Technical Manual

Reactive Oxygen Species (ROS) Fluorometric Assay Kit

- Catalogue Code: MAES0112
- Size: 96 Assays
- Research Use Only

1. Key features and Sample Types

Detection method:

Fluorimetric method

Specification:

96T

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 followed strictly, 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

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 development of many diseases, such as cancer, cardiovascular disease and diabetes.

3. Intended Use

This kit can be used to measure reactive oxygen species (ROS) in fresh tissue and alive cell samples.

4. 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.

5. Kit components & storage

Component	Specification	Storage
DCFH-DA (10 mmol/L)	0.1 mL × 1 vial	-20˚C, 6 months, avoid direct sunlight
Positive Control	1 mL × 1 vial	2-8˚C, 6 months
Buffer Solution	60 mL× 2 vials	2-8˚C, 6 months
Black Microplate	96 wells × 2	No requirement
Plate Sealer	4 pieces	

Materials required but not supplied

- Micropipettor
- Water bath
- Vortex mixer
- Centrifuge
- Fluorescence Microplate reader (Ex/Em=500 nm/525 nm)
- Fluorescence Spectrophotometer (Ex/Em=500 nm/525 nm)
- Flow Cytometry(Ex/Em=500 nm/525 nm)
- Tips (10 µL, 200 µL, 1000 µL)
- EP tubes (1.5 mL, 2 mL)
- Double distilled water

6. Assay Notes

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 much as possible to reduce experimental error.

4. Set a positive control (Positive Control working solution) and a negative control (only cells without 10 mmol/L DCFH-DA working solution).

7. Reagent Preparation

1. Preparation of **Buffer Solution working solution:** Dilute the Buffer Solution with double distilled water at the ratio of 1:9 and mix fully. The prepared solution can be stored at 2-8˚C for 6 months.

2. Preparation of **10 mmol/L DCFH-DA working solution:** Dilute the 10 mmol/L DCFH-DA with Buffer Solution working solution, the recommended working concentration is 0.1-20 µM. (Note: DMSO is harmful to cells, so the dilution ratio must be more than 500.)

3. Preparation of **Positive Control working solution:** Dilute the Positive Control with Buffer Solution working solution, the recommended working concentration of TBHP is 50-250 µM, dilute Positive Control and Buffer Solution working solution 40-200 times. Prepare fresh solution before use.

8. Assay Protocol

Ambient Temperature: 25-30°C

Optimum detection wavelength: Ex/Em=500 nm/525 nm

9. Operation Steps

1. Detection of cell sample:

(1) Add the fluorescent probe:

a. Add **DCFH-DA (10 mmol/L) working solution to the cells**. The DCFH-DA working concentration can be 0.1-20 μM for different cells and treatment. A pre-experiment is recommended 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˚C 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 **Buffer Solution working solution** 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 **Buffer Solution working solution** for 1~2 times. Centrifuge and collect cell precipitation for fluorescence detection.

(2) Fluorescence detection:

a. Re-suspend collected cells with **Buffer Solution working solution** 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: 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. 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 **Buffer Solution working solution** immediately and clean the blood and other contaminants. Remove the massive composition, fibre, 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 **Buffer Solution working solution** to remove the cell debris.

c. Add an appropriate amount of enzyme digestion, incubate in 37˚C 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 **Buffer Solution working solution** 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 pre-treatment 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 **Buffer Solution working solution** 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 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 **DCFH-DA working solution (10 mmol/L) 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˚C 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 **Buffer Solution working solution** for 1~2 times. Centrifuge and collect the cell precipitation for fluorescence detection.

(3) Fluorescence detection:

a. Re-suspend collected cells with **Buffer Solution working solution** 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.

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

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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.

Fluorescent substances are sensitive to light and should avoid light during detection.

DCF is easy to be quenched, and the samples after incubation must be detected within 2 hours.

Results were expressed as fluorescence intensity or geometric average fluorescence intensity (flow cytometry).

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.

Set a positive control (Positive Control working solution) and a negative control (only cells without 10 mmol/L DCFH-DA working solution).

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.

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