（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Catalog No：E-BC-K138-F

Specification：96T

Measuring instrument：Fluorescence Microplate reader, Fluorescence microscope, Flow Cytometry

Elabscience® Reactive Oxygen Species (ROS) Fluorometric Assay Kit (Green)

This manual must be read attentively and completely before using this product.
If you have any problem, please contact our Technical Service Center for help：

Toll-free: 1-888-852-8623
Tell: 1-832-243-6086
Fax: 1-832-243-6017
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Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

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Intended use

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

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	Size (96 T)	Storage
Reagent 1	10 mmol/L DCFH-DA	0.1 mL × 1 vial	-20°C, 12 months, shading light
Reagent 2	Positive Control	1 mL × 1 vial	-20°C, 12 months, shading light
	Black Microplate	96 wells	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. For a small volume of reagents, please centrifuge before use, so as not to obtain sufficient amount of reagents.

Materials prepared by users

Instruments:

Fluorescence Microplate reader (Ex/Em=488 nm/525 nm), Fluorescence Microscope (Ex/Em=488 nm/525 nm), Flow Cytometry(Ex/Em=488 nm/525 nm), Vortex mixer, Micropipettor, Water bath, Incubator, Centrifuge

Reagents:

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

Reagent preparation

① The preparation of DCFH-DA working solution：
Dilute the 10 mmol/L DCFH-DA with serum-free medium, the recommended working concentration is 0.1-20 μM. (Note: DMSO is harmful to cells, so the dilution ratio must be more than 500.) The DCFH-DA working solution should be prepared on spot.

② The preparation of positive control working solution：
Dilute the positive control (contain 10 mM TBHP) with serum-free medium, the recommended working concentration of TBHP is 50-250 μM. The positive control working solution should be prepared on spot.

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

Operating steps

Detection of culture cell sample

1. Add the fluorescent probe:

① Add DCFH-DA 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 500 in order to avoid effects of DMSO on cells. DMSO should be set as solution control.

② 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.

③ 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:

① Re-suspend collected cells with serum-free medium for detection.

② Wavelength: the excitation wavelength is 488 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.

① 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).

② 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.

③ Add an appropriate amount of enzyme digestion, incubate in 37°C water bath for 20~30 min and gently oscillate the mixture intermittently.

④ 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.

① The pretreatment is the same as step a and step b in the enzyme digestion method.

② 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.

③ 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:

① Add DCFH-DA 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 in order to avoid effects of DMSO on cells. DMSO should be set as solution control.

② 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.

③ 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:

① Re-suspend collected cells with serum-free medium for detection.

② Wavelength: the excitation wavelength is 488 nm, the emission wavelength is 525 nm. It can also be detected according to the fluorescence detection conditions of FITC.

Statement

1. This assay kit is for Research Use Only. We will not response for any arising problems or legal responsibilities causing by using the kit for clinical diagnosis or other purpose.
2. Please read the instructions carefully and adjust the instruments before the experiments. Please follow the instructions strictly during the experiments.
3. Protection methods must be taken by wearing lab coat and latex gloves.
4. If the concentration of substance is not within the detection range exactly, an extra dilution or concentration should be taken for the sample.
5. It is recommended to take a pre-test if your sample is not listed in the instruction book.
6. The experimental results are closely related to the situation of reagents, operations, environment and so on. Elabscience will guarantee the quality of the kits only, and NOT be responsible for the sample consumption caused by using the assay kits. It is better to calculate the possible usage of sample and reserve sufficient samples before use.