JC-1
Mitochondrial Membrane Potential Detection Kit.

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CELL TECHNOLOGY

Introduction

The loss of mitochondrial membrane potential ($\Delta\Psi$) is a hallmark for apoptosis. The APO LOGIX JC-1 Assay Kit measures the mitochondrial membrane potential in cells. In non-apoptotic cells, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) exists as a monomer in the cytosol (green) and also accumulates as aggregates in the mitochondria which stain red. Whereas, in apoptotic and necrotic cells, JC-1 exists in monomeric form and stains the cytosol green

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Background

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 (1-4).

The JC-1 Assay Kit uses a unique cationic dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) to signal the loss of the mitochondrial membrane potential (5). In healthy cells, the dye stains the mitochondria bright red (6). The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. When the critical concentration is exceeded, J-aggregates form which become fluorescent red. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria.

In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells which show red and green fluorescence. The aggregate red form has absorption/emission maxima of 585/590 nm (5). The green monomeric form has absorption/ emission maxima of 510/527 nm. Both apoptotic and healthy cells can be visualized simultaneously by fluorescence microscopy using a wide band-pass filter suitable for detection of fluorescein and rhodamine emission spectra. The JC-1™ reagent is easy to use. Simply dilute the reagent in cell culture medium and add to the cells. After a 15 minute incubation, wash the cells and analyze by flow cytometry or fluorescence microscopy or fluorescence plate reader.

Warnings and Precautions

1. For Research Use Only. Not for use in diagnostic procedures.
2. We are not aware of any toxicity data for JC-1. Gloves, protective clothing and eyewear should be worn and safe laboratory practices followed.

Storage and Shelf Life

1. Store the kit at 2°C to 8°C until first use. The performance of this product is guaranteed for six months from the date of purchase if stored and handled properly.
2. Reconstituted JC-1 reagent should be aliquoted in small amounts sufficient for one day of experimental work and stored at -20°C, protected from light and moisture (preferably in a desiccator).
3. Avoid multiple freeze-thaw cycles.

Kit Components

1. JC-1 Reagent (lyophilized)
2. 10X Assay Buffer.

Materials Required But Not Supplied

1. Solutions
a. Phosphate-Buffered Saline (PBS)
b. Dimethyl Sulfoxide (DMSO)
2. Equipment
a. Flow cytometer, equipped with a 15 mW, 488 nm argon excitation laser, with appropriate filters.
or
b. Fluorescence microscope with appropriate filters.
or
c. Fluorescence plate reader and black 96-well plates.

Preparation and Setup

Dilution of JC-1 Reagent
1. Reconstitute the lyophilized vial with 500 µL DMSO to obtain a 100X stock solution.
2. Mix by inverting the vial several times at room temperature until contents are completely dissolved.
3. Aliquot the resuspended JC-1 reagent in small amounts sufficient for one day of experimental work and store the remainder at -20°C in amber vials.
4. Immediately prior to use, dilute the 100X JC-1 reagent to 1X: Dilute the JC-1 1:100 in 1X assay buffer or per warmed media of your choice. Vortex the solution.
To remove any undissolved particulate matter, centrifuge the dye/media solution for 1 minute at 13,000 x g and carefully transfer the supernatant without disturbing pelleted debris into a fresh tube.
Protect reagent from light at all times.

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

A. Staining Protocol For Flow Cytometry

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL.
Each cell line should be evaluated on an individual basis to determine optimal cell density for apoptosis induction.
2. Induce apoptosis according to your specific protocol.
3. Transfer 0.5 mL cell suspension into a sterile centrifuge tube.
4. Centrifuge for 5 minutes at room temperature at 400 x g.
5. Remove the supernatant.
6. Resuspend cells in 0.5 ml 1X JC-1 Reagent solution prepared in step 4, above, under Dilution of JC-1 Reagent.
7. Incubate the cells at 37°C in a 5% CO_2 incubator for 15 minutes.
8. Centrifuge for 5 min at 400 x g and remove supernatant.
9. Resuspend the cell pellet in 2 mL cell culture medium or 1X Assay Buffer followed by centrifugation.
Remove the supernatant.
10. Repeat step 9.
11. Resuspend the cell pellet in 0.5 mL fresh cell culture medim or 1X Assay Buffer.
Cells are now ready for flow cytometry analysis.

Quantification by Flow Cytometry

Analyze cells **immediately** following step 11 by flow cytometry. Mitochondria containing red JC-1 aggregates in healthy cells are detectable in the FL2 channel,

and green JC-1 monomers in apoptotic cells are detectable in the FITC channel (FL1).

A. Instrument Set Up

Two Parameter Analysis

1. Run the uninduced control sample first. Generate a log FL1 (X-axis) versus log FL2 (Y-axis) dot plot. Add regions R2 and R3 (as in Figure 2) to the dot plot.
2. Adjust the FL1and FL2 PMT voltages to register a dual positive population in region 2 (R2). The peak of the dual positive population should fall within the second and third log decade scale of FL1and FL2.
3. The region 2 (R2) gate should be adjusted to include >95% of events. This number will vary depending on the condition of the cells.
4. Run the induced sample, using the PMT settings established above for the uninduced control sample. One should see a population of cells that appears in the region 3 (R3). This reflects a loss of red emission on the FL2 axis, which corresponds to the loss of mitochondrial membrane potential in induced cells.
5. If the induced sample exhibits only a minimal decrease in red emission, increase the FL2-%FL1 compensation.
6. Repeat steps 3 and 4.

See Section 3: TECH NOTE #1: *Flow Cytometer Settings*
See Section 3: TECH NOTE #2: *Quadrant*

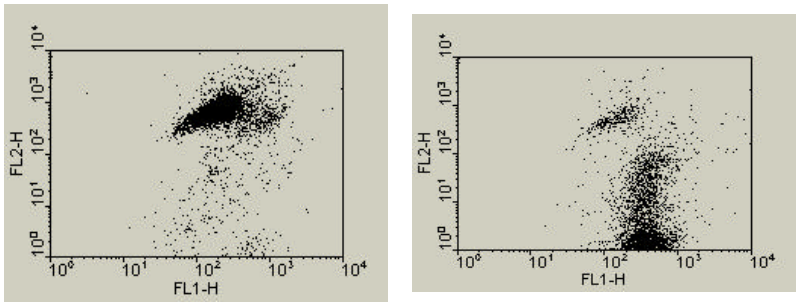

Figure 1. Mitochondrial Staining in Jurkat Cells Using JC-1. Flow Cytometry (Two Parameter Analysis)
Jurkat cells were treated with DMSO (Left) or 1.5 uM staurosporine (Right) for 3 hours. Cells were labeled with APO-LOGIC JC-1 reagent for 15 minutes. After washing, cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer. A dot plot of red fluorescence (FL2) versus green fluorescence (FL1) resolved live cells with intact mitochondrial membrane potential (Left) from apoptotic and dead cells with lost mitochondrial membrane potential (Right). Note the increase in cells numbers with decreased red fluorescence (Right).

B. Staining Protocol for Fluorescence Microscopy

a. Staining of Cells in Suspension

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL.

Each cell line should be evaluated on an individual basis to determine the optimal cell density for apoptosis induction.

2. Induce apoptosis according to your specific protocol.
3. Transfer 0.5 ml cell suspension into a sterile centrifuge tube.
4. Centrifuge for 5 minutes at room temperature at 400 x g.
5. Remove the supernatant.
6. Resuspend cells in 0.5 mL 1X JC-1 reagent prepared in step 4:

Dilution of JC-1 Reagent

7. Incubate the cells at 37°C in a 5% CO_2 incubator for 15 minutes.
8. Centrifuge for 5 min at 400 x g and remove supernatant.

9. Resuspend the cell pellet in 2 mL 1X Assay Buffer followed by centrifugation. Remove supernatant.
10. Resuspend the cell pellet in 0.3 mL Assay Buffer.
11. Observe **immediately** with a fluorescence microscope using a "dual-bandpass" filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red™*. In live non-apoptotic cells, the mitochondria will appear red following aggregation of the JC-1 reagent. The red aggregates emit at 590 nm. In apoptotic and dead cells, the dye will remain in its monomeric form and will appear green with an emission at 530 nm
See Section 3: TECH NOTE #3: *Fluorescence Filters*
*Texas Red™ is a Trademark of Molecular Probes, Inc.

B. Staining of Monolayer Cells

1. Grow cells on a glass cover slip in a petri dish or in a chamberslide. Induce cells according to your specific protocol.
2. Dilute JC-1 reagent to 1X immediately prior to use. As in step 4 under Dilution of JC-1 Reagent
Note: To remove undissolved particles, centrifuge the dye/media solution for 1 minute at 13,000 x g and carefully transfer the supernatant without disturbing pelleted debris into a fresh tube.
3. Remove the cell culture media and replace with enough diluted 1X JC-1 reagent sufficient to cover the cells.
4. Incubate the cells at 37°C in a 5% CO_2 incubator for 15 minutes.
5. Remove media and wash once with 1X Assay Buffer.
6. Add a drop of PBS and cover with a coverslip.
7. Observe **immediately** with a fluorescence microscope using a "dual-bandpass" filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red. In live non-apoptotic cells, the mitochondria will appear red following aggregation of the JC-1 reagent. The red aggregates emit at 590 nm. In apoptotic and dead cells the dye will remain in its monomeric form and will appear green with an emission at 530 nm.

C. Staining Protocol for Fluorescence Ratio Detection (7,8)

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL.
Each cell line should be evaluated on an individual basis to determine the optimal cell density for apoptosis induction.
2. Induce apoptosis according to your specific protocol.
3. Transfer 0.5 mL cell suspension into a sterile centrifuge tube.
4. Centrifuge for 5 minutes at room temperature at 400 x g.
5. Remove the supernatant.
6. Resuspend cells in 0.5 mL 1X JC-1 reagent prepared in step 4 under: Dilution of JC-1 Reagent
7. Incubate the cells at 37°C in a 5% CO_2 incubator for 15 minutes.
8. Centrifuge for 5 min at 400 x g and remove supernatant.
9. Resuspend the cell pellet in 2 mL 1X Assay Buffer followed by centrifugation. Remove supernatant.
10. Repeat step 9.
11. Resuspend the cell pellet in 300 mL Assay Buffer.
12. Transfer 100 mL cell suspension into each of three wells of a black 96-well plate.
13. Measure red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) using a fluorescence plate reader.
14. Determine the ratio of red fluorescence divided by green fluorescence.

The ratio of red to green fluorescence is decreased in dead cells and in cells undergoing apoptosis compared to healthy cells.

Tech Notes

TECH NOTE #1: *Flow Cytometer Settings.*
A typical setting for the analysis of JC-1 staining on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer is as follows:
FL1 PMT voltage 511
FL2 PMT voltage 389
Compensation: FL1 – 10.5% FL2
FL2 – 25.9% FL1
TECH NOTE #2: *Quadrants*.
On instruments where it is not possible to add regions to the dot plot, quadrants are added instead, using the following protocol:
1. Run the uninduced control sample first. Generate a log FL1 (X-axis) versus log FL2 (Y-axis) dot plot. Add quadrants to the dot plot.
2. Adjust the FL1and FL2 PMT voltages to register a dual positive population in quadrant 2 with the dual positive population falling within the second and third log decade scale of FL1and FL2.
3. The quadrant 2 markers should be adjusted so that the statistics read >95% on gated events.
4. Run the induced sample, using the PMT settings established above for the uninduced control sample. One should see a population of cells that appears in the 4th quadrant. This reflects a loss of red emission on the FL2 axis.
5. If the induced sample exhibits only a minimal decrease in red emission, increase the FL2-%FL1 compensation.
6. Repeat steps 3 and 4.
TECH NOTE #3: *Fluorescence Filters for Fluorescence Microscopy.*
a. Both the red JC-1 aggregate and the green monomer can be viewed with a "double-bandpass" filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red™*.
b. JC-1 aggregates can be viewed with a bandpass filter designed to detect rhodamine (excitation 540 nm, emission 570 nm) or Texas Red (excitation 590 nm, emission 610 nm).
c. JC-1 monomers are dected with a bandpass filter used for the detection of fluorescein (excitation 490 nm, emission 520 nm).

References Cited in Manual

1. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J.C. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J. Cell Biol. 144 (5): 891-901 (1999).

2. Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Tsujimoto, Y. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc. Natl. Acad. Sci. USA 95: 14681-14686 (1998).

3. Basanez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K. A., Hsu, Y. T., Zimmerberg, J., and Youle, R. J. Bax , but not Bcl-X_L decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. Proc. Natl. Acad. Sci. USA 96: 5492-5497 (1999).

4. Luo, X., Budihardio, I., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94: 481-490 (1998).

5. Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T. W., Steele, G.D., and Chen, L. B. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate forming lipophilic cation JC-1. Proc. Natl. Acad. Sci. USA 88: 3671-3675 (1991).

6. Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G., and Franceschi, C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). Biochem. Biophys. Res. Commun. 197 (1): 40-45 (1993).

7. Reers, M., Smith, T. W., and Chen, L. B. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 30: 4480-4486 (1991).

8. White, R. J., and Reynolds, I. J. Mitochondrial depolarization in glutamatestimulated neurons: an early signal specific to excitotoxin exposure. Journal of Neuroscience 16: 5688-5697 (1996).