MitoProbe™ Transition Pore Assay Kit (M34153)

Quick Facts

Storage upon receipt:

- ≤–20°C
- Desiccate
- Protect from light

Ex/Em: 494/517 nm

Number of Assays: 100, based on labeling volumes of 1.0 mL

Introduction

The mitochondrion plays a vital role in the processes of apoptotic and necrotic cell death. The mitochondrial permeability transition pore is a nonspecific channel formed by components from the inner and outer mitochondrial membranes, and appears to be involved in the release of mitochondrial components during cell death. The MitoProbe™ Transition Pore Assay Kit is based on published experimentation for mitochondrial transition pore opening.[1,2] This assay employs calcein AM, a colorless and nonfluorescent esterase substrate, and $CoCl_2$, a quencher of calcein fluorescence, to selectively label mitochondria.

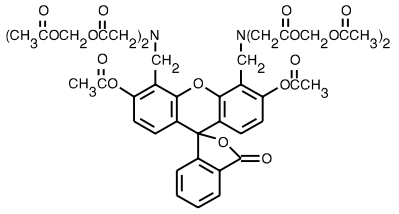

Figure 1. Structure of calcein AM. MW = 994.87.

In a healthy cell, the inner mitochondrial membrane is responsible for maintaining the electrochemical gradient that is essential for respiration and energy production. As Ca^{2+} is taken up and released by mitochondria, a low conductance permeability transition pore appears to flicker between open and closed states.[3] During cell death, the opening of the mitochondrial permeability transition pore dramatically alters the permeability of mitochondria. Continuous pore activation results from mitochondrial Ca^{2+} overload, oxidation of mitochondrial glutathione, increased levels of reactive oxygen species in mitochondria, and other pro-apoptotic conditions.[4] Cytochrome *c* release from mitochondria and loss of mitochondrial membrane potential are observed subsequent to continuous pore activation.

The MitoProbe Transition Pore Assay Kit provides a more direct method of measuring mitochondrial permeability transition pore opening than assays relying on mitochondrial membrane potential alone. Cells are loaded with the acetoxymethyl ester of calcein dye, calcein AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, intracellular esterases cleave the acetoxymethyl esters to liberate the very polar fluorescent dye calcein, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time. The fluorescence from cytosolic calcein is quenched by the addition of $CoCl_2$, while the fluorescence from the mitochondrial calcein is maintained. As a control, cells that have been loaded with calcein AM and $CoCl_2$ can also be treated with an ionophore, ionomycin, to allow entry of excess Ca^{2+} into the cells to trigger mitochondrial pore activation and subsequent loss of mitochondrial calcein fluorescence. The MitoProbe Transition Pore Assay Kit has been tested with Jurkat, MH1C1, and bovine pulmonary artery endothelial cells. The ionomycin response can be blocked with cyclosporine A, a compound reported to prevent mitochondrial transition pore formation by binding cyclophilin D.

Materials

Kit Contents

- Calcein AM, 5 vials, each containing 50 µg of dry powder
- $CoCl_2$, 1 vial containing 1.2 mL $CoCl_2$, 80 mM, in saline
- Ionomycin, 1 vial containing 55 µg ionomycin
- DMSO, 1.5 mL dimethylsulfoxide

Storage and Handling

Upon receipt, components should be stored desiccated at ≤–20°C until required for use. Before opening each vial, allow the product to warm to room temperature. When stored properly, solid calcein AM, DMSO, and solutions of $CoCl_2$ and ionomycin should be stable for at least six months. Once prepared, solutions of calcein AM in DMSO should be used within a short time period for one series of experiments. Sufficient materials are provided for 100 assays, based on labeling volumes of 1.0 mL.

Spectral Characteristics

The approximate excitation and emission peaks of this product after hydrolysis are 494 nm and 517 nm, respectively. Cells labeled with calcein AM can be analyzed by flow cytometry in an instrument equipped with a 488 nm excitation source.

Experimental Protocol

The reagents in the MitoProbe Transition Pore Assay Kit are used to follow mitochondrial transition pore opening following a given experimental treatment, such as induction of apoptosis. The following protocol describes the preparation calcein AM–stained cells, including the appropriate use of $CoCl_2$ (to quench cytosolic fluorescence) and ionomycin (to facilitate mitochondrial calcium overload and subsequent pore formation). The protocol was optimized using Jurkat cells. To achieve optimal results, experimental parameters such as the amount of reagent used for staining should be adjusted depending on cell type and culture conditions used. This protocol can also be adapted for use in conjunction with other probes.

Reagent Preparation

Prepare a 1 mM calcein AM stock solution by dissolving the contents of one vial in 50 µL of the DMSO provided. Mix well and protect from light. Once prepared, solutions of calcein AM in DMSO should preferably be used within a short time period for one series of experiments.

Prepare a 100 µM solution of ionomycin by adding 735 µL of DMSO (Component D) to the Component C vial. Mix well and store at 4˚C when not in use. This solution is stable for 6 months when stored as directed.

Labeling Cells for Analysis in Flow Cytometry

Before you begin, have the following:

- cells of interest as a single-cell suspension in balanced salt solution including calcium
- balanced salt solution including calcium, (e.g. Hanks' Balance Salt Solution (HBSS) with calcium, HBSS/Ca)
- 1 mM stock solution of calcein AM (see *Reagent Preparation*)
- $CoCl_2$ and ionomycin solutions at room temperature
- flow cytometer with 488 nm argon-ion laser and emission filters appropriate for fluorescein

1.1 Resuspend cells of interest in prewarmed HBSS/Ca at a final concentration of 1×10^6 cells/mL.

Note: It is important that the buffer used for the ionomycin control sample includes Ca^{2+}. Calcein AM loading should be performed in the absence of serum. Also, to ensure uniform labeling, it is important that you begin with a well-dispersed-cell suspension.

1.2 Prepare 1 mL aliquots of the cell suspension in separate flow cytometry tubes. For each cell sample, prepare 3 aliquots: one will contain calcein AM only (tube 1), one will contain calcein AM and $CoCl_2$ (tube 2), and the final one will contain calcein AM, $CoCl_2$, and ionomycin (tube 3). Also prepare a sample of the cells containing no added reagents for instrument set up.

1.3 Dilute the 1 mM stock calcein AM 1:500 in HBSS/Ca to make a 2 µM working solution. For example, mix 5 µL of 1 mM calcein AM stock with 2.5 mL of HBSS/Ca. Mix well.

1.4 To tubes 1, 2, and 3, add 5 µL of the working solution of calcein AM (prepared in step 1.3) and mix well.

1.5 To tubes 2 and 3, add 5 µL of $CoCl_2$ (supplied with the kit) and mix well.

1.6 To tube 3, add 5 µL of ionomycin (see *Reagent Preparation*) and mix well.

1.7 Incubate the samples at 37ºC for 15 minutes, protected from light.

1.8 Add ~3.5 mL of HBSS/Ca to the tubes and pellet the cells by centrifugation. This step serves to remove excess staining and quenching reagents.

1.9 Resuspend the pellet in ~400 µL of buffer suitable either for flow cytometric analysis or for further staining, if appropriate. For example, it is possible to further process the sample by staining with labeled annexin V to mark phosphatidylserine translocation, with a the viability stain propidium iodide, or with a mitochondrial vitality stain such as MitoTracker® Red dye. Throughout subsequent steps, the samples should be protected from light as much as possible.

Note: Reagents used for additional staining should be spectrally resolved from calcein fluorescence.

1.10 After staining, place samples on ice and analyze within one hour.

1.11 Analyze the samples using a flow cytometer with 488 nm excitation and emission filters appropriate for fluorescein. The sample containing no added reagents is useful for instrument set up. Tube 1, containing only calcein AM, should show high fluorescence (Figure 2, panel A). Tube 2 containing calcein AM and $CoCl_2$ corresponds to mitochondrial calcein staining alone and shows intermediate fluorescence (Figure 2, panel B). Tube 3 contains all three reagents. The calcein signals from both the cytosol and mitochondrial are quenched in this tube and the sample shows the lowest fluorescence of the three (Figure 2, panel C). The change in fluorescence intensity between tubes 2 and 3 indicates the continuous activation of mitochondrial permeability transition pores.

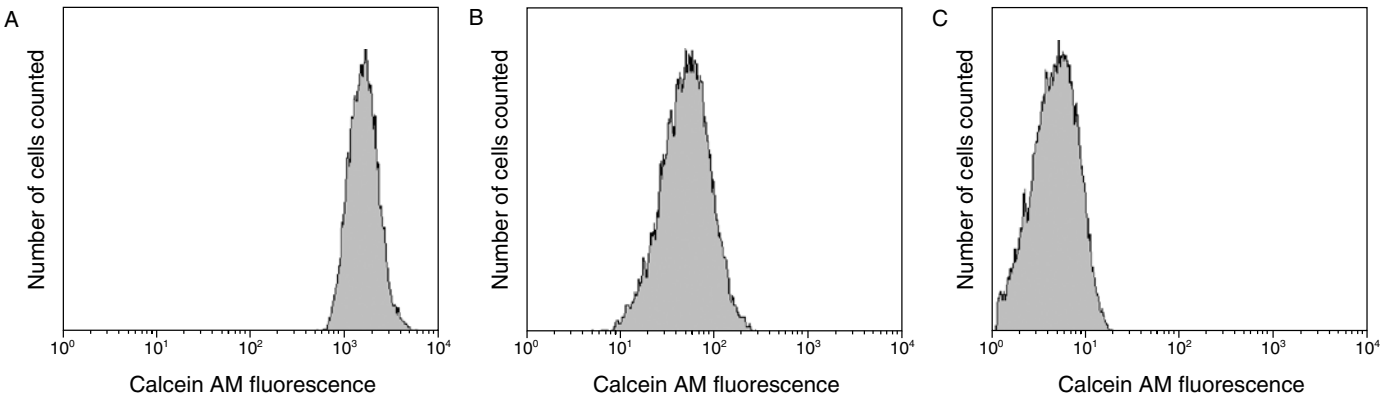

Figure 2. Jurkat cells were incubated with the reagents in the MitoProbe Transition Pore Assay Kit and analyzed by flow cytometry. In the absence of $CoCl_2$ and ionomycin, fluorescent calcein is present in the cytosol as well as the mitochondria, resulting in a bright signal (panel A). In the presence of $CoCl_2$, calcein in the mitochondria emits a signal, but the cytosolic calcein fluorescence is quenched; the overall fluorescence is reduced compared to calcein alone (panel B). When ionomycin, a calcium ionophore, and $CoCl_2$ are added to the cells at the same time as calcein AM, the fluorescence signals from both the cytosol and mitochondria are largely abolished (panel C). The change in fluorescence between panels B and C indicates the continuous activation of mitochondrial permeability transition pores.

References

1. Biophys J 76, 725 (1999); **2.** Biofactors 8, 263 (1998); **3.** Am J Physiol Cell Physiol 279, C852 (2000); **4.** Biochem J 341, 233 (1999).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
M34153	MitoProbe™ Transition Pore Assay Kit *for flow cytometry* *100 assays*	1 kit

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