Disruption of the mitochondrial transmembrane potential is one of the earliest intracellular events that occur following induction of apoptosis. The Mitochondrial Apoptosis Staining Kit provides a simple, fluorescent-based method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial transmembrane potential. The kit utilizes MitoStain, a cationic dye that fluoresces differently in healthy versus apoptotic cells. In non-apoptotic healthy cells, the dye exists as a monomer in the cytosol (green) but also accumulates and aggregates in the mitochondria, emitting a bright red fluorescence. Whereas in apoptotic and necrotic cells, MitoStain cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form fluorescing green. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells which show red and green fluorescence. The fluorescent signals can be easily detected by fluorescence microscopy using a band-pass filter (detects FITC and rhodamine) or analyzed by flow cytometry using the FITC channel. The aggregate red form has an absorption/emission maxima of 585/590 nm while the green monomeric form has absorption/emission maxima of 510/527 nm.

### Components

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity PK-CA577-K250-25</th>
<th>Quantity PK-CA577-K250-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>MitoStain Reagent</td>
<td>125 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>10 x Assay Buffer</td>
<td>2.5 ml</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

### Applications / Assay Protocol

**A. Reagent Preparation**

**Preparation of 1X Assay Buffer**

Note: Use of Assay Buffer is optional, you can also perform MitoStain staining in the buffer or cell culture medium of your choice.

1. If necessary, warm the 10X Assay Buffer until any salt crystals are completely dissolved.
2. Dilute the Assay Buffer 1:10 with dH₂O (e.g. 1 mL 10X assay buffer + 9 mL dH₂O).

**Preparation of MitoStain working solution**

Immediately before use, dilute the 100X MitoStain reagent to 1X by adding 10 µL of 100X MitoStain per mL of 1X Assay Buffer or pre-warmed media of your choice to generate MitoStain Reagent working solution.

**B. Cell staining**

1. Culture cells to the desired density (generally not to exceed 10⁶ cells/mL for suspension cells).
2. Induce apoptosis according to your specific protocol. Include an uninduced control sample.
3. For adherent cells, remove cells from the substrate to generate a single cell suspension before staining. Transfer 0.5 mL cell suspension to a centrifuge tube.
4. Pellet cells by centrifugation for 5 minutes at room temperature at 400 xg.
5. Remove the supernatant.
6. Resuspend cells in 0.5 mL 1X MitoStain Reagent working solution.
7. Incubate the cells in a 37°C cell culture incubator for 15 minutes.
8. Centrifuge for 5 minutes at 400 xg and remove supernatant.
9. Wash the cells by resuspending the cell pellet in 2 mL PBS or cell culture medium followed by centrifugation. Remove the supernatant. Repeat once.
10. Resuspend the cell pellet in 0.5 mL PBS or cell culture medium. Cells are now ready for flow cytometry analysis.

**Quantification by flow cytometry**

Analyze cells immediately following step 10 by flow cytometry. Mitochondria containing red MitoStain aggregates in healthy cells are detectable in the PE or PI channel (FL2), and green MitoStain monomers in apoptotic cells are detectable in FITC channel (FL1).
**Instrument set-up for 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 to the dot plot.
2. Adjust FL1 and 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 FL1 and FL2.
3. 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 **Tech Note 1: Quadrants**

**Staining of cells in suspension**

1. Stain cells according to the protocol for flow cytometry.
2. Resuspend the cell pellet in 0.3 mL PBS or cell culture medium.

**Staining of adherent cells**

1. Grow cells to the desired density on a glass cover slip in a petri dish or in a chamber slide.
2. Induce cells according to your specific protocol. Include an uninduced control sample.
3. Remove the cell culture media and replace with MitoStain Reagent working solution sufficient to cover the cells.
4. Incubate the cells in a 37°C cell culture incubator for 15 minutes.
5. Remove media and wash once with PBS or cell culture medium.
6. Mount coverslips on a slide with a drop of PBS or cell culture medium. For chamber slides, add PBS or cell culture medium sufficient to cover the cells.

**Imaging by fluorescence microscopy**

Observe cells immediately after staining with a fluorescence microscope using a dual band-pass filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red®. In cells with intact mitochondrial membrane potential, the mitochondria will fluoresce red with emission at 590 nm, and the cytoplasm will fluoresce green. In apoptotic and dead cells, the dye will remain in its monomeric form and will appear green with an emission at 530 nm. (See **Tech Note 2: Filters for fluorescence microscopy**).

**Staining protocol for fluorescence ratio detection**

1. Plate cells in a 96-well plate and grow to the desired density.
2. Induce cells according to your specific protocol. Include an uninduced control sample.
3. Stain cells according to the staining protocol for fluorescence microscopy.
4. Measure red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) using a fluorescence microplate reader.
5. Determine the ratio of red fluorescence divided by green fluorescence.
6. 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: Quadrants**

On instruments where it is not possible to add regions to the dot plot, quadrants can be 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 FL1 and 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 FL1 and 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 2: Filters for fluorescence microscopy
Red MitoStain aggregates and green monomers can be viewed simultaneously with a dual band-pass filter for fluorescein/rhodamine or fluorescein/Texas Red®. MitoStain aggregates can be imaged with a band-pass filter designed to detect rhodamine (Ex/Em: 540/570 nm) or Texas Red® (Ex/Em: 590/610 nm). MitoStain monomers can be imaged with a band-pass filter used for the detection of fluorescein (Ex/Em: 490/520 nm).

Intended Use
For in vitro research use only. Not for diagnostic or therapeutic procedures.

Storage & Stability
Store at 4°C or -20°C, protected from light. Product is stable for at least 1 year from date of receipt when stored as recommended.