh tissue and cell samples.

▲Background

Reactive oxygen species (ROS) are active chemical substances produced in the metabolic process of the body, including oxygen free radicals, hydrogen peroxide and its downstream products, such as peroxides and hydroxides. ROS are both necessary and harmful to organisms, and are involved in cell growth, proliferation, development and differentiation, aging and apoptosis, as well as many physiological and pathological processes. Excessive ROS will lead to oxidative stress and oxidative damage of cells, further promoting the occurrence and development of many diseases, such as cancer, cardiovascular disease and diabetes.

▲Detection principle

DCFH-DA (2,7-dichlorofuorescin diacetate) is a fluorescent probe without fluorescence that can freely cross the membrane. After entering the cell, it can be hydrolyzed by intracellular esterase to form DCFH (dichlorofluorescin). In the presence of reactive oxygen species (ROS), DCFH is oxidized to DCF (dichlorofluorescein) which is a strong green fluorescent substance that cannot penetrate the cell membrane. DCF has a maximum wave peak near the excitation wavelength of 502 nm and the emission wavelength of 525 nm, and the intensity is proportional to the level of intracellular reactive oxygen species.

▲Kit components & storage

Item	Component	Specification	Storage
Reagent 1	10 mmol/L DCFH-DA	0.1 mL × 1 vial	-20℃ , 12 months, shading light
Reagent 2	Positive Control	1 mL × 1 vial	2-8℃ , 12 months
	Black Microplate	96 wells × 2	No requirement
	Plate Sealer	4 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=500 nm/525 nm), Flow Cytometry(Ex/Em=500 nm/525 nm), Vortex mixer, Micropipettor, Water bath, Incubator, Centrifuge

Reagents

Double distilled water、PBS（0.01 M，pH 7.4）、Serum-free medium

⚠ 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. After the incubation of the probe, it is important to wash out residual probes that have not entered the cells, otherwise the background will be higher.
2. Avoid repeated freezing and thawing of DCFH-DA.
3. The time of detection is shortened as far as possible to reduce the experimental error.
4. Set a positive control (reagent 2 working solution) and a negative control (only cells without reagent 1 working solution).

Pre-assay preparation

1. Preparation of reagent 1 working solution

Dilute the reagent 1 with serum-free medium, the recommended working concentration is 0.1-20 μM. Prepare the fresh solution before use.

(Note: DMSO is harmful to cells, so the dilution ratio must be more than 500.)

2. Preparation of reagent 2 working solution

Dilute the reagent 2 (contain 10 mM TBHP) with serum-free medium, the recommended working concentration of TBHP is 50-250 μM. Prepare the fresh solution before use.

Assay protocol

▲Detection of culture cell sample

1. Add the fluorescent probe:

a. Add Reagent 1 working solution to the cells. The DCFH-DA working concentration can be 0.1-20 μM for different cells and treatment. Pre-experiment is suggested to determine the appropriate concentration. The total dilution ratio should be more than 1:500-1:1000 in order to avoid effects of DMSO on cells. DMSO should be set as solution control.

b. Incubate at 37℃ for 30 min ~ few hours, generally 30~60 min. The incubation time is related to cell types, stimulation conditions, and DCFH-DA concentration.

c. Cell collection:

Suspension cells: centrifuge the sample at 1000 g for 5~10 min and wash with serum-free medium for 2~3 times. Centrifuge and collect the cell precipitation for fluorescence detection.

Adherent cells: digest the cells with 0.25% trypsin, add medium that contain fetal bovine serum to terminate the digestion, thus to prepare the cell suspension. Centrifuge at 1000 g for 5~10 min and collect cells, then wash with serum-free medium for 1~2 times. Centrifuge and collect cell precipitation for fluorescence detection.

2. Fluorescence detection:

a. Re-suspend collected cells with serum-free medium for detection.

b. Wavelength: the excitation wavelength is 500 nm, the emission wavelength is 525 nm. It can also be detected according to the fluorescence detection conditions of FITC.

Note:

The density of re-suspension cell is determined by cell fluorescence intensity. If fluorescence is strong (weak), then decrease (increase) the cell density, but cell density of all samples should be consistent.

▲Detection of tissue sample

1. Preparation of single cell suspension:

Method 1: using the single cell suspension instrument.

Method 2: enzyme digestion.

a. Take the tissue into pre-cooled PBS（0.01 M，pH 7.4）immediately and clean the blood and other contaminants. Remove the massive composition, fiber, fat, and blood vessels (except for specialized cells).

b. Cut the tissue into about 1 mm^3 pieces with the ophthalmic scissors, then put these pieces to pre-cooled PBS（0.01 M，pH 7.4）to remove the cell debris.

c. Add an appropriate amount of enzyme digestion, incubate in 37℃ water bath for 20~30 min and gently oscillate the mixture intermittently.

d. Stop the digestion with medium that contain fetal bovine serum. Filter the mixture to remove the tissue massive component with nylon mesh (300 mesh) and collect the cells. Centrifuge at 500 g for 10 min and discard the supernatant, then wash with PBS（0.01 M，pH 7.4）for 1~2 times. Re-suspend to prepare the single cell suspension solution. The cell amount should be no less than 10^6.

Method 3: mechanical method.

a. The pretreatment is the same as step a and step b in the enzyme digestion method.

b. Tight the nylon mesh (300 mesh) on a small beaker, then place the tissue pieces on the mesh and gently rub the tissue with ophthalmic scissor or erasing knife. Wash the tissue with PBS（0.01 M，pH 7.4）at the same time.

c. Collect the cell suspension and centrifuge at 500 g for 10 min. Then discard the supernatant and wash with PBS（0.01 M，pH 7.4）for 1~2 times. Re-suspend to prepare the single cell suspension solution. The cell amount should be no less than 10^6.

2. Add the fluorescent probe:

a. Add Reagent 1 working solution to the cells. The DCFH-DA working concentration can be 0.1-20 μM for different cells and treatment. Pre-experiment is suggested to determine the appropriate concentration. The total dilution ratio should be more than 1:500-1:1000 in order to avoid effects of DMSO on cells. DMSO should be set as solution control.

b. Incubate at 37℃ for 30 min ~ few hours, generally 30~60 min. The incubation time is related to cell types, stimulation conditions, and DCFH-DA concentration.

c. Collect the incubated single cell suspension, centrifuge at 1000 g for 5~10 min to collect cells. Wash with serum-free medium for 1~2 times. Centrifuge and collect the cell precipitation for fluorescence detection.

3. Fluorescence detection:

a. Re-suspend collected cells with serum-free medium for detection.

b. Wavelength: the excitation wavelength is 500 nm, the emission wavelength is 525 nm. It can also be detected according to the fluorescence detection conditions of FITC.

Notes

1. The density of re-suspension cell is determined by cell fluorescence intensity. If fluorescence is strong (weak), then decrease (increase) the cell density, but cell density of all samples should be consistent.
2. Fluorescent substances are sensitive to light and should avoid light during detection.
3. DCF is easy to be quenched, and the samples after incubation must be detected within 2 hours.
4. Results were expressed as fluorescence intensity or geometric average fluorescence intensity (flow cytometry).
5. When using flow cytometry, in order to avoid the interference of cell debris and dead cells on the experimental results, it is necessary to eliminate them.
6. Set a positive control (reagent 2 working solution) and a negative control (only cells without reagent 1 working solution).
7. The timing of adding DCFH-DA or incubation time depends on whether the intracellular reactive oxygen species can be detected successfully. DCFH-DA can be added in advance or at the same time if the drug treatment time is short (<2 h) or the predicted ROS is weak. Conversely, DCFH-DA can be added later if the drug treatment time is long (>6 h) or predicted ROS is strong.

Appendix References

1. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction[J]. Annu Rev Plant Biol, 2004, 55: 373-399.
2. Gospodaryov D, Lushchak V, Oxidative Stress: Cause and Consequence of Diseases. 2012: InTech. 353-374.
3. Forstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies[J]. Nat Clin Pract Cardiovasc Med, 2008, 5(6): 338-349.