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

Catalog No：E-BC-F064

Specification：48T/96T

Measuring instrument：Flow Cytometry/Fluorescence microscope (FITC)

Elabscience® Mitochondrial Permeability Transition Pore (mPTP) 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
Tel: 1-832-243-6086
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Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

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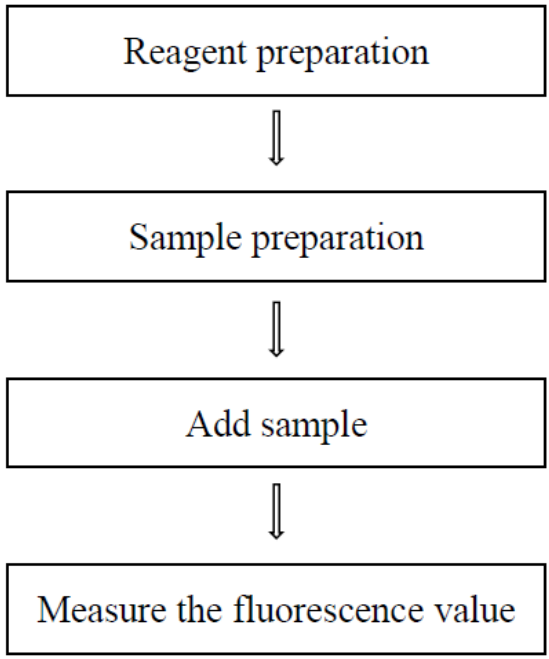
Assay summary



Intended use

This kit can be used to measure the changes in the opening degree of mitochondrial permeability transition pores (mPTP) in cells.

Detection principle

Mitochondria are the energy centers of cells and play a very important role in the processes of apoptosis and necrotic cell death. Mitochondrial permeability transition pore (mPTP), also known as mitochondrial megachannel (MMC), It is a non-specific channel composed of the inner and outer membranes of mitochondria, which participates in the release of substances within mitochondria during cell death. The opening of mitochondrial permeability transition pores is a key event causing cell death and plays an important role in the regulation of cell survival and apoptosis.

The detection principle of this kit: The kit uses Calcein AM to stain cells, causing the cytoplasm, including mitochondria, to show strong green fluorescence. Under physiological pH conditions, when Calcein complexes with Co^{2+}, the fluorescence signal is quenched. Under normal circumstances, the mPTP in mitochondria is off, and at this time, Co^{2+} cannot enter the mitochondria, resulting in only green fluorescence appearing within the mitochondria. The inner mitochondrial membrane of normal cells can maintain a normal mitochondrial potential gradient to ensure cellular respiration and energy supply. With the intake and release of Ca^{2+}, a low-conductivity permeability conversion pore switches back and forth between opening and closing. When cells undergo apoptosis and pathological death, the permeability of mitochondrial membrane potential conversion pores changes. Overload of Ca^{2+}, oxidation of mitochondrial glutathione, increase in reactive oxygen species levels, including

subsequent release of cytochrome C, and decrease in mitochondrial membrane potential can all lead to the activation of mitochondrial permeability conversion pores. Further treatment with the calcium ion carrier Ionomycin as a control to induce a large amount of extracellular Ca^{2+} to enter the intracellular and mitochondrial matrix and cause the opening of mPTP will weaken or disappear the green fluorescence in the mitochondria. In this way, the degree of opening of mitochondrial MPTP can be judged based on the intensity of the green fluorescence of Calcein in mitochondria. The stronger the green fluorescence, the lower the degree of opening; the weaker the green fluorescence, the higher the degree of opening.

Kit components & storage

Item	Component	Size1 (48 T)	Size2 (96 T)	Storage
Reagent 1	Probe (500×)	0.03 mL × 1 vial	0.06 mL × 1 vial	-20℃, 12 months, shading light
Reagent 2	Quencher (25×)	0.5 mL × 1 vial	1 mL × 1 vial	-20℃, 12 months, shading light
Reagent 3	Ionomycin (200×)	0.05 mL × 1 vial	0.1 mL × 1 vial	-20℃, 12 months, shading light

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:

Flow Cytometry/Fluorescence microscope (FITC), Incubator

Reagents:

PBS(0.01 mol/L，pH = 7.4), Phenol red-free basic medium

Reagent preparation

① Equilibrate all the reagents to 25℃ before use.

② The dilution of the probe working solution:

The recommended concentration for preparing the probe is 1× (dilute according to the volume ratio of probe to PBS = 1:499), and usually the fluorescence effect is better at this time. The concentration of the probe can also be appropriately optimized based on the type of cells used in the experiment and the sensitivity of the instrument to explore the best staining effect, and can be adjusted within the range of 1×~2.5×. The probe working solution should be prepared on spot. Keep it on ice during use protected from light and used up on the same day.

③ The dilution of the quencher working solution:

The recommended concentration for preparing the quencher is 1× (dilute according to the volume ratio of quencher to PBS = 1:24), and usually the fluorescence quenching effect is better at this time. The concentration of the quencher can also be appropriately optimized based on the type of cells used in the experiment to explore the best quenching effect, and can be adjusted within the range of 0.15×~2.5×. Store at -20°C for 2 days protected from light.

④ The dilution of the Ionomycin working solution:

The recommended concentration for preparing the Ionomycin is 1× (dilute according to the volume ratio of Ionomycin to PBS = 1:199), and usually the experimental effect is better at this time. The concentration of the Ionomycin can also be appropriately optimized based on the type of cells used in the experiment to explore the best experimental effect, and can be adjusted within the range of 0.1×~1×. Aliquoted storage at -20°C for 2 days protected from light.

The key points of the assay

① The probe is easy to quench, and should be stored protected from light to slow down the fluorescence quenching.

② The probe should be aliquoted storage at -20°C, and avoid repeated freeze/thaw cycles is advised.

③ All reagents added to the cell wells need to be preheated to 37°C before use.

Operating steps

Suspension cells: Collect cells and centrifuge at 400×g for 5 min at 4℃ to remove supernatant. Resuspend the cells in phenol red-free basic medium. It is recommended that the cell density be 2×10^5 cells/mL. For example, if there are 2×10^5 cells, resuspend them in 1 mL of phenol red-free basic medium. Design blank control tubes, negative control tubes, sample tubes and positive control tubes, and add 200 μL of cell suspension to each tube.

① Blank control tube: Add 30 μL of phenol red-free basic medium to the tubes.

Negative control tube: Add 10 μL of probe working solution and 20 μL of phenol red-free basic medium to the tubes.

Sample tube: Add 10 μL of probe working solution, 10 μL of quencher working solution and 10 μL of phenol red-free basic medium to the tubes.

Positive control tube: Add 10 μL of probe working solution, 10 μL of quencher working solution and 10 μL of Ionomycin working solution to the tubes.

② Incubate at 37℃ for 30-60 min protected from light (the optimal incubation time varies for different cells. Take 30 minutes as the initial incubation time and appropriately optimize the incubation time based on the cells used to achieve the best results). Centrifuge at 400×g for 5 min at room temperature to remove supernatant and slowly resuspend the cells in preheated phenol red-free basic medium at 37°C. Repeat the above centrifugation and medium change steps twice and then replace with fresh 37°C preheated phenol red-free basic medium.

③ Incubate at 37℃ for 10-30 min protected from light (the optimal incubation time varies for different cells. Take 10 minutes as the initial incubation time and appropriately optimize the incubation time based on the cells used to achieve the best results). After incubation, observe with

a fluorescence microscope or detect with a flow cytometer.

Adherent cells: Take a 24-well plate as an example. Set up the wells of the cell plate according to the experimental requirements to ensure that the cells are healthy and do not grow excessively. Carefully aspirate the culture medium to avoid cell shedding. Add 500 μL of phenol red-free basic culture medium to each well.

① Blank control well: Add 75 μL of phenol red-free basic medium to the wells.

Negative control well: Add 25 μL of probe working solution and 50 μL of phenol red-free basic medium to the wells.

Sample well: Add 25 μL of probe working solution, 25 μL of quencher working solution and 25 μL of phenol red-free basic medium to the wells.

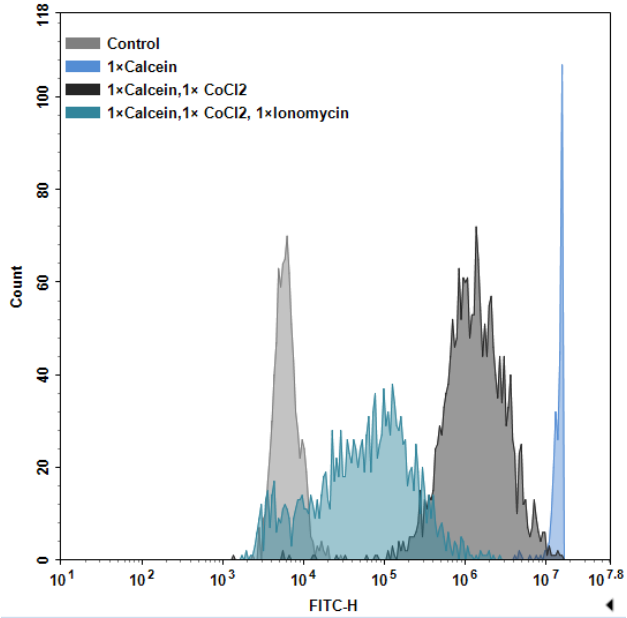
Positive control well: Add 25 μL of probe working solution, 25 μL of quencher working solution and 25 μL of Ionomycin working solution to the wells.

② Incubate at 37℃ for 30-60 min protected from light (the optimal incubation time varies for different cells. Take 30 minutes as the initial incubation time and appropriately optimize the incubation time based on the cells used to achieve the best results). Absorption culture medium and slowly add the preheated phenol red-free basic medium at 37°C. Repeat the above aspiration and medium change steps twice and then replace with fresh 37°C preheated phenol red-free basic medium.

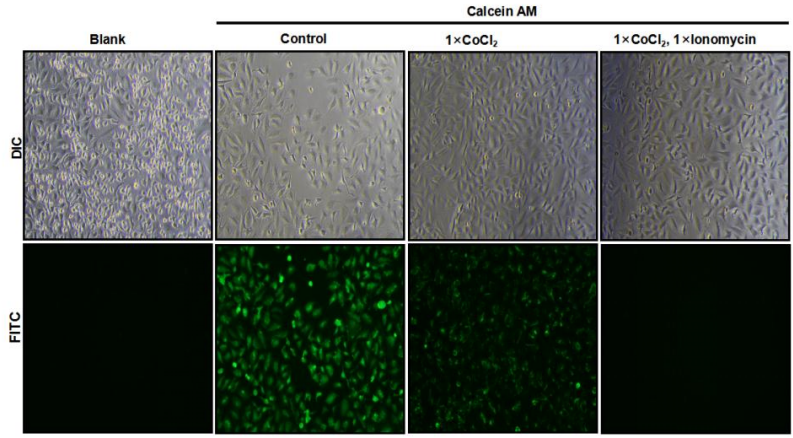
③ Incubate at 37℃ for 10-30 min protected from light (the optimal incubation time varies for different cells. Take 10 minutes as the initial incubation time and appropriately optimize the incubation time based on the cells used to achieve the best results). After incubation, observe with a fluorescence microscope or detect with a flow cytometer after digestion and collection.

Appendix I Performance Characteristics

1. Flow cytometry data for 293T cells:



2. Fluorescence microscope data for Hela cells:



The probe working solution (1×) was incubated with Hela cells, and the cytoplasm, including mitochondria, emitted strong green fluorescence. After the cells were further incubated with the quencher working solution (1×), the green fluorescence of Calcein in the cytoplasm was quenched, leaving only the green fluorescence in the mitochondria. Cells were treated with Ionomycin working solution (1×) to induce a large amount of extracellular Ca^{2+} to enter the cells. Excessive Ca^{2+} entered the mitochondrial matrix, causing mPTP to open and resulting in the release of some Calcein from the mitochondria. At the same time, Co^{2+} entered the mitochondria and led to the green fluorescence quenching of Calcein.

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.