# FlowCellect™ Cytochrome *c* Kit

100 Tests

## Cat. No. FCCH100110

**FOR RESEARCH USE ONLY**
**Not for use in diagnostic procedures.**

USA & Canada Phone: +1 (800) 437-7500 Fax: +1 (951) 676-9209
www.millipore.com

## Application

Mitochondria play a pivotal role in the generation of energy for the cell and the cascade of events associated with cell death pathways such as apoptosis. The release of key mitochondrial proteins such as Cytochrome c is an important hallmark in the pathway of apoptosis and is considered a point of no return in the apoptosis process. [1-4]

Cytochrome *c*, is a soluble heme protein localized in the mitochondrial intermembrane space which plays an indispensable role as an electron carrier in oxidative phosphorylation during respiration, transferring electrons from the cytochrome $bc_1$ complex to cytochrome oxidase on the surface of the inner mitochondrial membrane. On induction of apoptosis, and accompanying events such as mitochondrial depolarization, cardiolipin peroxidation,, Cytochrome *c* along with other pro-apoptotic proteins such as proCaspase-9, Smac/DIABLO, APF-1 and Endo F is released into the cytosol signaling the commitment to the intrinsic pathway of cell death. When released, Cytochrome *c* acts with other intermembrane mitochondrial proteins such as procaspase 9 and Smac, as well as cytosolic factors, including apopototic protease activating factor-1 and ATP to activate a cascade of caspases that promote cell death. The quantification of the release of Cytochrome *c* from the mitochondria of apopotic cells can be used to characterize the mitochondrial-dependent pathway to cell death. [5-11]

Current techniques for evaluation of release of Cytochrome *c* include methods such as Western blots, ELISA and fluorescence microscopy, which are time consuming and laborious and make it cumbersome to asses multiple samples in parallel. Further, since methods like Western blots or ELISA typically involve lysis of cells, the Cytochrome *c* translocation information in a per cell basis is lost. Further, in methods involving fractionation and isolation of mitochondrial proteins which often employ harsh mechanical means such as homogenization, there may be incomplete or excessive rupture of mitochondria which can cause the amount of Cytochrome *c* to be over or under-estimated.

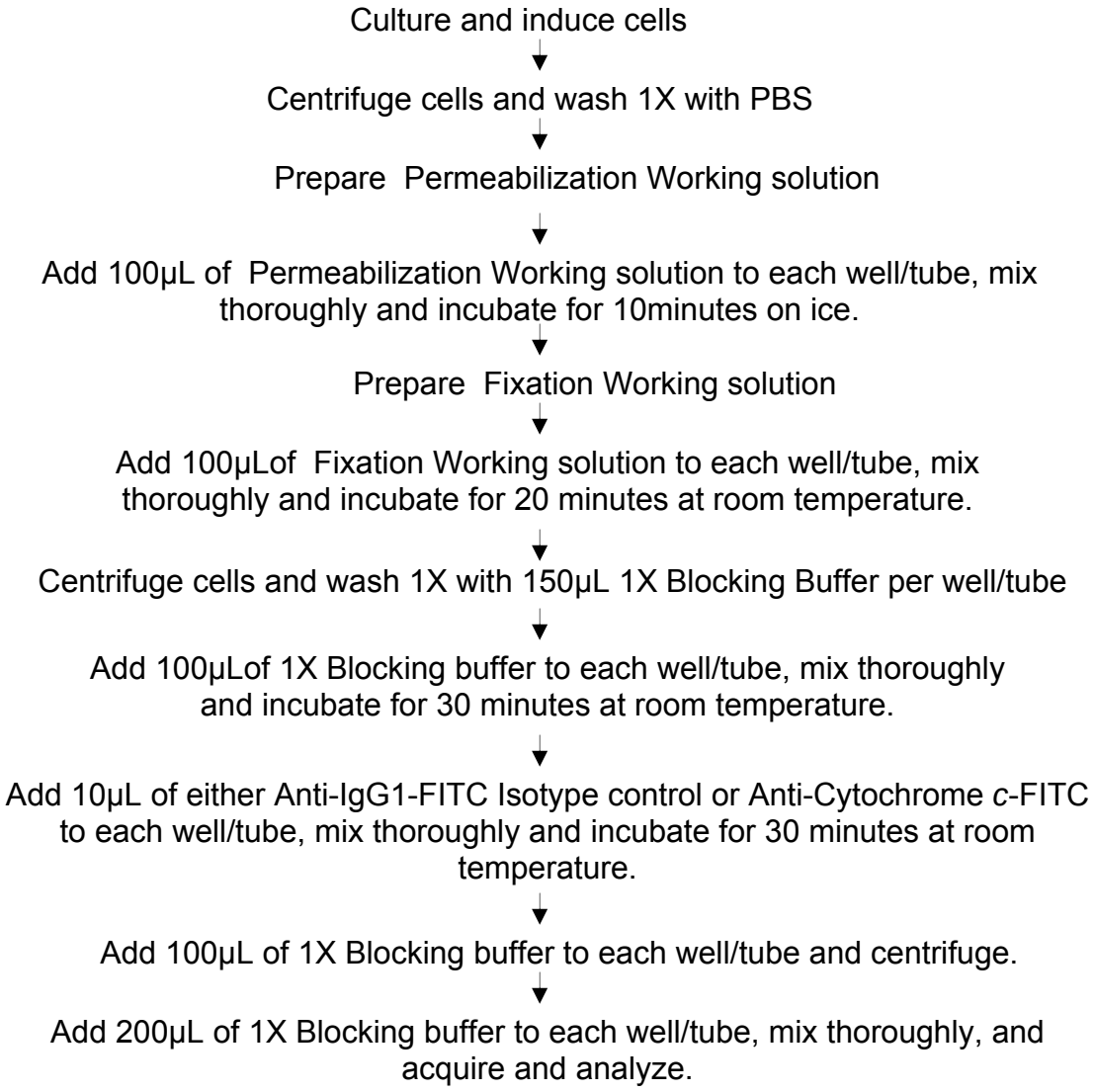
Millipore's FlowCellect Cytochrome *c* kit provides for a simpler, gentler and a faster method to asses levels of Cytochrome c in mitochondria of cells undergoing apoptosis on a per cell basis using flow cytometry. The kit provides a means of identifying apoptotic cells and provides as a method for inhibitor /compound screening and assessing the regulation of apoptotic signaling in cells. In combination with analysis by flow cytometry the Cytochrome *c* kit provides researchers an important tool in assessing regulation of apoptotic signaling in cells.

## Test Principle

Millipore's FlowCellect™ Cytochrome *c* Kit assesses the loss of mitochondrial Cytochrome *c* in cells that have been induced to undergo apoptosis. The kit includes a directly labeled Anti-Cytochrome *c*-FITC Antibody, Anti-IgG1-FITC Isotype control, along with optimized fixation, permeabilization, and blocking buffers to allow the detection of Cytochrome *c* using flow cytometry. The assay kit may be used on a flow cytometer equipped with a 488 nm laser which allows the detection of FITC probes. The kit uses specialized buffer sets to achieve selective permeabilization of mitochondira while leaving the mitochondrial membrane intact. Viable or live cells will demonstrate higher levels of Cytochrome *c* staining while apoptotic cells which have released their Cytochrome *c* from the mitochondria to the cytoplasm will demonstrate reduced staining intensity when probed with an anti-Cytochrome *c* FITC antibody. Stained cells can then be analyzed by flow cytometry. The entire staining can be performed in 2 hours once cells are ready as shown in the flow chart in Figure 1.

Sufficient reagents are provided for 100 samples. The kit includes all optimized fluorescently labeled antibodies and buffers necessary for cell preparation and analysis. The flow cytometry method described here can be can be stained and analyzed within 3 hours of completion of induction for as many as 96 samples.

**Figure 1: FlowCellect™ Cytochrome *c* Kit Workflow**



## Kit Components

- Anti-Cytochrome *c*- FITC Antibody (Part No. 4700-1320) One vial containing 1mL of antibody.
- Anti-IgG1-FITC isotype Control (Part No. 4700-1325) One vial containing 150µL of antibody.
- 10X Permeablization Buffer (Part No. 4200-0636) One vial containing 1mL buffer.
- 5X Fixation Buffer (Part No. 4300-0340) Two vials containing 3mL buffer.
- 1X Blocking buffer (Part No. 4200-0633) One bottle containing 55mL buffer.

## Materials Not Supplied

1. guava System (EasyCyte™, EasyCyte™ Mini, or easyCyte HTSystem) with Guava CytoSoft™ or guavaSoft Software containing the ExpressPro or InCytoe software module or equivalent flow cytometry system with ability to detect FITC.
2. ViaCount™ reagent (Catalog No. 4000-0041) or ViaCount Flex reagent (Catalog No. 4700-0060)
3. Cell line of interest
4. Media for cell line of interest
5. Tissue culture instruments and supplies (including 37°C incubator, growth media, plates, detachment buffer, etc.)
6. Polypropylene tubes and or bottles for sample and buffer preparation and storage.
7. 96 well plates, if desired for sample preparation.
8. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 µL
9. Tabletop centrifuge capable of exceeding x300G.
10. Vortex mixer
11. 1X Phosphate buffered saline (PBS) without calcium or magnesium.
12. Reagent reservoirs, optional
13. Guava® Instrument Cleaning Fluid (ICF) (Cat. No. 4200-0140), optional
14. guava easyCheck Kit (Cat. No. 4500-0025), optional
15. 20% bleach solution

## Precautions

- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this product
- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. The kit includes a formaldehyde solution (fixation solution). Please refer to the MSDS sheet for specific information on hazardous materials.

- All fluorochrome conjugated antibodies are light sensitive and must be stored in the dark at 4°C.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of product, centrifuge vial briefly prior to removing cap.
- The FlowCellect Cytochrome *c* Kit contains sodium azide, which is toxic.
- Avoid microbial contamination of the solution, which may cause erroneous results.

## Storage

Upon receipt, store the kit as follows:

1) -20$^0$C dessicated
    - 10X Permeablization Buffer

**Caution:** *Permeabilization Buffer is highly hygroscopic avoid multiple freeze thaws and needs to be stored dessicated. Aliquot 10X Permeablization Buffer into appropriate test sizes.*

2) 2-8$^0$C
    - Anti-Cytochrome *c*- FITC Antibody , Anti-IgG1-FITC isotype Control, 1X Blocking Buffer

***Caution:*** *Fluorochrome conjugated antibodies should always be stored at 4°C. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.*

*3)* Room temperature
    - 5X Fixation Buffer

## Preparation of Buffers

Prepare each of these buffers fresh each day of your experiment. Each buffer is only stable for 4 hours at room temperature. To ensure there is enough buffer for your samples mix n+2 samples, where n is the number of tests you wish to run for each experiment, plus one for the isotype control, plus one extra.

1. **Permeabilization buffer Working Solution:** Mix the following reagents together. Make sure to add the 5X Fixative Solution first, followed by 1X PBS without calcium and magnesium, and the 10X Permeablization Buffer last.

| Component | 1 Test | 25 Tests | 100 Tests |
|---|---|---|---|
| 5X Fixation Solution | 2.5µL | 62.5µL | 0.25mL |
| 1X PBS | 87.5µL | 2187.5µL | 8.75mL |
| 10X Permeabilization Buffer | 10µL | 250µL | 1mL |
| Total Volume | 100µL | 2500µL | 10mL |

**NOTE:** Make sure that 10X Permeabilization buffer is completely thawed before mixing. Pipette material up and down to ensure proper mixing.

2. **Fixation buffer Working Solution:** Mix the following reagents together.

| Component | 1 Test | 25 Tests | 100 Tests |
|---|---|---|---|
| 5X Fixation Solution | 40µL | 1000µL | 4mL |
| 1X PBS | 60µL | 1500µL | 6mL |
| Total Volume | 100µL | 2500µL | 10mL |

## Before You Begin

This protocol was developed to allow direct determination of the percent of cells that have released their Cytochrome c from the mitochondria into the cytoplasm. For optimal throughput during acquisition, final cell concentrations should be between 2 x $10^4$ and 1 x $10^5$ cells/well (or 1 x $10^5$ to 5 x $10^5$ cells/mL), although apoptosis can be detected in cultures with as few as 2 x $10^3$ cells/well (or 1 x $10^4$ cells/mL). Care should be taken to keep cell concentrations as constant as possible in all samples of an experiment. The mean fluorescent intensity of Cytochrome *c*-FITC bound to cells can vary significantly with a two-fold change in cell concentration, although the percentage of cells bound by Cytochrome c-FITC remains constant. However, if the cell concentration exceeds 5 x $10^5$ cells/mL, the Cytochrome *c*-FITC reagent may be in limiting concentration and will therefore bind to fewer cells, resulting in lower percentages for Cytochrome *c*-FITC negative cells.
Cells should be acquired shortly after the sample preparation had been completed. While cells are fixed, and some cell lines have been shown to yield stable results for up to 24 hours, post preparation stability may be different for your particular cell line.
The following procedures for cell staining are guidelines. Different cell types have varying Cytochrome c within their Mitochondria. Different cell lines may have more optimal permeabilization and staining conditions. You may need to adjust the amount of reagents used for optimal staining of your cell samples. If this is the case, please follow the recommendations described in Cell Staining Procedure.

**Time considerations:** The process of staining cells with the FlowCellect Cytochrome c Kit takes approximately 2 hours. Acquiring data on your guava system usually takes approximately 1 hour but can vary depending on your cell concentration. However, preparing cells for testing requires periodic maintenance and cultivation several days in advance. Once you cultivate the proper number of cells for your experiment, it can take an additional 2 to 48 hours of culture with various inducers to stimulate detectable apoptosis.
Make sure to include proper controls for instrument set up and accurate results. Millipore recommends, including an isotype control for both your positive and negative controls.
**NOTE: For details on how to culture and prepare cell samples, including positive and negative control samples, for the FlowCellect Cytochrome c Kit, see Appendix A.**

## Cell Staining Procedure

1. Allow FlowCellect Cytochrome c Reagents warm to room temperature.
2. Prepare required buffers as described under **Preparation of Buffers**.
3. Prepare samples for testing. For instructions on making cell suspensions, see Appendix A.
4. Add 1.5x$10^4$ to 1.5x$10^5$ cells in 200µL (7.5x$10^4$ to 7.5x$10^5$ cells/mL) of cells in suspension to each well or tube.
5. Centrifuge cells at 300xg for 5 to 7 minutes.
6. Aspirate off the supernatant and add 200µL of 1X PBS to each well or tube. Mix each sample thoroughly.
7. Centrifuge cells at 300xg for 5 to 7 minutes and aspirate off supernatant.
8. Add 100 µL of Permeabilzation Buffer Working Solution to each well or tube and mix thoroughly
9. Cover the plate with a plate sealer or cap each tube and incubate for 10 minutes on ice.

10. After permeabilzation incubation add 100µL of the Fixation buffer Working Solution prepared to each sample and mix each sample thoroughly.
11. Incubate at room temperature for 20 minutes.
12. After incubation, centrifuge the cells for 5 to 7 minutes at 300xg.
13. Aspirate off the supernatant and add 150µL of 1X Blocking Buffer to each sample.
14. Centrifuge cells for at least 5 to 7 minutes at 300xg.
15. Aspirate off the supernatant and add 100µLof 1X Blocking Buffer to each sample and mix thoroughly.
16. Incubate the plate for 30 minutes at room temperature.
17. After incubation add 10µL of either the Anti-IgG1-FITC Isotype Control or Anti-Cytochrome c-FITC Antibody to each sample and mix thoroughly.
18. Incubate the plate at room temperature for 30 minutes in the dark.
19. After incubation, add 100µL of 1X Blocking Buffer to each sample.
20. Centrifuge sample for at 5 to 7 minutes at 300xg.
21. Aspirate off the supernatant, and add 200 µL of 1X Blocking Buffer to each well.
22. Samples are now ready for acquisition on a flow cytometer.
23. Read within 4hours.

## Sample Data

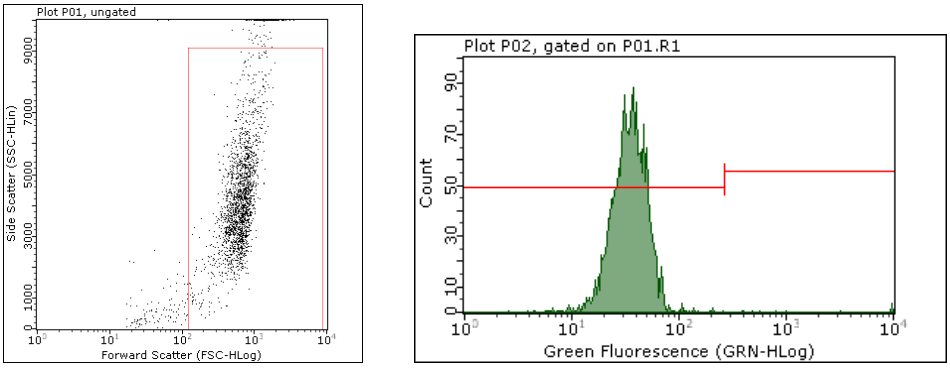



**Figure 2A**

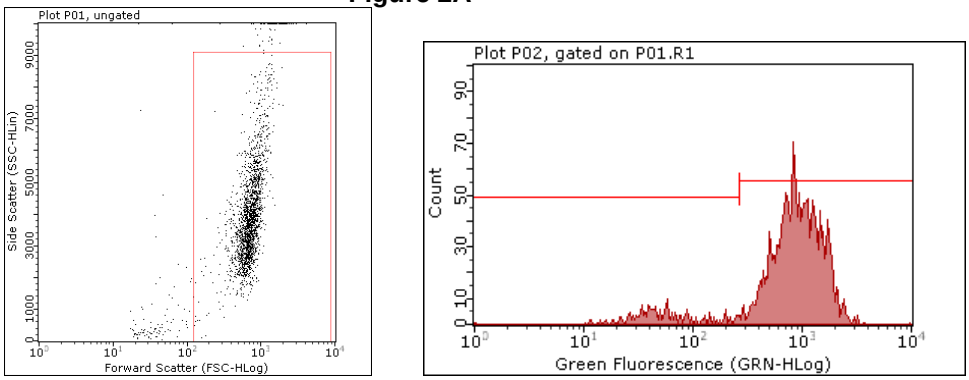



**Figure 2B**

**Figure 2 Display of Plots for Sample Acquisition:** Set up of plots for data acquisition for samples treated with the Cytochrome c Kit. Two plots are typically set up for data acquisition. The first dot plot on the left, FSC (log) vs. SSC is typically used as a counting gate to eliminate debris. The second plot shown on the right is a histogram of the green (FITC) channel. Panel A shows cells stained with the Isotype Control. Cells stained with the Isotype control show a low green fluorescence. Center your cells on the histogram so it is between $10^1$ and $10^2$. Plots in Panel B show live cells (negative control) stained with Cytochrome *c*-FITC. Cells stained with Cytochrome *c*-FITC show a high fluorescence. Ensure permeabilization and staining of the cells has occurred properly by checking that there is an upward shift when compared to the isotype control. Set one marker to include live or uniduced cells and a second marker to include apoptotic or dead cells, and the isotype control.

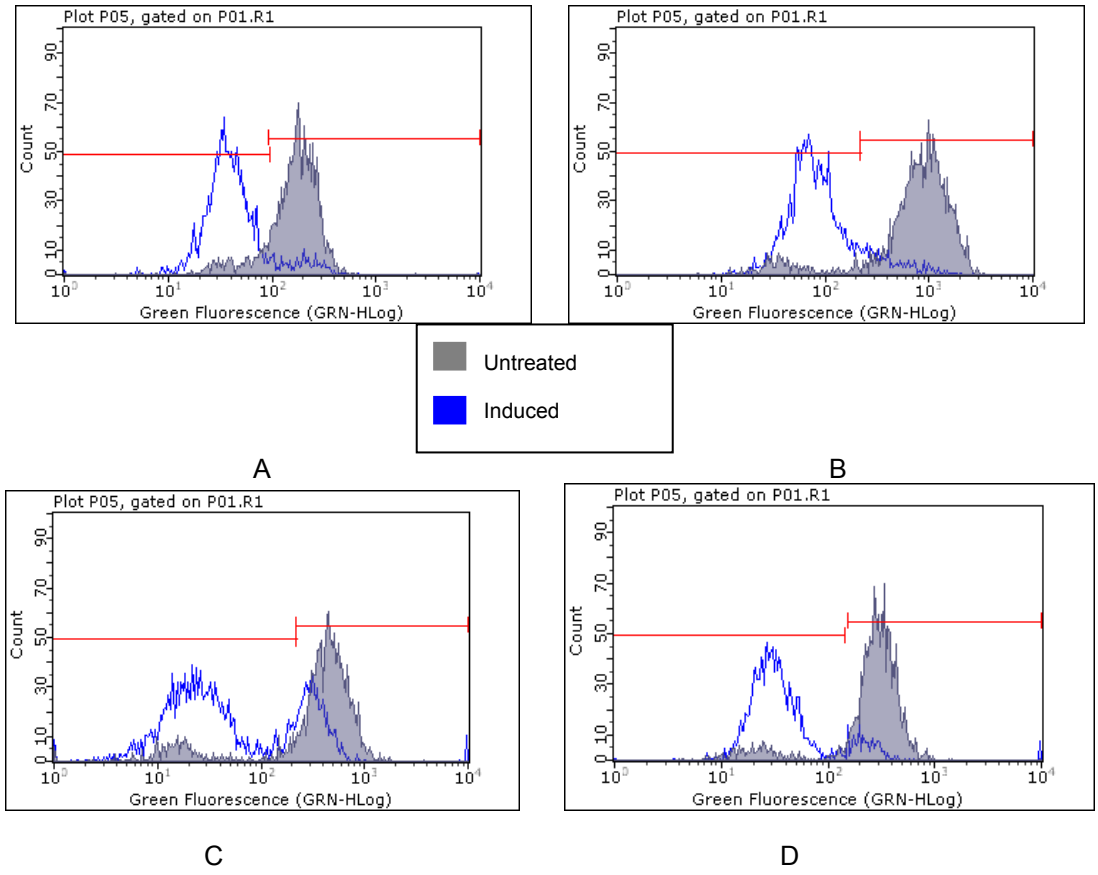



**Figure 3:** Histograms depicting cells stained using the FlowCellect Cytochrome c Kit. Plot **A** shows Jurkat cells untreated or induced with 1µM staurosporine for 2 hours. Plot **B** shows HeLa cells untreated or induced with 3µM staurosporine for 4 hours. Plot **C** shows Hl-60 cells untreated or induced with 33 µM anisomycin for 4 hours. Plot **D** shows HB cells untreated or induced with 100 µM for 4 hours. Plots show downward shifts in fluorescence of levels of Cytochrome c for multiple cell lines and inducers for the FlowCellect Cytochrome c Kit.

## Technical Hints

- If minor precipitate is detected in the 10X Permeabilization Buffer place the tube in a 56$^0$C water bath for 30 minutes, followed by mixing the contents on a mechanical vortex. Before adding solution to cells allow cooling to room temperature for 1 hour.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- For certain cell cultures cell pellets may become hazy or transparent following the fixation step, making it difficult to see. If sampling a small collection of cells for flow analysis, it is recommended that after each centrifugation step, 20µL is left behind.
- To ensure optimal staining, avoid mixing of multiple kit lots.

## Troubleshooting

| Potential Problem | Experimental Suggestions |
|---|---|
| Acquisition rate decreases dramatically Instrument clogging Too many cells | • Cell concentration too high - Decrease the number of cells per microliter by diluting sample to 300 – 500 cells per microliter. The Guava EasyCyte™ Plus or guava easyCyte HT systems gives the most accurate data when the flow rate is less 500 cells per microliter.<br>• Run a Clean and Rinse to clean out capillary. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls. |
| Too few cells | • Spin down cells and resuspend in a smaller volume. The assay instructions are optimized to give you a range of cells between 100-500 cells/μL in the final sample volume. However, cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to difficulty in adjusting settings. Make sure to leave the cell pellet intact when discarding buffer. If the cells are not generating a compact pellet after centrifugation, increase the time to 7 minutes and/or increase the speed by 300 xg until a compact and visible cell pellet forms. |
| Background staining and/or non-specific staining of cells | • Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure. |
| High background staining for adherent cells | • The cells may be damaged. Avoid damaging adherent cells when removing them from their substrate. |
| Staining is weak | • Cells may not be fully induced. To ensure proper induction conduct a time-course or concentration study in order to achieve the best results for Cytochrome c loss. |
| Staining is weak | • Some cells may require a longer permeabilization time. Optimization of permeabilization time may need to be optimized. |
| Uninduced Cells show now upward shift when compared to the isotype control | • If live or negative control cells show no upward shift when compared to the isotype control, permeabilization may not have occurred correctly. Ensure complete permeabilization by making sure no precipitate is present when diluting the 10X permeabilization solution, mixing the Permeabilization working solution is prepared in the correct order, and there is thorough mixing when adding the permeabilization working solution. |
| No downward shift | • Live cells may have been permeabilized for too long, ensure that 10 minutes is the maximum permeabilization as to not kill the uninduced cells. |

| | |
|---|---|
| | • Cells may not have been induced. Positive controls should be included for each experiment to ensure accurate staining protocol. Treatments to induce apoptosis in various cell lines include, but are not limited to a) serum starvation, b) activation of cell surface receptors such as Fas, TNFR1, or TCR, c) UV irradiation, and d) treatment with a compound that is known to induce apoptosis in your sample. |
| Samples appear to be induced when low level of induction is expected | • Cell cultures may be compromised. Negative controls should be a sample from your cell culture, not treated to induce apoptosis. Negative control samples show low levels of Cytochrome c loss. Sub-optimal culture conditions may stress cells in culture, causing them to undergo apoptosis in the absence of experimental induction treatment. The negative control from a stressed culture often shows a downward shift in Cytochrome c. |

*****_For further support, please contact Millipore's Technical services at 1-800-645-5476_

## References


1. Newmeyer DD and Ferguson-Miller S Mitochondria: releasing power for life and unleashing the machineries of death. 2003: Cell 112: 481–490
2. Campos C, Paim B, Cosso R, Castilho R, Rottenberg H, Vercesi A. Method for Monitoring of Mitochrondrail Cytochrome c release during cell death: Immunodetection of Cytochrome c by Flow Cytometry after selective permeabilization of the plasma membrane. Cytometry: 2006. 69A:515-523
3. Finkel E. The mitochondrion: is it central to apoptosis? Science.2001;292:624-626.
4. Zamzami N, Kroemer G. The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol. 2001;2:67-71.
5. King M, Eddaoudi A, Davies D. A Comparison of Three Flow Cytometry Methods for Evaluating Mitochondrial Damage During Staurosporine_Induced Apopotosis in Jurkat Cells. Cytometry. 2007: A: 668-674
6. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP,Wang X. Prevention of apoptosis by Bcl-2: Release of Cytochrome c from mitochondria locked. Science 1997;275:1129–1132.522 CAMPOS ET AL.
7. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of Cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. Science 1997;275:1132–1136.
8. Khosravi-Far R, Esposti MD. Death receptor signals to mitochondria. Cancer Biol Ther 2004;3:1051–1057.
9. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004;305:626–629.
10. Jiang X, Wang X. Cytochrome c-mediated apoptosis. Annu Rev Biochem 2004;73:87–106.
11. Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeffner A, Hirsch F, Geuskens M, Kroemer G. Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 1996;184:1155–1160.

## Related Kit Information

1. FlowCellect™ MitoPotential Red Kit (Catalog No. FCCH100105)
2. FlowCellect™ MitoDamage Kit (Catalog No. FCCH100106)
3. FlowCellect™ MitoLive Kit (Catalog No. FCCH100107)
4. FlowCellect™ Annexin Red Kit (Catalog No. FCCH100108)
5. FlowCellect™ MitoStressKit (Catalog No. FCCH100109)
6. FlowCellect™ Cytochrome *c* Kit (Catalog No. FCCH100110)
7. Guava® EasyCyte™ MitoPotential™ Kit (Catalog No. 4500-0250)
8. Guava Nexin® Reagent (Catalog No. 4500-0450, 4500-0455)
9. Guava Caspase Kits (Catalog Nos. 4500-0500 to 4500-0650)

## Appendix A:
## Cell Sample Preparation
### Preparing Controls

Regardless of the type of cells (adherent or nonadherent) or culture vessel (microplate, tube, or flask) used, each experiment should include the proper negative and positive control samples as indicated below.

- Negative control sample: The negative control should be a sample from your cell culture, not treated to induce apoptosis. The stained negative control sample should be run at the beginning of the experiment, and used to adjust the instrument settings for background level staining.
- Positive control sample: The positive control should be a sample of apoptotic and dead cells from a culture treated using a known apoptosis induction method for your cell line.

### Preparing Non-Adherent and Adherent Cells

The following protocols describe how to harvest non-adherent or adherent cells cultured in 96-well plates, as well as non-adherent or adherent cells cultured in flasks or other tissue culture vessels. Each of the culturing conditions requires different protocols to harvest the cells. Thereafter, the staining protocols are identical.

### Preparing non-adherent cells cultured in 96-well plates

1. Set up initial culture conditions, such that after culture and treatment, cells are at a concentration of 2 x $10^4$ to 1.5 x $10^5$ cells in 200 µL of serum- or albumin containing medium (2 x $10^5$ to 7.5 x $10^5$ cells/mL).
2. Proceed to Cell Staining Procedure on page 6.

### Preparing adherent cells cultured in 96-well plates

For harvesting adherent cells, we suggest using Guava ViaCount Cell Dispersal Reagent (Cat. No. 4700-0050) instead of other enzymatic treatments. ViaCount Cell Dispersal Reagent contains proteases that cleave proteins, nucleases that cleave DNA and RNA, and collagenases that cleave collagen, among other enzymes. Hence, ViaCount Cell Dispersal Reagent is preferred for detaching adherent cells and dispersing cell clumps over other enzymatic treatments, such as trypsin. Alternately, some cells can be dislodged mechanically or by using an EDTA solution.

1. Set up initial culture conditions, such that after culture and treatment, cells are at a concentration of 2 x 104 to 1.5 x $10^5$ cells in 100 µL (2 x $10^5$ to 7.5 x $10^5$ cells/mL).
2. Centrifuge the cells at 300 x g for 5 to 7 minutes.
3. Adherent cells may detach from the plate as they start to enter into apoptosis. Spinning the cells down prior to detaching ensures that all cells are at the bottom of the well before aspirating.
4. Aspirate off the culture medium and rinse each well once with 50 µL of PBS.
5. Pipette the PBS wash, which will contain any detached apoptotic cells, into a fresh 96-well plate.
6. Dilute the ViaCount Cell Dispersal Reagent 1:3 with PBS.
7. Add 50 µL of diluted ViaCount Cell Dispersal Reagent to each well and incubate at 37°C for 3 to 5 minutes (or until cells begin to detach).
8. Add 100 µL of medium (containing at least 5% BSA or serum) to each well and pipet repeatedly to release cells from the well bottoms.
9. Add the 50 µL of PBS from step 5 back into each well.
10. Centrifuge the cells at 300 x g for 5 to 7 minutes.
11. Aspirate the culture medium, being careful not to disturb the cell pellet.
12. Add 200 µL of fresh serum- or albumin-containing medium to each well.
13. Proceed to Cell Staining Procedure on page 6.

**Preparing non-adherent cells cultured in flasks or other tissue culture vessels**

1. Transfer between 2 x $10^4$ and 1.5 x $10^5$ cells in 200 µL of serum- or albumin containing medium (2 x $10^5$ to 7.5 x $10^5$ cells/mL) to each well in a 96-well round bottom plate or into a 1.5-mL microcentrifuge tube.
2. Proceed to Cell Staining Procedure on page 6.

**Preparing adherent cells cultured in flasks or other tissue culture vessels.**

For harvesting adherent cells, we suggest using Guava ViaCount Cell Dispersal Reagent (Cat. No. 4700-0050) instead of other enzymatic treatments. ViaCount Cell Dispersal Reagent contains proteases that cleave proteins, nucleases that cleave DNA and RNA, and collagenases that cleave collagen, among other enzymes. Hence, ViaCount Cell Dispersal Reagent is preferred for detaching adherent cells and dispersing cell clumps over other enzymatic treatments, such as trypsin. Alternately, some cells can be dislodged mechanically or by using an EDTA solution.

1. Remove culture medium from flask and place in a 50-mL conical screw cap tube.

**NOTE:** This is to retain any detached apoptotic or dead cells present in the flask.

2. Wash cells with 10 mL of 1X PBS.
3. Remove PBS and place in 50-mL conical screw cap tube used in step 1.
4. Dilute the ViaCount Cell Dispersal Reagent 1:3 with PBS.
5. For a T-75cm2 flask, add 3 mL of diluted ViaCount Cell Dispersal Reagent and incubate at 37°C for 3 to 5 minutes (or until cells being to detach).
6. Add 6 mL of medium with serum and pipet repeatedly to release cells from the flask bottom.

**NOTE:** If the cells are typically grown in serum- or protein-free medium, then serum or BSA must be added to the medium to a final concentration of at least 5%. The addition of the protein is necessary to “quench” the activity of the enzymes in the ViaCount Cell Dispersal Reagent.

7. Transfer released cells to the 50-mL conical screw cap tube used in step 1.
8. Centrifuge cells at 300 x g for 5 to 7 minutes.
9. Aspirate the culture medium, being careful not to disturb the cell pellet.
10. Add fresh serum- or albumin-containing medium to the 50-mL tube.
11. Count the cells in suspension with either Guava ViaCount Reagent or Guava ViaCount Flex Reagent and adjust the cell concentration, if necessary.
12. Transfer 2 x $10^4$ to 1.5 x $10^5$ cells in 100 µL of serum- or albumin-containing medium (2 x $10^5$ to 7.5 x $10^5$ cells/mL) to each well in a 96-well microplate or into a 1.5-mL microcentrifuge tube.
13. Proceed to Cell Staining Procedure on page 6.

## Warranty

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03/2010
Revision A: 4600-3300MAN