NEUROMICS

MitoPT™

Catalog#: KF17305

Mitochondrial Permeability Transition Detection Kit

non-apoptotic cells appear red

apoptotic cells appear green

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1. Introduction

Detection of the mitochondrial permeability transition event (PT) provides an early indication of the initiation of cellular apoptosis. This process is typically defined as a collapse in the electrochemical gradient across the mitochondrial membrane, as measured by the change in the membrane potential ($\Delta\Psi$).

Changes in the mitochondrial $\Delta\Psi$ lead to the insertion of proapoptotic proteins into the membrane and possible oligomerization of BID, BAK, BAX or BAD. This could create pores, which dissipate the transmem-brane potential, thereby releasing cytochrome c into the cytoplasm (1-5).

Loss of mitochondrial $\Delta\Psi$, indicative of apoptosis, can be detected by a unique fluorescent cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarbocyanin iodide, commonly known as JC-1 (6). This dye has been incorporated into the user-friendly MitoPT™ kit for the simple and reproducible detection of the PT event in apoptotic cells.

The structure of Immunochemistry Technologies' MitoPT™ reagent allows it to easily penetrate cells and healthy mitochondria. Once inside a healthy non-apoptotic cell, the lipophilic MitoPT™ reagent, bearing a delocalized positive charge, enters the negatively charged mitochondria where it aggregates and fluoresces red (7). These aggregates, first described by Jelley in 1937, are referred to as J-aggregates (8). When the mitochondrial $\Delta\Psi$ collapses in apoptotic cells, the MitoPT™ reagent no longer accumulates inside the mitochondria. Instead, it is distributed throughout the cell. When dispersed in this manner, the MitoPT™ reagent assumes a monomeric form, which fluoresces green (9). Use of the MitoPT™ kit allows the easy distinction between non-apoptotic red fluorescent cells and apoptotic green fluorescent cells.

The MitoPT™ kit is used in conjunction with your existing apoptosis protocols. Grow your cells in culture and induce apoptosis according to your existing procedure (also reserve a non-induced population of cells as a negative control). Once you have induced apoptosis in your cells, add the 1X MitoPT™ solution to each population and incubate the cells for an additional 15 minutes. During this incubation time, the MitoPT™ reagent will enter each cell and the mitochondria inside. If the cell is not undergoing apoptosis, the mitochondrial $\Delta\Psi$ will be intact, and the MitoPT™ reagent will aggregate inside the mitochondria and fluoresce red. If the cell is apoptotic, the mitochondrial $\Delta\Psi$ will be breaking down, thereby causing the MitoPT™ reagent to be dispersed throughout the entire cell and fluoresce green.

The MitoPT™ reagent excites at 488-490 nm. The monomeric dye structure emits at 527 nm, whereas the J-aggregates in healthy (non-apoptotic) mitochondria emit at 590 nm (7).

The MitoPT™ kit can evaluate apoptosis using three different technologies: flow cytometers; fluorometric plate readers; and fluorescence microscopes.

Following the flow cytometer and fluorescence microscope protocols, each sample to be stained requires only 0.5 mL of 1X MitoPT™ solution (equal to 5 μL of 100X MitoPT™ stock). The MitoPT™-25 Kit will test 25 samples; the MitoPT™-100 Kit will test 100 samples. Following the fluorescence plate reader protocol, each sample requires 1 mL of 1X MitoPT™ solution (equal to 10 μL of 100X MitoPT™ stock). The MitoPT™-25 Kit will test 12 samples; the MitoPT™-100 Kit will test 50 samples.

When cells stained with MitoPT™ are run through a flow cytometer, the instrument will measure apoptosis by monitoring the amount of red fluorescence in each region. Healthy cells, which fluoresce red, will appear in R2. As the mitochondrial $\Delta\Psi$ collapses and cells enter apoptosis, the amount of red fluorescence will drop. An increasing number of cells will fall into R3 corresponding to a loss of red fluorescence as the dispersed MitoPT™ dye coverts to a monomeric form and fluoresces green (see Section 18 for sample data).

When cells stained with MitoPT™ are analyzed with a fluorescence plate reader, the instrument will measure apoptosis by monitoring the amount of red fluorescence. Healthy cells will give a high OD reading of red fluorescence. As the mitochondrial $\Delta\Psi$ collapses, indicating apoptosis, an increasing number of cells will lose red fluorescence and give a lower OD reading as the dispersed MitoPT™ dye coverts to a green monomeric form (see Section 21 for sample data).

Looking at the cells under a fluorescence microscope, non-apoptotic cells will appear to have red fluorescent spots (the MitoPT™ dye aggregates) within healthy mitochondria. In contrast, apoptotic cells will appear mostly green. Cells still undergoing apoptosis will contain less and less red dye aggregate spots in the mitochondria, and the entire cell will appear more and more green as the mitochondrial $\Delta\Psi$ disintegrates, thereby dispersing the MitoPT™ dye (see Section 25 for sample data).

2. Contents of the MitoPT™ Kit:

- MitoPT™ Reagent, lyophilized, part# 684 (25) or 657 (100).
- 10X Assay Buffer, part# 686 (15 mL), or 685 (60 mL).

- Assay Manual with protocols for 3 applications: Flow Cytometer; Fluorometer; and Fluorescence Microscope.

3. Recommended Materials and Equipment (not all are required):

- Cultured cells and media
- Protocol and reagents to induce apoptosis
- 15 mL polystyrene centrifuge tube (1 per sample)
- Microfuge at 13,000 X g, Clinical centrifuge at 400 – 1,000 X g
- Pipette(s) capable of dispensing at 10 µL, 500 µL, and 1 mL
- Graduated cylinder
- Dimethyl Sulfoxide - DMSO (125 µL or 500 µL)
- Vortexer
- Amber vials or polypropylene tubes for storage at –20°C
- 37°C CO_2 incubator
- Hemocytometer

4. Instrumentation (not all are required):

- Flow cytometer with excitation between 488 - 490nm, and emission at 527 and 590 nm.
- 96-well fluorescence plate reader with excitation between 488 -490 nm, and emission at 590 nm (dual emission at 527 nm and 590 - 600 nm is best), with endpoint reading and black round or flat bottom 96-well microtiter plates.
- Fluorescence microscope with broad band path filters and slides.

5. Storage and Shelf-Life

- Store the unopened kit (and each unopened component) at 2°C to 8°C until the expiration date.
- Once reconstituted, store the 100X MitoPT™ stock at –20°C for 6 months. It may be frozen twice.
- Once diluted, store the 1X assay buffer at 2°C to 8°C up to 7 days.

6. Safety Information

- Use gloves while handling the MitoPT™ reagent, and the 10X assay buffer.
- Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be tossed in standard trash bins.

7. Overview of the MitoPT™ Protocol

Staining cells with MitoPT™ takes only about 15 minutes. However, the MitoPT™ kit is used with living cells, which may take several hours to prepare. In addition, once the cells are grown, they must be induced to undergo apoptosis, which may also take several hours to complete. As the

1X MitoPT™ solution must be used immediately, prepare the MitoPT™ reagents at the end of your apoptosis induction process. Here is a quick overview of the MitoPT™ protocol:

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol, but not to exceed 10^6 cells/mL.
2. At the same time, culture a non-induced negative control cell population (at the same density as the induced population).
3. Induce apoptosis following your protocol (2 sample protocols are mentioned in Section 8).
4. Prepare 1X assay buffer and warm to 37°C (Section 9).
5. Prepare 100X MitoPT™ stock (Section 10).
6. Prepare 1X MitoPT™ solution (Section 11 or 13).
7. Stain cells with 1X MitoPT™ solution (Section 14, 19, 22, or 23).
8. Analyze data (Section 15, 20, or 24).

8. Induction of Apoptosis

The MitoPT™ kit works with your current apoptosis protocols. 2 examples of protocols to induce apoptosis in suspension culture are: a) treating Jurkat cells with 2 μg/ml camptothecin (or 1 μM staurosporine) for 3 hours; and b) treating HL-60 cells with 4 μg/ml camptothecin (or 1 μM staurosporine) for 4 hours.

9. Preparation of 1X Assay Buffer

The assay buffer is formulated for use as reaction buffer, and for washing the cells. It is supplied as a 10X concentrate which must be diluted to 1X with DI H_20 prior to use.

1. If necessary, gently warm the 10X concentrate to completely dissolve any salt crystals that may have come out of solution.
2. For the MitoPT™-25 Kit, add the entire bottle (15 mL) of 10X assay buffer to 135 mL of DI H_2O.
3. Or, for the MitoPT™-100 Kit, add the entire bottle (60 mL) of 10X assay buffer to 540 mL of DI H_2O
4. Or, if not using the entire bottle, dilute the 10X assay buffer 1:10 in DI H_2O. For example, add 10 mL 10X assay buffer to 90 mL DI H_2O.
5. Stir the solution for at least 5 minutes.
6. If using the 1X assay buffer the same day it was prepared, warm it to 37°C prior to use (it is used in Sections 8, 10, 12, 18, 22, and 23).
7. Or, if not using the 1X assay buffer the same day it was prepared, store it at 2°C to 8°C up to 7 days.

10. Reconstitution of the 100X MitoPT™ Stock

The MitoPT™ dye reagent is supplied as a highly concentrated lyophilized powder. It must first be reconstituted, forming a 100X stock concentrate, and then diluted 1:100 to form a final 1X working solution. The 1X working solution must be prepared immediately prior to use; however, the reconstituted 100X stock can be stored at –20°C for 6 months, and used twice during that time.

- **The newly reconstituted 100X MitoPT™ stock must be used or frozen immediately after it is prepared and protected from light during handling.**

1. For the 25 test kit, reconstitute the 25-test vial (part# 684) with 125 µL DMSO at room temperature (RT), forming a 100X stock.
2. Or, for the 100 test kit, reconstitute the 100-test vial (part# 657) with 500 µL DMSO at room temperature (RT), forming a 100X stock.
3. Re-cap the vial and invert it several times to fully dissolve the MitoPT™ dye reagent.
4. Immediately use the 100X stock by diluting it to 1X (Section 11).
5. Or, aliquot and store it at –20°C (Section 12).

11. Preparation of 1X MitoPT™ Solution for Immediate Use

Using the freshly reconstituted 100X MitoPT™ stock, prepare the 1X working strength MitoPT™ solution by diluting the stock 1:100 with 37°C 1X assay buffer (or, you may substitute your own cell culture media, warmed to 37°C, in place of the 1X assay buffer). Each sample to be stained requires only 0.5 mL of 1X MitoPT™ solution (equal to 5 µL of 100X MitoPT™ stock).

- **The 1X working strength MitoPT™ solution must be used immediately – prepare it as apoptosis induction is completed.**

1. Warm the 1X assay buffer (or cell culture media) to 37°C.
2. For the 25-test kit, add 125 µL of the 100X MitoPT™ stock from each vial to 12.375 mL of the 37°C 1X assay buffer or cell culture media.
3. Or, for the 100-test kit, add 500 µL of the 100X MitoPT™ stock to 49.5 mL of the 37°C 1X assay buffer or cell culture media.
4. Or, if not using the entire vial of 100X MitoPT™ stock, dilute it 1:100 in 37°C 1X assay buffer or cell culture media. For example, add 10 µL of 100X MitoPT™ stock to 990 µL of 1X assay buffer or cell culture media.
5. Vortex the 1X working strength MitoPT™ solution thoroughly.
6. If particulate matter is present, the solution should be clarified by centrifugation at 13,000 X g in a microfuge for 3 minutes, or 15 minutes

in a clinical centrifuge at 1,000 X g at RT. This is especially important when using a fluorometer.

- **Clarification of the 1X solution is absolutely necessary when performing the fluorometer protocol. Any free dye aggregates in the solution will interfere with the OD reading, leading to a falsely increased reading of red fluorescence. (The aggregates do not interfere with a flow cytometer, and are easily identifiable under a fluorescence microscope.)**

7. If centrifuged, transfer the clarified supernatant to a clean tube and discard particulates.
8. Go on to the staining protocol (Section 14, 19, 22, or 23).

- **To avoid photo-bleaching and degradation of the dye, protect the MitoPT™ dye reagent from light while handling.**

12. Storage of 100X MitoPT™ Stock for Future Use

If not all of the 100X MitoPT™ stock will be used the same time it is reconstituted, the unused portion may be stored at -20°C for 6 months. During that time, the 100X MitoPT™ stock may be thawed and used twice. After the second thaw, discard any remaining 100X MitoPT™ stock. If you anticipate using it more than twice, make small aliquots in amber vials or polypropylene tubes.

Following the flow cytometer and fluorescence microscope protocols, each sample to be stained requires only 0.5 mL of 1X MitoPT™ solution (equal to 5 μL of 100X MitoPT™ stock). Following the fluorescence plate reader protocol, each sample requires 1 mL of 1X MitoPT™ solution (10 μL of 100X MitoPT™ stock). When ready to use, follow Section 13.

13. Preparation of 1X MitoPT™ Solution from a Frozen Aliquot

If some of the 100X MitoPT™ stock was previously reconstituted and then stored at –20°C, it may be used 2 more times within 6 months.

1. Thaw the 100X MitoPT™ stock and protect from light.
2. Once the aliquot has become liquid, dilute the 100X MitoPT™ stock 1:100 in 37°C 1X assay buffer or cell culture media. For example, mix 10 μL of 100X MitoPT™ stock with 990 μL of 37°C 1X assay buffer or cell culture media.
3. If the 100X MitoPT™ stock was frozen immediately after reconstitution and never been thawed, return it to the freezer. If the stock has been thawed once before, discard it.

4. Vortex the 1X working strength MitoPT™ solution thoroughly.
5. If particulate matter is present, the solution must be clarified by centrifugation at 13,000 X g in a microfuge for 3 minutes, or 15 minutes in a clinical centrifuge at 1,000 X g at RT.

- **Clarification of the 1X solution is especially important when performing the fluorometer protocol. Any free dye aggregates in the solution will interfere with the OD reading, leading to a falsely increased reading of red fluorescence. (The aggregates do not interfere with a flow cytometer, and are easily identifiable under a fluorescence microscope.)**

6. If centrifuged, transfer the clarified supernatant to a clean tube and discard particulates.
7. Go on to the staining protocol (Section 14, 19, 22, or 23).

14. Flow Cytometry Staining Protocol

Following the flow cytometer protocols, each sample to be stained requires only 0.5 mL of 1X MitoPT™ solution (equal to 5 μL of 100X MitoPT™ stock). The MitoPT™-25 Kit will test 25 samples; the MitoPT™-100 Kit will test 100 samples.

1. As discussed in Section 8, culture cells to a density optimal for apoptosis induction according to your specific induction protocol.
2. Induce apoptosis following your protocol.
3. At the same time, culture a non-induced negative control cell population.
4. After the induction process, transfer 0.5 mL of each cell suspension into a sterile 15 mL polystyrene centrifuge tube.

- **Cell density in the cell culture flasks should not exceed 10^6 cells per mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Optimal cell concentration will vary depending on the cell line used. Density can be determined by counting cell populations on a hemocytometer.**

5. Centrifuge cells at 400 X g for 5 minutes at RT.
6. Carefully remove and discard the supernatant.
7. Gently vortex the cell pellets, or use a pipette, disrupt any cell-to-cell clumping.
8. Resuspend cells in 0.5 mL of RT 1X MitoPT™ solution.
9. Incubate the cells (which are now being stained with the MitoPT™ dye reagent) at 37°C for 10-15 minutes in a CO_2 incubator.
10. Warm the 1X assay buffer to 37°C (Section 9).
11. Add 2 mL of 1X assay buffer to each tube.

12. Mix each tube.
13. Centrifuge the stained cells at 400 X g for 5 minutes at RT.
14. Carefully remove and discard supernatant.
15. Gently vortex the pellet to disrupt any cell-to-cell clumping.
16. Resuspend the cells in 1 mL of 1X assay buffer.
17. Centrifuge the stained cells at 400 X g for 5 minutes at RT.
18. Carefully remove and discard supernatant.
19. Gently vortex the pellet to disrupt any cell-to-cell clumping.
20. Resuspend the cells in 1 mL of 1X assay buffer.
21. Centrifuge the stained cells at 400 X g for 5 minutes at RT.
22. Carefully remove and discard supernatant.
23. Gently vortex the pellet to disrupt any cell-to-cell clumping.
24. Resuspend the cell pellet in 0.5 mL of 1X assay buffer.
25. Analyze cells by flow cytometry (Sections 15-18).

15. Flow Cytometer Set Up

Healthy cells containing the aggregated MitoPT™ dye reagent within their mitochondria (which fluoresces red) can be detected in the FL2 channel. Apoptotic cells, which contain non-aggregated green MitoPT™ monomers, can be detected in the FL1 channel.

16. Single-Parameter Analysis Using a Flow Cytometer

1. Generate a log FL2 (X-axis) versus cell count.
2. Adjust the FL2 PMT voltage to allow the peak to fall within the third log decade when running the negative control sample.
3. When running the induced positive samples, use the same adjusted PMT voltage as was determined for the negative control.
4. Observe the mean fluorescence of both the apoptotic positive cell population, as well as the mean fluorescence of the treated negative control cell population.

- **The apoptotic cell population presents a lower red fluorescence signal intensity (FL2 axis) than the negative control population.**

17. Multi-Parameter Analysis Using a Flow Cytometer

1. Create a log FL1 (X-axis) verses log FL2 (Y-axis) scatter plot.
2. Add two regions, R2 and R3, as shown in Figure 1.
3. Run the negative control sample first (non-induced cells). Adjust the FL1 and FL2 PMT voltages so that the majority of the cell population falls within the upper right hand region. The peak of the dual fluorescent population should fall within the second and third log decade scale of FL1 (X-axis) and FL2 (Y-axis) as seen in Figure 1.
4. Adjust R2 so that greater than 95% of the dual fluorescent cell population falls within this region. The number of cells falling into this region will vary depending on the condition of the culture and cell type.
5. Next, adjust R3 so that it falls directly below R2, as in Figure 1.
6. Using the same PMT settings established for the non-induced negative control sample in Step 3, run the induced positive sample. If a change in mitochondrial $\Delta\Psi$ has occurred, an increase in the number of cells falling in R3 is observed as shown in Figure 2.

- **This reflects a reduction in red fluorescence.**

7. If the induced sample exhibits only a minimal change in red emission, increase the FL2-FL1 compensation and repeat Steps 3-6.

18. Flow Cytometry Sample Data

When cells stained with MitoPT™ are run through a flow cytometer, the instrument will measure apoptosis by monitoring the amount of red

fluorescence in each region. Healthy cells, which fluoresce red, will appear in R2. As the mitochondrial $\Delta\Psi$ collapses and cells enter apoptosis, the amount of red fluorescence will drop. An increasing number of cells will fall into R3 corresponding to a loss of red fluorescence as the dispersed MitoPT™ dye coverts to a monomeric form and fluoresces green.

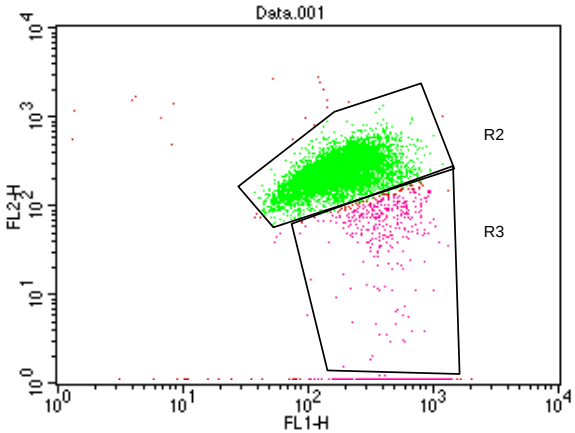

Figure 1: Negative cell control, Log FL1 (X-axis) and log FL2 (Y-axis) R2 = 91.07% R3 = 8.08%.

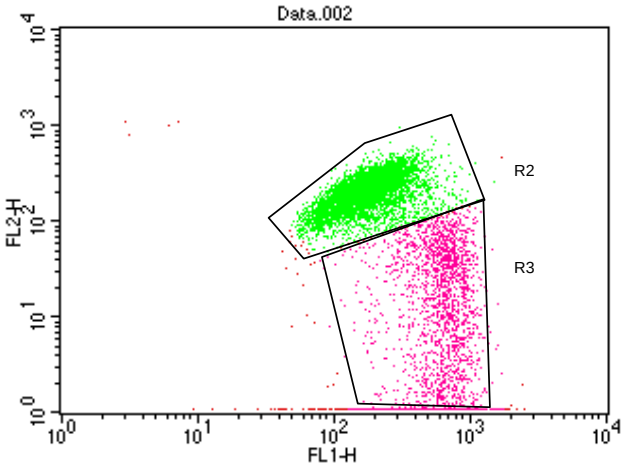

Figure 2: Positive cell control, Log FL1 (X-axis) and log FL2 (Y-axis) R2 = 60.93% R3 = 38.13%.

In Figures 1 and 2, cells were analyzed in a FACS Caliber Becton Dickinson Flow Cytometer. Jurkat cells were either treated with DMSO (negative, non-induced cells) or with staurosporine (apoptotic, induced cells) for 3 hours at

37°C and then labeled with MitoPT™ for 15 minutes. Collapse of the mitochondrial $\Delta\Psi$ is indicated by an increase in the number of cells falling into R3 corresponding to a loss of red fluorescence, indicative of the onset of apoptosis. (In these figures of flow cytometer data, non-apoptotic cells are in R2, which prints in green, and apoptotic cells are in R3, which prints in pink.)

19. 96-Well Fluorescence Spectroscopy Staining Protocol

Following the fluorescence plate reader protocol, each sample requires 1 mL of 1X MitoPT™ solution (equal to 10 µL of 100X MitoPT™ stock). The MitoPT™-25 Kit will test 12 samples; the MitoPT™-100 Kit will test 50 samples.

1. As discussed in Section 8, culture cells to a density optimal for apoptosis induction according to your specific induction protocol.

- **Cell density in the cell culture flasks should not exceed 10^6 cells per mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Optimal cell concentration will vary depending on the cell line used. Density can be determined by counting cell populations on a hemocytometer.**

2. Induce apoptosis following your protocol.
3. At the same time, culture an equal volume of non-induced cells for a negative control cell population. Make sure that both tubes of cells contain similar quantities of cells.
4. Pellet cells by centrifugation at 400 X g for 5 minutes at RT.

- **When concentrated, enough cells should have been grown to form a 0.5 mL concentrated pool between 1 - 2 X 10^6 cells/mL.**

5. Carefully remove and discard the supernatants.
6. Resuspend at between 0.5 – 2 X 10^6 cells in 1 mL of 1X working strength MitoPT™ solution (Sections 11 and 13).
7. Gently vortex the cell pellets, or use a pipette, disrupt any cell-to-cell clumping.
8. Incubate the cells (which are now being stained with the MitoPT™ dye reagent) at 37°C for 10-15 minutes in a CO_2 incubator.
9. Warm the working strength 1X assay buffer to 37°C (Section 9).
10. Add 2 mL of 1X assay buffer to each tube.
11. Mix each tube.
12. Centrifuge the stained cells at 400 X g for 5 minutes at RT.
13. Carefully remove and discard supernatants.
14. Gently vortex the pellets to disrupt any cell-to-cell clumping.

15. Resuspend the cells in 1 mL of 1X assay buffer.
16. Take out a small aliquot to determine the concentration of both the induced and non-induced cell populations.
    a. Remove a 50 µL aliquot of each cell populations.
    b. Add to 450 µL PBS (forming a 1:10 dilution of each).
    c. Count the cells (a hemocytometer may be used).
    d. After counting, compare the density of each. The non-induced population may have more cells than the induced population, as some induced cells may be lost during the apoptotic process. If necessary, adjust the volume of the induced cell suspension to match that of the non-induced suspension (Step 20).
17. Centrifuge the remaining stained cells at 400 X g for 5 minutes at RT.
18. Carefully remove and discard supernatants.
19. Gently vortex the pellets to disrupt any cell-to-cell clumping.
20. Adjust the volume of the induced cell suspension to match that of the non-induced suspension.

- **At least 0.5 X 10^6 cells/mL are required to generate an adequate signal using a fluorometer.**

    a. Resuspend the non-induced cell pellets in 500 µL - 1 mL of 1X assay buffer to produce a cell suspension at least 5 X 10^5 cells/mL. The volume may vary depending upon cell density.
    b. Resuspend the induced cell population in a volume of 1X assay buffer to yield the same concentration of cells as the non-induced cell suspension.

21. For each sample to be tested, dispense 100 µL into each of 2 wells in a black round or flat bottom 96-well microtiter plate.

- **At least 5 X 10^4 cells/well are required to generate an adequate signal using a fluorometer, with best results obtained using 1 X 10^5 cells/well.**

- **The 96-well microtiter plate used to analyze the cells must be black. A clear plate will interfere with the OD readings.**

22. Analyze cells using a fluorescence plate reader (Section 20).

20. 96-Well Fluorescence Plate Reader Set Up

1. Set the plate reader to perform an endpoint read.
2. Set the excitation wavelength at 488 - 490 nm
3. Set the emission wavelengths to 527 nm for green fluorescence and 590 - 600 nm for red fluorescence. If your plate reader cannot read dual emission wavelengths at the same time, use the red fluorescence setting of 590 - 600 nm to perform the analysis.
4. Read the samples.

21. 96-Well Fluorescence Spectroscopy Sample Data

When cells stained with MitoPT™ are analyzed with a fluorescence plate reader, the instrument will measure the amount of red fluorescence. Healthy cells will give a high OD reading of red fluorescence; apoptotic cells will generate a lower reading of red fluorescence.

By comparing the average 590-600 nm OD signal in stimulated versus non-stimulated sample wells, loss of mitochondrial $\Delta\Psi$ can be monitored. As the mitochondrial $\Delta\Psi$ collapses (indicating apoptosis) and the dispersed MitoPT™ dye coverts to a green monomeric form, more and more cells will lose red fluorescence (Figures 3 and 4).

Using the dual fluorescence characteristic of the dye, the changes in the mitochondrial $\Delta\Psi$ can be most accurately assessed by comparing the ratios of 590-600 nm (red) / 527 nm (green) ODs. When apoptosis is induced, the red/green OD ratio drops compared to the negative (non-stimulated) control wells (the red OD decreases and the green OD increases). This drop corresponds to a reduction in the number of healthy mitochondria able to maintain the negative potential necessary to concentrate the MitoPT™ dye in the red aggregate form.

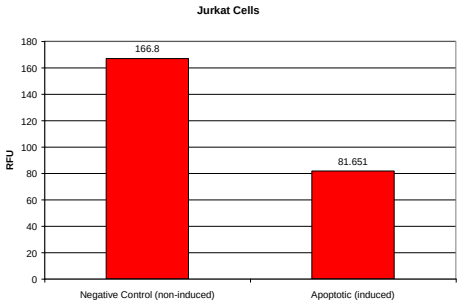

Figure 3: Red fluorescence of Jurkat cells.

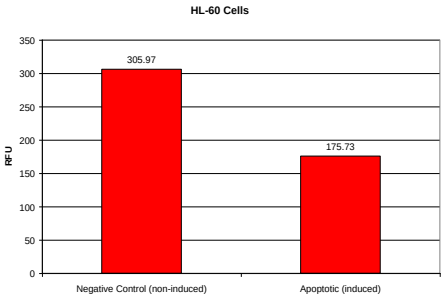

Figure 4: Red fluorescence of HL-60 cells.

In Figures 3 and 4, the cells were either treated with DMSO (negative, non-induced cells – bars on the left side of each graph) or with staurosporine (apoptotic, induced cells - bars on the right side of each graph) for 4 hours and then labeled with the 1X MitoPT™ solution for 15 minutes. The samples were then read on a 96-well fluorescence plate reader using the settings described above. As the mitochondrial $\Delta\Psi$ collapses, indicating apoptosis, the amount of red fluorescence drops by 51% in the Jurkat cells and 43% in HL-60 cells.

If there are free aggregates of the MitoPT™ dye in solution with the cells, they will fluoresce red and interfere with the data, leading to a falsely increased reading of red fluorescence (Sections 11 and 13).

22. Fluorescence Microscopy Staining Protocol for Adherent Cells

Following the fluorescence microscope protocol, each sample to be stained requires only 0.5 mL of 1X MitoPT™ solution (equal to 5 μL of 100X MitoPT™ stock). The MitoPT™-25 Kit will test 25 samples; the MitoPT™-100 Kit will test 100 samples.

1. Culture cells on a sterile coverslip or chamberslide to a cell density optimal for apoptosis induction according to your specific induction protocol.

- **Cell density should not exceed the threshold where cell sloughing occurs.**

2. As discussed in Section 8, induce apoptosis following your protocol.

3. At the same time, culture an equal volume of non-induced cells for a negative control cell population.
4. Make sure that both the negative control and induced positive cell populations contain similar quantities of cells.
5. Remove the media from induced and non-induced monolayer cultures.
6. Add enough 1X MitoPT™ solution (Sections 11 and 13) to cover the cells on the slide.
7. Incubate the cells (which are now being stained with the MitoPT™ dye reagent) at 37°C for 15 minutes in a CO_2 incubator.
8. Warm the 1X assay buffer to 37°C (Section 9).
9. Carefully remove and discard staining media.
10. Wash the monolayer cultures with 1 to 2 mL of 1X assay buffer.
11. Discard wash.
12. Add a drop of 1X assay buffer plus coverslip.
13. Examine using a fluorescence microscope (Sections 24 and 25).

23. Fluorescence Microscopy Staining Protocol for Suspension Cells

Following the fluorescence microscope protocol, each sample to be stained requires only 0.5 mL of 1X MitoPT™ solution (equal to 5 μL of 100X MitoPT™ stock). The MitoPT™-25 Kit will test 25 samples; the MitoPT™-100 Kit will test 100 samples.

1. Culture cells to a cell density optimal for apoptosis induction according to your specific induction protocol.

- **Optimal cell densities will vary with the cell line; cell concentrations may be determined using a hemocytometer.**

2. As discussed in Section 8, induce apoptosis following your protocol.
3. At the same time, culture an equal volume of non-induced cells for a negative control cell population.
4. Make sure that both the negative control and induced positive cell populations contain similar quantities of cells.
5. Once induced, count cells (a hemocytometer may be used).
6. Transfer 0.5 X 10^6 cells to a centrifuge tube.
7. Centrifuge cells at 400 X g for 5 minutes at RT.
8. Carefully remove and discard the supernatants.
9. Gently vortex the cell pellets, or use a pipette, disrupt any cell-to-cell clumping.
10. Resuspend cells in 0.5 mL 1X MitoPT™ solution (Sections 11 and 13).
11. Incubate the cells (which are now being stained with the MitoPT™ dye reagent) at 37°C for 10 to 15 minutes in a CO_2 incubator.
12. Warm the 1X assay buffer to 37°C (Section 9).
13. Resuspend cells in 2 mL 1X assay buffer.

14. Centrifuge cells at 400 X g for 5 minutes at RT.
15. Carefully remove and discard the supernatants.
16. Gently vortex the cell pellets to disrupt any cell-to-cell clumping.
17. Resuspend cells in 1 mL of 1X assay buffer.
18. Centrifuge the stained cells at 400 X g for 5 minutes at RT.
19. Carefully remove and discard the supernatants.
20. Gently vortex the cell pellets to disrupt any cell-to-cell clumping.
21. Resuspend cells in 1 mL of 1X assay buffer.
22. Centrifuge the stained cells at 400 X g for 5 minutes at RT.
23. Carefully remove and discard the supernatants.
24. Resuspend cells in 0.5 mL 1X assay buffer.
25. Examine a drop of cell suspension under a cover slip using a fluorescence microscope (Sections 24 and 25).

24. Fluorescence Microscope Set Up

This scope should contain a long band path filter (Ex 490 nm, Em >510 nm) capable of detecting both fluorescein and rhodamine fluorescence.

25. Fluorescence Microscopy Sample Data

The MitoPT™ dye will be concentrated in the mitochondria of healthy cells, thereby creating red fluorescent regions within the cell. The MitoPT™ dye will become dispersed in apoptotic cells; these cells will not have red aggregates in the mitochondria, rather the entire cell will appear green. Cells in varying stages of apoptosis will contain less and less red dye aggregate and appear mostly green.

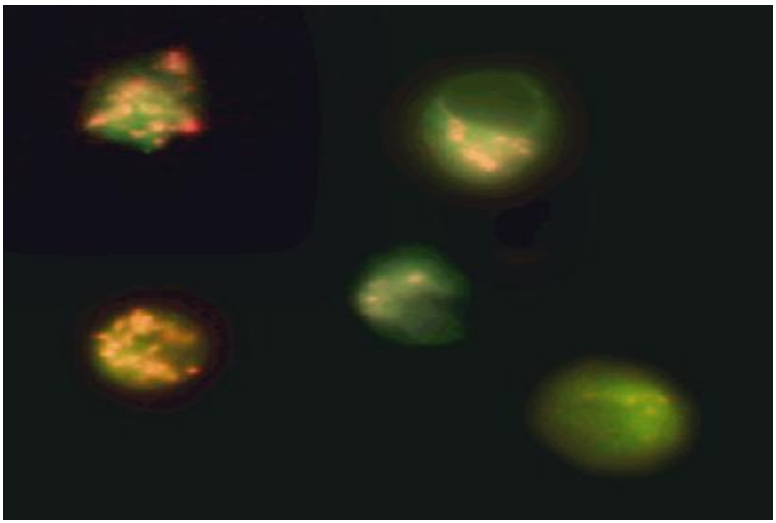

Figure 5: Jurkat cells were stained with MitoPT™ and viewed through a fluorescence microscope using a broad band path filter. Non-apoptotic cells exhibit red stained mitochondria (2 cells at left). Apoptotic cells at varying stages of mitochondrial $\Delta\Psi$ appear green (3 cells at right).

If there are free aggregates of the MitoPT™ dye in solution with the cells, these aggregates will appear as bright red spots on the slide.

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