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

Catalog No：E-BC-F021

Specification：96T

Measuring instrument：Flow Cytometry

Elabscience® Nitric Oxide (NO) Fluorometric Assay Kit

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
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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

The kit is suitable for detecting NO level in alive cell sample.

Detection principle

Nitric oxide (NO) is a nonpolar free radical gas molecule and serves as a key participant in biological signal transduction. It can rapidly diffuse and transmit signals between cells, playing important roles in promoting vasodilation, facilitating neurotransmission, and regulating immune and metabolic functions.

DAF-FM DA probe can enter cells and react with NO to produce fluorescence. The NO levels in the cell can be evaluated by measuring the fluorescence intensity.

Kit components & storage

Item	Component	Size (96T)	Storage
Reagent 1	5 mmol/L DAF-FM DA	0.025 mL × 1 vial	-20°C, 12months, shading light

Note: All the reagents should be stored according to the table. The reagents from different kits can not be mixed or used interchangeably. For liquid reagents with small volumes or powders, centrifuge them before use to prevent loss.

Instruments

Flow Cytometry, Incubator

Materials required but not provided

Phenol red-free basal medium or phenol red-free Hank's balanced salt solution (Hank's), PBS (0.01 M, pH 7.4)

Reagent preparation

① Equilibrate all the reagents to 25°C before use.

② Probe Working Solution preparation:
Dilute the 5 mmol/L DAF-FM DA with phenol red-free basal medium or Hank's.The recommended initial concentration in the system of DAF-FM DA is 5 μmol/L. For different samples and treatments, the DAF-FM DA concentration in the system is suggested to be 1–10 μM. Preliminary experiments are required to determine the appropriate concentration. The Working Solution should be freshly prepared before use. Keep it protected from light and use within 8 h.

③ Recommended solution volumes in different culture dishes:

Cell types	adherent cell				Suspended cell
Plate types	6-well plates	24-well plates	96-well plates	35 mm culture dish	2 mL centrifuge tube
Volume of working solution required per well	1 mL/well	0.5 mL/well	0.2 mL/well	1.5 mL/well	0.3 mL/tube

The key points of the assay

① Wash away any residual probes after probe incubation. Otherwise it will lead to elevated background levels.

② DAF-FM DA fluorescent is easily quenched. Therefore, samples should be measured within 2 hours after incubation to prevent fluorescence decay and minimize potential errors.

③ Since DAF-FM DA tends to settle at low temperatures, equilibrate DAF-FM DA to 25°C before use and mix it thoroughly.

④ Phenol red and BSA interfere with DAF-FM DA detection and should be avoided.

⑤ Avoid DAF-FM DA repeated freeze-thaw cycles

Operating steps

Instrument parameter	
Flow Cytometry	FITC

Suspension cells

① Centrifuge at 1000×g for 5-10 min to collect cells. A recommended cell density is 2×10^5 cells/mL. Set up control tubes, and experimental tubes. Cells in the control tube are resuspended in phenol red-free basal medium or Hank's, while cells in the experimental tube are resuspended in Probe Working Solution.

② Incubate at 37°C for 20-60 min. The optimal incubation time varies for different cell types, stimulus condition and the comcentration of DAF-FM DA in the working solution.

③ Centrifuge at 1000×g for 5-10 min to collect cells. Wash cells 2-3 times with PBS (0.01 M, pH 7.4) to thoroughly remove DAF-FM DA that did not enter the cells.

④ Resuspend the collected cell pellet in phenol red-free basal medium or Hank's.
⑤ Detect by Flow Cytometry in the FITC channel.

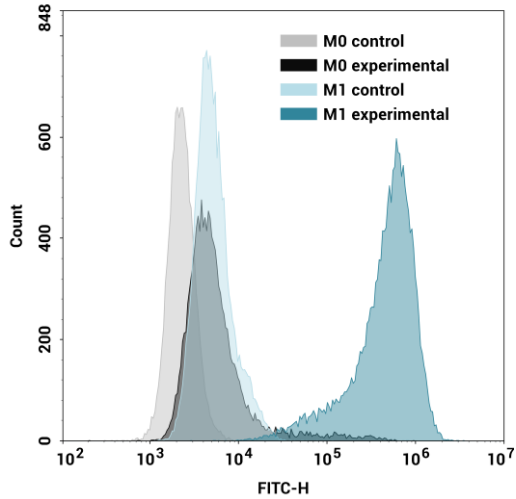
Adherent cells

① Set up control wells, and experimental wells. A recommended cell number is 2–5 × 10^6/well.
② Control wells: add phenol red-free basal medium or Hank's.
Experimental wells: add Probe Working Solution.
③ Incubate at 37°C for 20-60 min. The optimal incubation time varies for different cell types, stimulus condition and the comcentration of DAF-FM DA in the working solution.
④ Remove the solution in the well and wash 2–3 times with PBS (0.01 M, pH 7.4) to thoroughly remove DAF-FM DA that did not enter the cells.
⑤ Collect cells after digestion and centrifuge at 1000×g for 5-10 min to collect cell pellet.
⑥ Resuspend the collected cell pellet in phenol red-free basal medium or Hank's.
⑦ Detect by Flow Cytometry in the FITC channel.

Note: If drugs are used to stimulate cells, the concentration of drug and stimulation duration needs to be determined based on the type of the cell type. For example, RAW 264.7 cells can be co-stimulated with LPS and IFN-γ for 12–48 hours. For stimuli that produce large amounts of NO within a short timeframe (≤2 h), drugs can be added simultaneously with the Working Solution for incubation. For long-time stimulation, it is recommended to add the Working Solution for incubation after drug treatment.

Appendix I Case Analysis

Stimulate RAW 264.7 M0-type cells with LPS and IFN-γ to induce differentiation into the M1 phenotype. Then, remove the culture medium and proceed according to the operating steps. Set up the M0 control group, M0 experimental group, M1 control group, and M1 experimental group respectively. The concentration of DAF-FM DA in the M0 control group and M1 control group is 0 μM, while in the M0 experimental group and M1 experimental group is 10 μM. After incubation at 37°C for 30 min in the dark, the result is shown as follows:



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.