Dual Sensor: MitoCasp ™

A simultaneous dual parameter Assay For: Mitochondrial Membrane Potential Detection and Caspase Activity.

Patent Pending*

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Introduction

Cell Technology introduces MitoCasp a cell permeable two-color stain to simultaneously detect caspase activity and mitochondrial membrane potential ($\Delta\Psi$) in cells.

Caspase Detection

Apoptosis is an evolutionarily conserved form of cell suicide, which follows a specialized cellular process. The central component of this process is a cascade of proteolytic enzymes called caspases. These enzymes participate in a series of reactions that are triggered in response to pro-apoptotic signals and result in cleavage of protein substrates, causing the disassembly of the cell (1).

Caspases have been identified in organisms ranging from C. *elegans* to humans. The mammalian caspases play distinct roles in apoptosis and inflammation. In apoptosis, caspases are responsible for proteolytic cleavages that lead to cell disassembly (effector caspases), and are involved in upstream regulatory events (initiator caspases). An active caspase consists of two large (~20 kD) and two small (~10 kD) subunits to form two heterodimers which associate in a tetramer (2-4). As is common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity (5).

Mitochondrial Membrane Potential Detection

The loss of mitochondrial membrane potential ($\Delta\Psi$) is a hallmark for apoptosis.

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the electrochemical gradient (referred to as $\Delta\Psi$) across the mitochondrial membrane collapses. The collapse is thought to occur through the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm (11-14).

Assay Principle

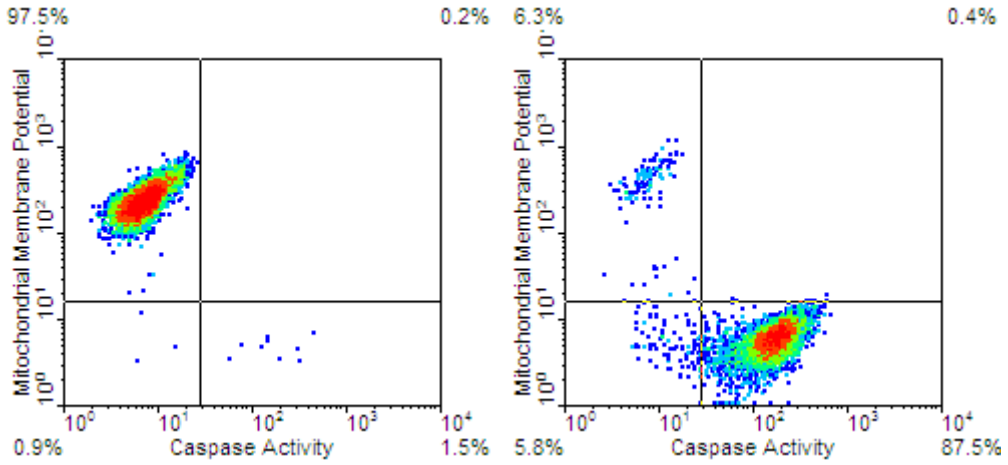
Caspase Detection

Caspase enzymes specifically recognize a 4 amino acid sequence (on their substrate) which necessarily includes an aspartic acid residue. This residue is the target for the cleavage reaction, which occurs at the carbonyl end of the aspartic acid residue(6). Caspases can be detected via immunoprecipitation, immuno-blotting techniques using caspase specific antibodies, or by employing fluorogenic substrates which become fluorescent upon cleavage by the caspase. MitoCasp uses a novel approach to detect active caspases (7-9). The methodology is based on carboxyfluorescein (FAM) labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor binds covalently to the active caspase (10). Cells that contain bound inhibitor can be analyzed by flow cytometry or fluorescence microscopy.

Mitochondrial Membrane Potential Detection

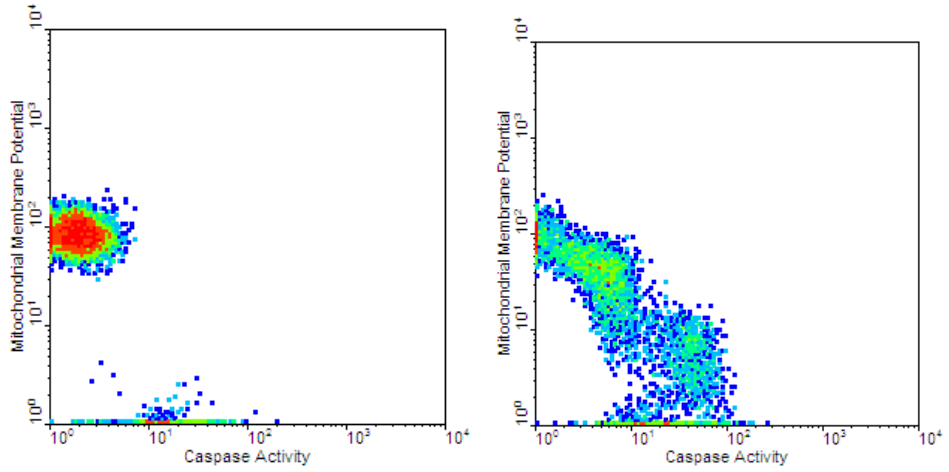
Cell Technology utilizes a cationic dye to visualize mitochondrial membrane potential (15-17). The cationic dye is cell permeable and has a strong fluorescent signal in the red region and exhibits low membrane potential independent (non specific) binding and toxicity. In healthy cells the cationic dye is accumulated by the mitochondria in proportion to the DeltaPsi (membrane potential). In most cell lines, accumulation of the cationic dye in the mitochondria results in a higher fluorescence intensity. In apoptotic cells, where the mitochondrial membrane potential is compromised, the cationic dye does not accumulated in the mitochondria and these cells exhibit a lower fluorescence signal.

Utilizing these two reagents in combination Caspase activity and mitochondrial membrane potential can be analyzed simultaneously.



A **B**

Figure 1. Jurkat cells were stimulated with Staurosporine for 3 hours (B) or DMSO (A). The cells were then stained with the MitoCasp kit according to the protocol. The cells were then washed twice and analyzed by flow cytometry: Ex:488nm Em: FL1 and FL2.
A. Healthy cells show a strong red fluorescence indicating intact mitochondria and no green fluorescence, indicating no active caspases. **B.** Apoptotic cells show a loss of red fluorescence (y axis) indicating loss of mitochondrial membrane potential and positive green fluorescence (x axis) indicating active caspases.



A **B**

Figure 2
HeLa Cells activated with Staurosporine (B) or DMSO (A) for 3 hours. The cells were then stained with MitoCasp kit according to the protocol. After staining the cells were washed twice and analyzed by flow cytometry: Ex:488nm Em: FL1 and FL2.
A. Healthy cells show a strong red fluorescence indicating intact mitochondria and no green fluorescence, indicating no active caspases. **B.** Cells in transition to apoptosis, loss of red fluorescence (y axis) indicating loss of mitochondrial membrane potential accompanied by positive green fluorescence (x axis) indicating active caspases.

A. Warnings and Precautions

1. For Research Use Only. Not for use in diagnostic procedures.
2. We are not aware of any toxicity data for Mito Flow Reagent. Gloves, protective clothing and eyewear should be worn and safe laboratory practices followed.
3. Some cells over expressing multidrug resistance proteins may show a low mitochondrial membrane potential. See Technical note #1 below.

B. Storage and Shelf Life

1. Store the kit at 2-4°C until first use.
2. Reconstituted cationic dye and FAM-PEPTIDE-FMK should be aliquoted in small amounts sufficient for one day of experimental work and stored at -20°C. Protect from light.
3. Avoid multiple freeze-thaw cycles.

C. Kit Components

Note: All MITCAPx00-2 kits (100 Tests) would be shipped as 4 x MITCAPx00-1 (25 Tests).

1.

MitoCasp: Poly caspase Detection	Cat# MITCAP100-1: 25 Tests Cat# MITCAP100-2: 100 Tests	Part# 5031 Part# 5032
1. Mitochondria Membrane Potential Dye (Cationic Dye)		Part# 4015
2. 1X Dilution Buffer		Part# 3032
3. FAM-VAD-FMK(Poly Caspase)		Part# 8001
4. 10 X Wash Buffer		Part# 3028

2.

MitoCasp : Caspase 3/7 Detection	Cat# MITCAP200-1: 25 Tests Cat# MITCAP200-2: 100 Tests	Part# 5033 Part# 5034
1. Mitochondria Membrane Potential Dye (Cationic Dye)		Part# 4015
2. 1X Dilution Buffer		Part# 3032
3. FAM-DEVD-FMK (Caspase 3/7)		Part# 8002
4. 10 X Wash Buffer		Part# 3028

3.

MitoCasp : Caspase 8 Detection	Cat# MITCAP300-1: 25 Tests Cat# MITCAP300-2: 100 Tests	Part# 5050 Part# 5051
1. Mitochondria Membrane Potential Dye (Cationic Dye)		Part# 4015
2. 1X Dilution Buffer		Part# 3032
3. FAM-LETD-FMK (Caspase 8)		Part# 8004
4. 10 X Wash Buffer		Part# 3028

4.

MitoCasp : Caspase 9 Detection	Cat# MITCAP400-1: 25 Tests Cat# MITCAP400-2: 100 Tests	Part# 5036 Part# 5037
1. Mitochondria Membrane Potential Dye (Cationic Dye)		Part# 4015
2. 1X Dilution Buffer		Part# 3032
3. FAM-LEHD-FMK (Caspase 9)		Part# 8003
4. 10 X Wash Buffer		Part# 3028

5.

MitoCasp : Caspase 1 Detection	Cat# MITCAP600-1: 25 Tests Cat# MITCAP600-2: 100 Tests	Part# 5052 Part# 5053
1. Mitochondria Membrane Potential Dye (Cationic Dye)		Part# 4015
2. 1X Dilution Buffer		Part# 3032
3. FAM-YVAD-FMK (Caspase 1)		Part# 8005
4. 10 X Wash Buffer		Part# 3028

D. Materials Required But Not Supplied

1. Solutions
    a. Dimethyl Sulfoxide (DMSO)
2. Equipment
    a. Flow Cytometer with 488nm laser for excitation and emission in FL1 and FL2 channels.
    b. FACS tubes

E. Preparation and Setup

Note: Protect reagent from light at all times. Each investigator should titrate out the reagents to optimize staining.

1. Dilution of Mitochondria Membrane Potential Dye Reagent (Cationic Dye).

A. Reconstitute the Mitochondria Membrane Potential Dye Reagent by adding 50 µL DMSO to the vial to obtain a 150X stock solution.

B. Mix by vortexing the vial several times at room temperature until contents are completely dissolved.

C. Aliquot the the reconstituted reagent in small amounts sufficient for one day of experimental work and store the remainder at -20°C in amber vials.

D. Immediately prior to use, dilute the 150X reagent to 30X. This can be accomplished by diluting the Reagent 1:5 with the 1X dilution buffer. Vortex the solution until completely dissloved.
E. Add 10µL of 30X Reagent per 300 µL sample.

2. Dilution of FAM-VAD-FMK /FAM-DEVD-FMK / FAM-LEHD-FMK / FAM-LETD-FMK FAM-YVAD-FMK caspase detection reagent.
A. Reconstitute the reagent by adding 50 µL DMSO to the vial to obtain a 150X stock solution.
B. Mix by vortexing the vial several times at room temperature until contents are completely dissolved.

C. Aliquot the reconstituted reagent in small amounts sufficient for one day of experimental work and store the remainder at -20°C in amber vials.
D. Immediately prior to use, dilute the 150X reagent to 30X. This can be accomplished by diluting the Reagent 1:5 with the 1X dilution buffer. Vortex the solution until completely dissloved.
E. Add 10µL of 30X Reagent per 300 µL sample.

3. Dilution of 10X Wash Buffer.
A. If necessary warm the 10X Wash Buffer until any salt crystals are completely dissolved.
B. Dilute the Wash Buffer 1:10 with DI water (e.g. 1ml 10X assay buffer + 9ml DI water).

F. Staining Protocol For Suspension Cells

1. Read Section H Analyzing Samples: Flow Cytometer: in order to set up proper controls for compensation if needed.
2. Cells should be cultured to a density not to exceed 1x 10^6 cells/mL.
Note: Each cell line should be evaluated on an individual basis to determine optimal density for cell culture and apoptosis induction.
3. Induce apoptosis according to your specific protocol and or add test compounds.
A negative control should also be set up at this time point.
4. After the required activation time transfer 300 µL of cells for each test sample to a FACS tube prior to staining.
5. Add 10µL each of the 30X Mitochondria Membrane Potential Dye and 30X Caspase detection reagent to each tube. Vortex the samples.
6. Incubate the samples for 30 to 60 minutes at the same culture conditions as required in the experimental protocol (e.g. 37^0C at 10% CO_2).
7. Next wash the samples 2 times with 2mL of 1X Wash Buffer.
8. Analyze samples on flow cytometer using: Excitation: 488nm and detect emission: Caspase Detection Reagent: FL1 (e.g FITC filter) and Mitochondria Membrane Potential Dye: FL2 (Phycoerytherin: PE filter).

Do not fix samples. Fixing samples will lead in mitochondrial membrane potential collapse and erroneous results.

G. Staining Protocol For Monolayer Adherent Cells

1. Culture cells in 96 well plates at 100µL per well or by any other suitable culture dish.
2. Induce apoptosis according to your specific protocol and or add test compounds. A negative control should also be set up at this time point.
3. After the required activation time, add 10µL each of the 30X Mitochondria Membrane Potential Dye and 30X Caspase detection reagent to each well.

4. Incubate the samples for 30 to 60 minutes at the same culture conditions as required in the experimental protocol (e.g. 37^0C at 10% CO_2).
5 Next wash the wells with PBS. This can be accomplished by aspirating out the media and adding PBS to the wells. Save the aspirate as it may contain detached apoptotic cells.
6. Next aspirate out the PBS and again save the aspirate. Repeat this wash step one more time.
7. Now the cells can be detached via any common method (e.g. trypsin).
8. After detachment the cells and their respective saved aspirates should be transferred to a FACS tube, washed twice with the 1X wash buffer and analyzed via flow cytometry.
Do not fix samples. Fixing samples will lead in mitochondrial membrane potential collapse and erroneous results.

H. Analyzing Samples: Flow Cytometer

These controls are included as a suggestion. Each investigator should consult their instrument manufacture for further details.

1. Controls. Set up single stained controls for compensation.
   Control 1: Mitochondria Membrane Potential Dye stain only in non-apoptotic cells. This will allow to set compensation for FL2 bleed over into FL1.
   Control 2: Caspase Detection Reagent only in apoptotic cells. This will allow to set compensation for FL1 bleed over into FL2.
2. Other Controls if needed.
   Control 3: Caspase Detection Reagent only in non-apoptotic cells to set up FL1 background.
   Control 4: Mitochondria Membrane Potential Dye stain only in apoptotic cells. This will help determine background of FL2.
3. Set up flow cytometer so cells can be gated with a forward scatter vs side scatter plot as depicted in figure 3 below.

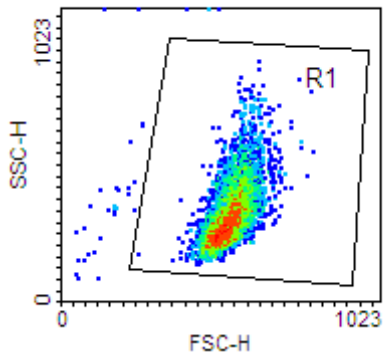


Figure 3.

4. Next set up dot plot histogram FL2 vs FL1 as depicted in figure 4 below.

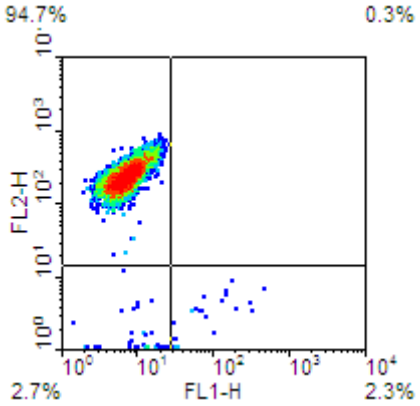


Figure 4.

5. Run controls to set compensation and background signal. Next run samples.

I. Analyzing Samples in Fluorescent Plate Reader or Fluorescent Microscope

1. Caspase Detection: Ex:488 nm / Em: 515-530 nm.
2. Mitochondrial membrane potential Detection: Ex: 488 to 549nm (optimal is 549nm)/ Emission: 574nm to 600nm (optimal is 574nm).

I. Technical Notes

1. Cell lines over expressing Multidrug Resistance proteins and p-glycoproteins.

Over expression of Multidrug Resistance proteins and p-glycoproteins is associated with a decrease in intracellular accumulation of certain compounds including the Mitochondria Membrane Potential dye. This will result in falsely low readings of mitochondrial membrane potential (8). Pre treatment of cells with inhibitors of Multidrug Resistance proteins and p-glycoproteins for 24 hours prior to staining may solve this (8).

2. Analyzing Cell via Flow Cytometry

Non-apoptotic healthy cells will have a strong FL2 fluorescence indicating accumulation of cationic dye due to mitochondrial membrane potential. They will be caspase negative, background FL1 signal. Cell lines have basal levels of cells undergoing apoptotic, which will vary between cell lines and culture conditions.
Apoptotic cells will show loss of FL2 due to mitochondrial membrane potential loss and lack of the cationic dye accumulation in the mitochondria. These apoptotic cells will become FL1 positive indicating caspase activity.

Ordering Information

Catalog #	Size
MitCap100-1 (Poly Caspase)	25 Tests
MitCap100-2 (Poly Caspase)	100 Tests
MitCap200-1 (Caspase 3/7)	25 Tests
MitCap200-2 (Caspase 3/7)	100 Tests
MitCap300-1 (Caspase 8)	25 Tests
MitCap300-2 (Caspase 8)	100 Tests
MitCap400-1 (Caspase 9)	25 Tests
MitCap400-2 (Caspase 9)	100 Tests
MitCap600-1 (Caspase 1)	25 Tests
MitCap600-2 (Caspase 1)	100 Tests

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