FlowCellect™ MitoLive Kit

100 Tests

Cat. No. FCCH100107

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Not for use in diagnostic procedures.

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Application

Mitochondria are important cellular organelles that maintain crucial cellular energy balance, contain key regulators of cell death processes such as apoptosis and play a significant role in cellular oxidative stress generation. Fluorescence-based assays designed to evaluate the functional status of mitochondria are emerging as useful tools to elucidate the role of mitochondrial activity[1-3] in the apoptosis cascade and other cellular processes. Loss of the mitochondrial inner transmembrane potential ($\Delta\Psi m$) is often [4-7], but not always [8,9], observed to be associated with the early stages of apoptosis and is also believed to have a role in caspase independent cell death. Collapse of mitochondrial membrane potential is believed to coincide with the permeabilization of outer mitochondrial membrane, and release of Cytochrome c and other pro-apoptotic proteins into the cytosol, which then triggers the downstream events in the apoptotic cascade. Another important parameter of cell health it's cell vitality, which is typically measured by assessing intracellular esterase activity. Approaches to assessment of cell vitality include the use of carboxylated non-fluorescent compounds modified with acetomethoxy ester groups that can permeate cell membranes. Once inside the cell, the ipophilic blocking groups are cleaved by nonspecific esterases, resulting in a highly fluorescent form that is retained in cells unless there is a loss of integrity of cellular membranes. Use of acetomethoxy esters such as Calcein AM thus reflect the status of intracellular esterases and membrane permeability providing important cell viability and health information.

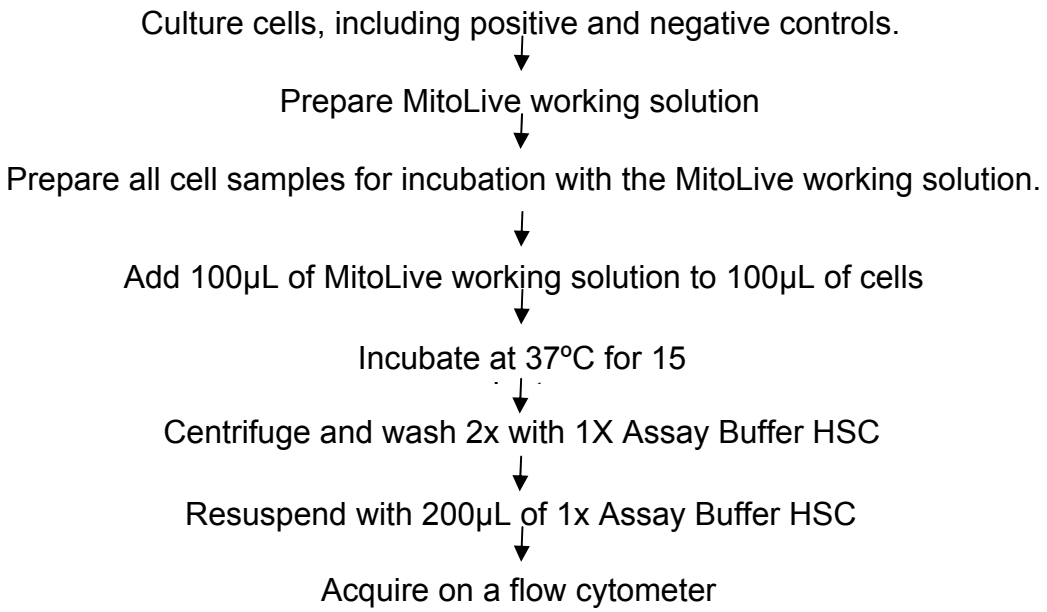
Mitochondria and mitochondrial function thus are highly sensitive indicators of cell health and stress and mitochondrial dysfunction can be a trigger for processes such as caspase-dependant and caspase-independent cell death, loss in cellular metabolism or vitality and eventual cell death. The simultaneous measurement of mitochondrial health as measured by mitochondrial membrane potential status and cellular vitality provides a quick assessment of the state of mitochondrial health and its correlation to overall cellular health. The FlowCellect MitoLive Kit allows for the simple, simultaneous measurement of 2 important cell health parameters; (1) change in mitochondrial membrane potential using MitoSense Red dye, and (2) change in cellular vitality (as measured by activity of intracellular esterases) using Calcein AM. The simultaneous measurement of the two parameters minimize assay workflow and time to results, utilizes less sample and ensures more precise measurements. Measurement of mitochondrial membrane potential along with cell vitality is of increasing importance in compound profiling during the drug discovery process, screening for mitochondrial dysfunction, toxicity and in the study of process and mechanisms of apoptosis and as a reflection of disease states [10,11].

Test Principle

Millipore's FlowCellect™ MitoLive 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 and (2) Calcein acetoxymethylester (calcein, AM) a non-fluorescent, cell permeant compound that is hydrolyzed by intracellular esterases into the fluorescent anion calcein and provides a measure of cellular vitality. The simultaneous use of the reagents allows researchers to obtain information on early and late apoptosis in one simple assay. MitoSense Red is excitable by a red laser and fluoresces maximally at 650 nm (Red2 channel 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. The cell permeant dye, Calcein, AM included in the kit, enters all cells and if the cell is vital is hydrolyzed by intracellular esterases into the fluorescent anion calcein which are retained in cells with intact membranes. Healthy cells thus exhibit high green fluorescence. Cells in which the intracellular esterases are impacted on treatment or have compromised membranes demonstrate low green fluorescence. The assay kit allows the identification of 3 populations of cells; healthy live cells with intact membrane potential, live cells with impaired mitochondria and non-vital or dead cells with impaired mitochondria. The entire assay can be performed in approximately 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 dyes and buffers necessary for cell preparation and analysis.

Figure 1. FlowCellect™ MitoLive Assay Workflow



Kit Components

- MitoSense Red Dye (Part No.4300-0315) One vial containing 200 uL of MitoSense Red Dye.
- Calcein, AM Reagent (Part No. 4300-0310) One vial containing 200 uL of Calcein, AM.
- 1X Assay Buffer (Part No.4700-1330) One bottle containing 100 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. 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. Milli-Q™ Distilled Water or DI water.
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. Please refer to the MSDS sheet for specific information on hazardous materials.
- All 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 dyes and buffers should be stored at 2-8°C.

Caution: *Dyes 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 and Calcein, AM are highly hydroscopic and need to be stored desiccated.*

Preparation of Reagents

1. Preparation of MitoLive Working Solution: Prepare a working solution by diluting the MitoSense Red Dye 1:50 and the Calcein, AM 1:50 in 1X Assay Buffer. Each sample to be tested requires 100 µL of the MitoLive Working Solution. MitoLive Working Solution must be made fresh each day of use.
   Note: MitoSense Red Dye and Calcein, AM are in DMSO and therefore solid at 2-8ºC. Allow the reagent to completely thaw prior to making the working solution.
   a. Dilute the stock solutions with 1X Assay Buffer 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 MitoLive Working Solution

	1 Test	10 Tests	25 Tests	100 Tests
MitoSense Red Dye	2 uL	20 uL	50 uL	200 uL
Calcein, AM	2 uL	20 uL	50 uL	200 uL
1X Assay Buffer	96 uL	9960 uL	2400 uL	9600 uL

   b. The MitoLive 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 depolarized cells induced in cultures and plated in 96-well microplates. For optimal performance, final cell concentration should be between 2 x 10^4 to 5 x 10^5 cells/mL (4 x 10^3 to 1 x 10^5 cells/well). Care should be taken to keep cell concentrations as constant as possible in all wells of an experiment. The mean fluorescent intensity of polarized and depolarized cells can vary significantly with a two-fold change in cell concentration, although the percentage of polarized and depolarized cells remains constant. However, if the cell concentration exceeds 5 x 10^5 cells/mL, the MitoSense Red Dye will be in limiting concentration and hence will be unable to concentrate in mitochondria, resulting in polarized cells appearing to be depolarized.

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 2 hour. This time variability is a consequence of using live, unfixed cells. You should determine the stability results for your own cells.

Time considerations: The process of staining cells with the FlowCellect™ MitoLive 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 may take an additional 15 minutes to 72 hours to induce the cells to depolarize.

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

Example Cell Staining Protocol

1. Prepare FlowCellect™ MitoLive Kit Working Solution as described under Preparation of Reagents.
2. Treat cells by desired method. Concurrently incubate a control culture without induction. For instructions on making cell suspensions, see Appendix A.
3. Centrifuge and resuspend cells at 1 x 10^6 cells/mL in 1x Assay Buffer
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, add 100 uL of MitoLive Working Solution to each well or tube.
6. Incubate the cells for 15 minutes in a 37°C CO_2 incubator.
7. Centrifuge at 300 x g for 5 minutes at RT. Discard supernatant.
8. Wash 2 more times with 200 µL of 1x Assay Buffer 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.
10. Samples are now ready for acquisition on a flow cytometer.

Sample Data

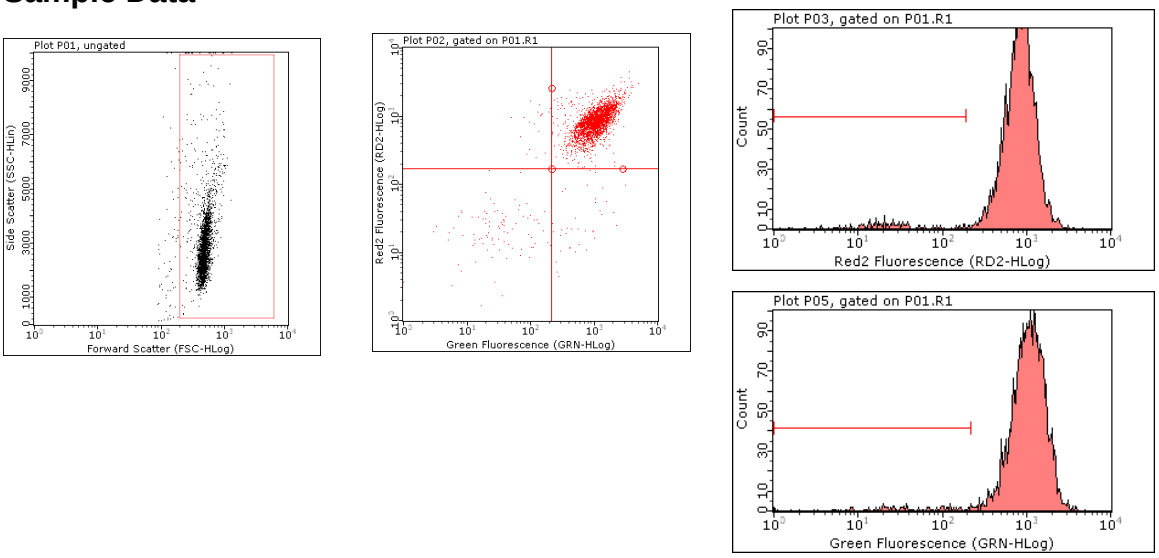


Figure 2. Display of Plots for Sample Acquistion: Set up of plots for data acquisition for samples treated with the MitoLive Kit. Plot 1 provides the plot of FSC (log) vs. SSC which s typically used to gate and count cells (x events are usually counted). Plot 2 provides comparison of MitoSense Red (y-axis, Red2 channel) vs. Calcein, AM (Green channel). If needed the user can set up histogram plots for MitoSense Red (Red2 channel, Plot 3) and for Calcein, AM (Green Channel, Plot 4). Use the uninduced sample to adjust settings for the Red2 and Green channels by placing the cells centered around 10^3 in each case.

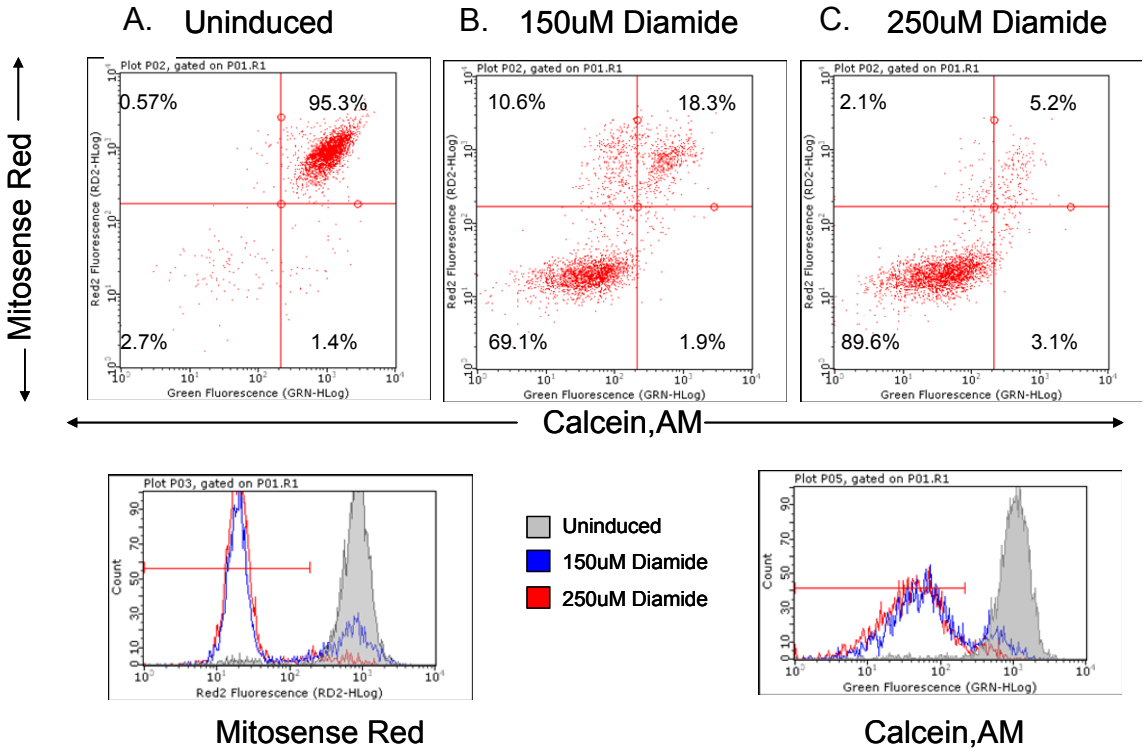


Figure 3. Analyzed Dual Parameter Data: Dot plots depicting Jurkat cells stained using MitoLive Kit. Jurkat cells were uninduced (Plot A), Induced with 150 uM Diamide (Plot B), Induced with 250 uM Diamide (Plot C), and then stained using the MitoLive Kit. Quadrant gates were set up on the uniduced cells and applied to the treated cells. The histogram plots show the change in both the Mitosense Red staining and the Calcein, AM staining with treatment. Samples which undergo a change in mitochondrial potential will show a downward shift in fluorescence and samples which demonstrate decreased cell vitality will show a decrease in green fluorescence.

Technical Hints

- All kit reagents, MitoSense Red Dye, 1 X Assay Buffer and Calcein ,AM 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™MitoLive 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 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 x g until a compact and visible cell pellet forms.
High background staining for adherent cells	• The cells may be damaged. Avoid damaging adherent cells when removing them from their substrate.
Low level of staining of MitoSense Red or Calcein, AM	• Possible reagent degradation. Verify that the reagent has been stored desiccated and is not past its expiry date. • Check if Live/uninduced control samples demonstrate good staining • Check if the right volume of MitoSense Red and Calcein AM was used in making up the MitoLive Working Solution and the solution was made fresh.

	• Dim staining may be a sign that the cell concentration was too high and the concentration of reagents was insufficient to stain the cells. Repeat experiment using lower number of cells per well. • 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 dye will need to be used during the staining procedure.
No downward shift in signal on induction	• Cells may not have undergone a change in membrane potential or vitality. Positive controls should be included for each experiment to ensure accurate staining protocol. Positive controls should be appropriate for comparison with the test procedure or test cell population. • Treatments to induce a change in membrane potential in various cell lines include CCCP, valinomycin, and staurosporine. Positive controls for vitality include Diamide as well as heat killed cells.
Poor resolution of stained populations.	• Poor resolution could indicate that the staining time was too short. Make sure that the cells were stained for 15 minutes in a 37 C CO2 incubator.
Variability in day to day experiments	• If the FlowCellect™ MitoLive Kit results are inconsistent, check that the samples were well mixed prior to acquisition. If using a ,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) 437-7500

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Related Kits

1. FlowCellect™ MitoPotential Red Kit (Catalog No. FCCH100105)
2. FlowCellect™ MitoDamage Kit (Catalog No. FCCH100106)
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 7.

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 7

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 7.

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 7.

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