FlowCellect™ MitoDamage Kit

100 Tests

Cat. No. FCCH100106

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: 1-800-645-5476 (phone), 1-800-645-5439 (fax)
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Application

Apoptosis, or programmed cell death, is an important and active regulatory pathway of cell growth and proliferation. Cells respond to specific induction signals by initiating intracellular processes that result in characteristic biological, morphological and physiological changes. Cells undergoing the apoptotic process display depolarization of the inner mitochondrial membrane electrochemical gradient, mitochondrial release of apoptogenic molecules, and activation of specific proteases termed caspases, blebbing of cytosolic vesicles from the cell surface and loss of plasma membrane asymmetry, condensation of nuclear material, and finally, DNA cleavage and ruptures of the plasma membrane [1–7]. However, some of these cell changes are not specific to the apoptotic process. Therefore, to validate the presence of apoptosis in a cell sample, one single assay is usually not considered sufficient. More recent studies have suggested multiple mechanisms of cell death such as caspase mediated and caspase independent cell death. Multiparametric evaluation of apoptosis markers allows detailed kinetic events in the sequence of events leading to apoptosis and provides broader information on events in the cell mechanism of mode of action. Characterizing the mechanistic machinery of apoptosis at molecular levels and changes that occur in different compartments during apoptosis provides for a greater understanding of compound mode of action and disease processes.

The FlowCellect™ MitoDamage Kit allows for the simultaneous measurement of 3 important cell health parameters; change in mitochondrial potential considered an early hallmark of apoptosis and cellular stress, phospatidyl serine expression on the cell surface of apoptotic cells as assessed by Annexin V binding and plasma membrane permeabilization or cell death using a single cellular sample. The simultaneous measurements of these parameters minimize assay workflow and time to results, utilizes less sample and ensures more precise measurements. Multiparametric evaluation of these 3 cell health markers can be of great utility in compound screening, understanding mechanistic machinery on treatment and disease and for conducting kinetic and dose response studies.

Test Principle

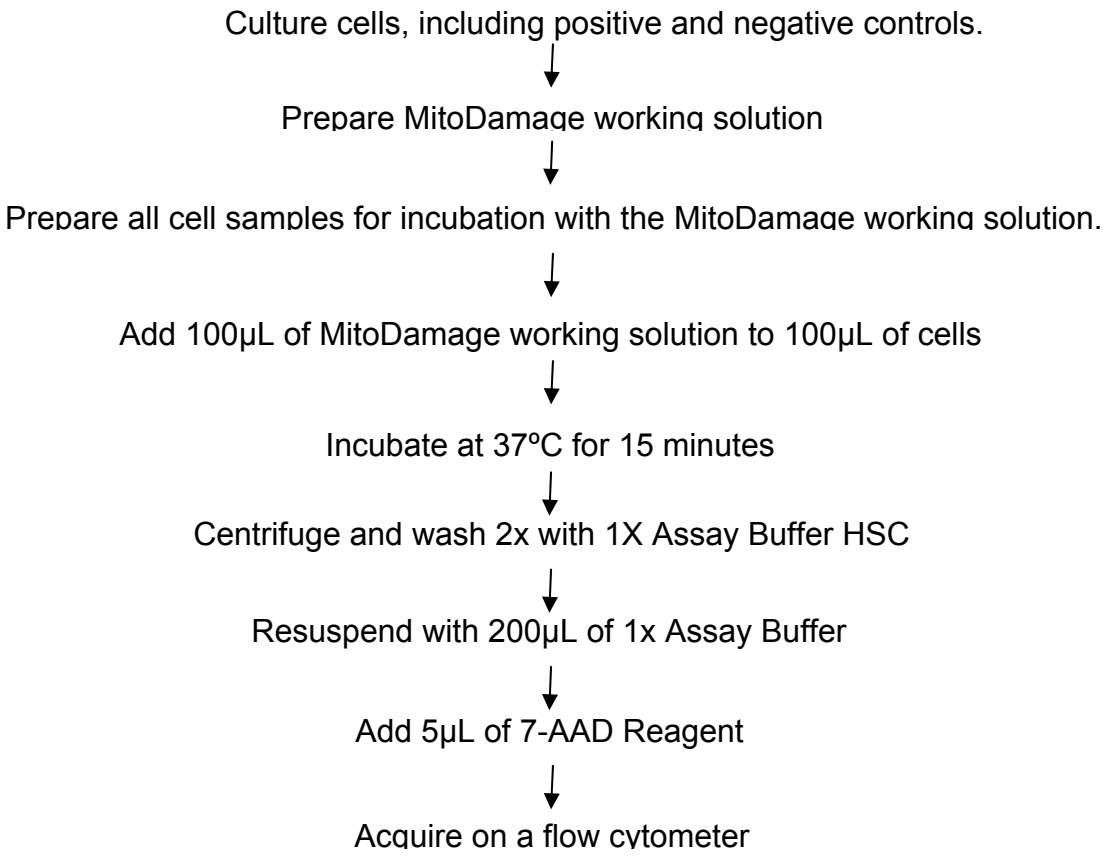
Millipore's FlowCellect™MitoDamage Kit includes (1) MitoSense Red (1,1',3,3,3',3' - Hexamethylindodicarbocyanine iodide), a fluorescent cationic dye that accumulates in the mitochondria and is responsive to mitochondrial potential changes. 2) Annexin V conjugated to a green sensitive dye CF488A which binds to phosphatidylserine on surface of apoptotic cells and (3) 7-AAD a membrane impermeant dead cell dye. The simultaneous use of the reagents allows researchers to obtain information on early, mid and late apoptosis in one simple assay.

MitoSense Red is excitable by a red laser and fluoresces maximally at 650 nm (Red2 fluorescence on the guava easyCyte 8HT). Uninduced cells with intact mitochondrial membrane potential demonstrate high Red2 fluorescence while cells which have impaired mitochondrial membrane potential depict lower Red2 fluorescence. Annexin V is calcium-dependent phospholipid binding protein with high affinity for PS, a membrane component normally localized to the internal face of the cell membrane. It is conjugated to CF488A which is excited by a 488 nm laser and emits at 525 nm. Control cells depict no green fluorescence while apoptotic cells exhibit positive green fluorescence. The cell impermeant DNA intercalator 7-Aminoactinomycin (7-AAD), dye included in the kit monitors cell membrane permeability changes typically observed later in apoptosis as well as necrotic cell death. 7-AAD is excluded from live, healthy cells as well as early apoptotic cells and these cells have low red fluorescence. The kit can thus distinguish multiple populations: 1) Live cells with intact mitochondrial membrane 2) Cells with dissipated membrane potential but no Annexin V or 7AAD staining 3) Early

apoptotic cells with dissipated membrane potential and Annexin V binding 4) Late Apoptotic Cells or dead cells 3) Cells with dissipated membrane potentials and The kit thereby provides a complete picture of mitochondrial health and cell health and allows the correlation of mitochondrial damage to cell health. The entire assay can be performed in <30 min once cellular samples are ready as shown in the flow chart

Sufficient reagents are provided for 100 tests. The kit includes all optimized fluorescently labeled antibodie, dyes and buffers necessary for cell preparation and analysis.

Figure 1. FlowCellect ™ MitoDamage Assay Workflow



Kit Components

- MitoSense Red Dye (Part No.4300-0315) One vial containing 200 uL of MitoSense Red Dye.
- Annexin V, CF488A Reagent (Part No. 4300-0320) One vial containing 500 µL Annexin V, CF488A.
- 7-AAD Reagent (Part No. 4000-0110) One vial containing 500 uL of 7-AAD.
- 10X Assay Buffer HSC (Part No.4700-1325) One bottle containing 10 mL of Assay Buffer

Materials Not Supplied

1. easyCyte HT System (guava® easyCyte 8HT or easyCyte 6HT-2L) with guavaSoft™ Software or equivalent flow cytometry system with ability to detect green, red1 and red2 fluorescence
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. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 μL
8. Tabletop centrifuge capable of exceeding x300G.
9. Vortex mixer
10. Milli-Q™ Distilled Water or DI water.
11. Reagent reservoirs, optional
12. Guava® Instrument Cleaning Fluid (ICF) (Cat. No. 4200-0140), optional
13. guava easyCheck Kit (Cat. No. 4500-0025), optional
14. 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. Please refer to the MSDS sheet for specific information on hazardous materials.
- All fluorochrome conjugated antibodies and dyes are light sensitive and must be stored in the dark at 2-8°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.
- Avoid microbial contamination of the solution, which may cause erroneous results.
- Do not use reagents beyond their expiration date.

Storage

Upon receipt, all antibodies, dyes and buffers should be stored at 2-8°C.

Caution: *Fluorochrome conjugated antibodies should always be stored at 2-8°C. Any deviation in temperature for long periods of time may compromise the performance of the antibodies.*

Caution: *MitoSense Red Dye is highly hygroscopic and needs to be stored dessicated.*

Preparation of Reagents

1. Preparation of 1X Assay Buffer HSC: The Assay buffer is supplied as a 10X concentrate, which must be diluted to 1X with deionized water prior to use. Approximately 1 mL of 1X Assay Buffer is required per sample to be stained.
    a. Mix 1 part of Assay buffer Buffer (10X) with 9 parts of deionized water. Mix thoroughly.
    b. **Note:** Prepared 1X Assay Buffer is stable up to one month if stored at 4ºC

2. Preparation of MitoDamage Working Solution: Prepare a working solution by diluting the MitoSense Red Dye 1:50 and the Annexin V, CF 488A stock solution 1:20 in 1X Assay Buffer HSC. Each sample to be tested requires 100 µL of the MitoDamage Working Solution. MitoDamage Working Solution must be made fresh each day of use.
    a. Dilute the stock solution with 1X Assay Buffer HSC as suggested in the following table: NOTE: Quantities below are for one or more extra tests to allow for sufficient volume for the desired number of tests.

Table 1. Preparation of MitoDamage Working solution

	1 Test	10 Tests	25 Tests	100 Tests
MitoSense Red Dye	2 uL	20 uL	50 uL	200 uL
Annexin V, CF488A	5 uL	50 uL	125 uL	500 uL
1X Assay Buffer HSC	93 uL	9950 uL	2375 uL	9500 uL

    b. The MitoDamage Working Solution must be used the same day it is prepared. Store at room temperature, protected from light until ready for use.

Before You Begin

This protocol was developed to allow direct determination of the percent of early and late apoptotic populations induced in cultures. For optimal throughput, 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.

Cells should be acquired shortly after the sample preparation had been completed. While some cell lines have been shown to yield stable results for up to 3 hours, others are stable for only 1 hour. This time variability is a consequence of using live, unfixed cells. You should determine the stability of results for your own cells. We strongly discourage fixing the cells after sample preparation to enhance stability, as the fixation will permeabilize all cells increasing the percentage of cells stained with 7-AAD, and resulting in an underestimation of the early apoptotic cells and an overestimation of the late apoptotic and dead cells.

The following procedures for cell staining are guidelines. Different cell types have varying phosphatidylserine (PS) content in their cell membranes.[9-11] Upon induction of apoptosis, different cell types vary in the amount of PS exposed on the cell surface.[8, 12] You may need to adjust the amount of Annexin V, CF488A 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™MitoDamage Kit takes approximately 30 minutes. 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 takes an additional 15 minutes to 72 hours of culture with various inducers to stimulate detectable apoptosis.

NOTE: For details on how to culture and prepare cell samples, including positive and negative control samples, for the FlowCellect™MitoDamage Kit, see Appendix A .

Example Cell Staining Protocol

1. Prepare FlowCellect™MitoDamage Kit Working Solution as described under Preparation of Reagents.
2. Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction. . For instructions on making cell suspensions, see Appendix A.
3. Resuspend cells at 1 x 10^6 cells/mL in 1x Assay Buffer HSC
4. Add 100 µL of cells in suspension to each well or tube. For instructions on making cell suspensions, see Appendix A.
5. For every cell sample (treated and untreated), add 100 uL of MitoDamage Working Solution to each well or tube.
6. Incubate the cells for 15 minutes in a 37°C CO_2 incubator.
7. Centrifuge at 300xG for 5 minutes at RT. Discard supernatant.
8. Wash 2 more times with 200 µL of 1x Assay Buffer HSC and centrifuge cells at 300xG for 5 minutes at RT. Discard supernatant.
9. Resuspend cells in each well with 200 µL of 1x Assay Buffer HSC..
10. Add 5 uL of 7-AAD reagent to each well.
11. Samples are now ready for acquisition on a flow cytometer.

Sample Data

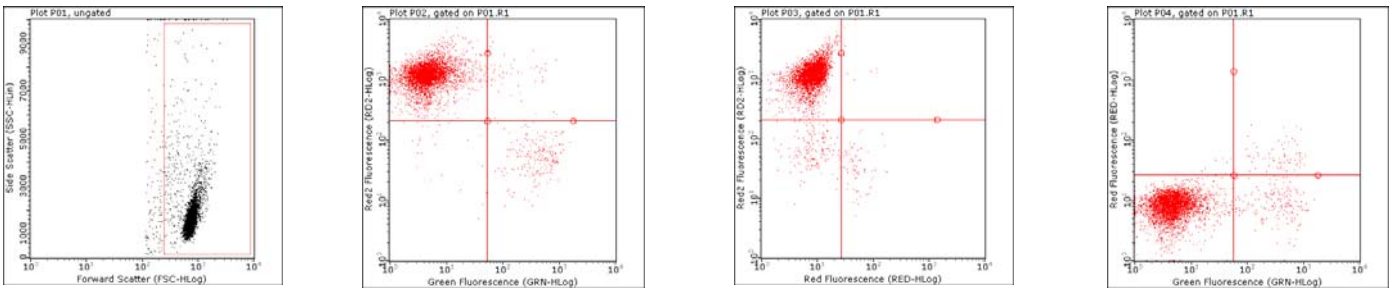


Figure 2. Display of Plots for Sample Acquistion: Set up of plots for data acquisition for samples treated with the MitoDamage Kit. Plot 1 provides the plot of FSC (log) vs. SSC which s typically used to gate and count cells. Plot 2 provides comparison of MitoSense Red (y-axis, Red2 channel) vs. Annexin V, CF488A (Green channel); Plot 3 provides comparison of MitoSense Red (y-axis, Red2 channel) vs. 7-AAD (Red channel); Plot 4 provides comparison of 7-AAD (Red Channel) vs. Annexin V, CF488A (green channel). Use the uninduced sample to adjust settings for green and red channels. Adjust settings for the Red2 channel so as to place the fluorescent population at top of the plot. Adjust the green and red settings so that they have minimal fluorescence. If needed the user can also set up histogram plots for the green, red and red2 channels.

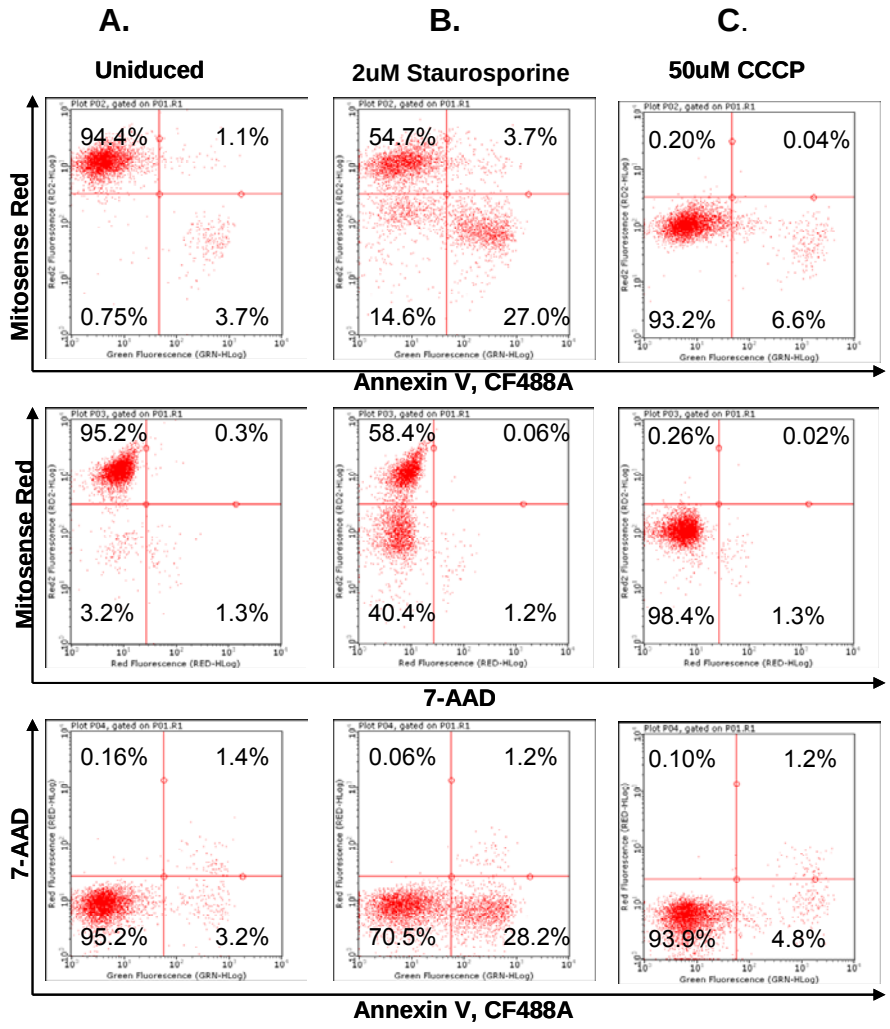


Figure 3. Analyzed Dual Parameter Data: Dot plots depicting Jurkat cells treated with multiple inducers and stained using MitoDamage Kit. Jurkat cells were treated with 0 (Plot A), 2 uM Staurosporine (Plot B) and CCCP (Plot C) and then stained using the MitoDamage Kit. Plots show the percentage of positive cells for 1) Apoptosis (Annexin V binding) and mitochondrial membrane potential change (Red2), 2) Cell death and mitochondrial membrane potential change, and 3) apoptosis and cell death.

Technical Hints

- All kit reagents, MitoSense Red Dye, Annexin V, CF488A , 10 X Assay Buffer HSC and 7AAD Reagent should be brought to room temperature prior to staining and washing.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- The easyCyte HT System and FlowCellect™MitoDamage Kit yield optimal results when the stained cell sample used for acquisition is between 1 x 10^4 to 5 x 10^5 cells/mL. To obtain the most accurate results, adjust the cell concentrations to within the recommended range. However, to optimize throughput, Millipore recommends using cellular concentrations between 1 x 10^5 to 5 x 10^5 cells/mL when possible.

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. • 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.
Cells do not show a downward shift in membrane potential.	• Cells may not have undergone mitochondrial membrane depolarization or the MitoSense Red Dye may not have not been taken up correctly by the cells. Positive control samples are recommended for each experiment. Positive controls should be appropriate for comparison with the test procedure or test cell population. Treatments to induce membrane potential changes in various cell lines include, but are not limited to CCCP and Valinomycin. • Although the assay procedure has been optimized to function utilizing many different cell types, further titrations may be necessary for some cell types to capture the ideal staining concentration. A lack of signal may indicate that excess antibody will need to be used during the staining procedure.
Dim MitoSense Red Dye Staining	• Dim or false negative staining obtained with the MitoSense Red Dye may indicate reagent degradation. Verify that the reagent is not past its expiration dates before using. • Dim staining may also be a sign that the cell concentration was too high (>500 cells/ μL) and thus the concentration of reagents was insufficient to properly stain the cells. Repeat the experiment, using a lower number of cells per well.

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. 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 for MitoSense Red Dye.
Low level of staining of Annexin V	• Cells may not have induced or the Annexin V may have not been taken up correctly by the cells. To determine optimal apoptotic induction, conduct a time-course study in order to achieve the best results for Annexin V, CF488A staining. Positive control samples are recommended for each experiment. Positive controls should be appropriate for comparison with the test procedure or test cell population. Use a cell line previously characterized as inducible for apoptosis. 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. • Although the assay procedure has been optimized to function utilizing many different cell types, further titrations may be necessary for some cell types to capture the ideal staining concentration. A lack of signal may indicate that excess antibody will need to be used during the staining procedure.
Background and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing many different cell types, further 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. • If all samples appear to be induced even when low levels of induction are expected, your cultures may be compromised. It is important to run negative control samples for each experiment. The negative control should be a sample from your cell culture, not treated to induce apoptosis. Typically, negative control samples show a low level of Annexin V and/or 7AAD positive cells that is distinct from that of induced cells because healthy cell cultures contain a small number of apoptotic and/or dead cells. However, 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 increased Annexin V and/or 7AAD reactivity. • If adherent cells have high background staining, the cells may be damaged. Avoid damaging adherent cells when removing them from their substrate.
Variability in day to day experiments	• If the FlowCellect™MitoDamage Kit results are inconsistent, check that the samples were well mixed prior to acquisition. If using an easyCyte 8HT System, be sure that the mixing option has been selected in the Worklist file used to collect data. Cells may quickly settle in your samples and your results will be inaccurate unless the cells are mixed just prior to acquisition. • Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results.

	• If there appears to be day-to-day variation of the staining pattern, ensure the easyCyte HT System is working properly. Run the easyCheck Procedure using the easyCheck Kit (Part No 4500-0025) to verify proper instrument function and accuracy.

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

References

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Related Kits

1. FlowCellect™ MitoPotential Red Kit (Catalog No. FCCH100105)
2. FlowCellect™ MitoLive Kit (Catalog No. FCCH100107)
3. FlowCellect™ Annexin Red Kit (Catalog No. FCCH100108)
4. FlowCellect™ MitoStressKit (Catalog No. FCCH100109)
5. FlowCellect™ Cytochrome *c* Kit (Catalog No. FCCH100110)
6. Guava® EasyCyte™ MitoPotential™ Kit (Catalog No. 4500-0250)
7. Guava Nexin® Reagent (Catalog No. 4500-0450, 4500-0455)
8. Guava Caspase Kits (Catalog No. 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 x 10^5 cells in 200 µL of serum- or albumin containing medium (2 x 10^5 to 10 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 x 105 cells in 100 µL (2 x 105 to 10 x 105 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 x 10^5 cells in 200 µL of serum- or albumin containing medium (2 x 10^5 to 10 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-75 cm2 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 pipette 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 x 10^5 cells in 100 µL of serum- or albumin-containing medium (2 x 10^5 to 10 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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