FlowCellect™ MitoPotential Red Kit

100 Tests

Cat. No. FCCH100105

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

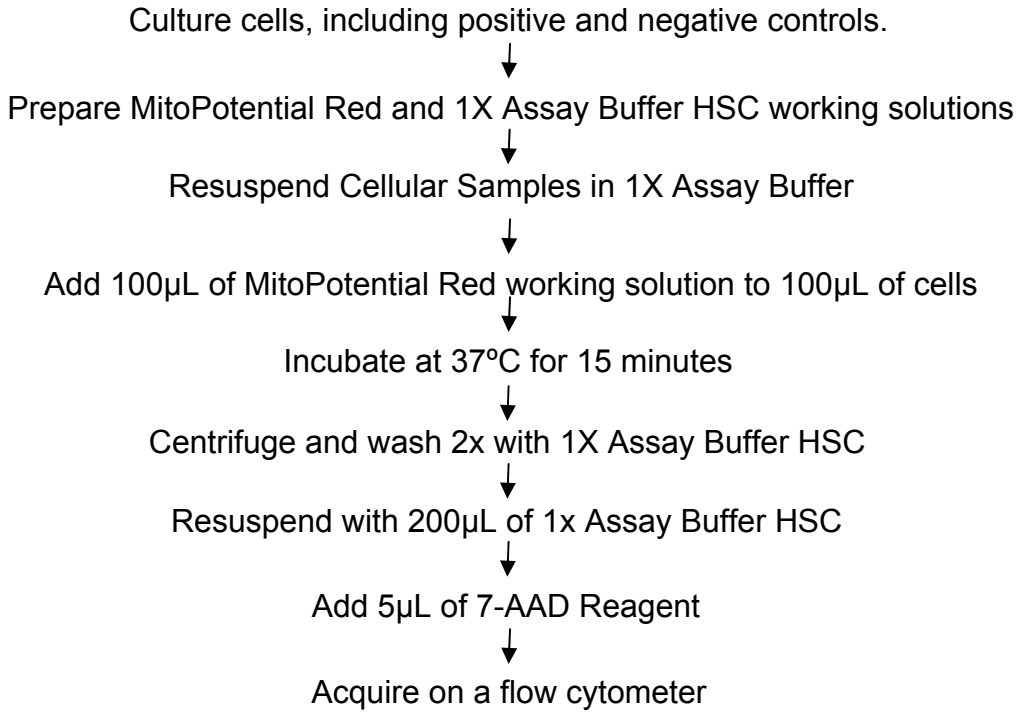
Mitochondria are important cellular organelles that maintain crucial cellular energy balance and in addition contain key regulators of cell death processes such as apoptosis. Mitochondria are also a primary site of production of free radicals and play a key role in oxidative stress generation for the cell. Mitochondria and mitochondrial function thus are highly sensitive indicators of cell health and stress. Cellular energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane and this accumulation of energy in healthy cells creates a mitochondrial trans-membrane potential, ($\Delta\Psi m$) that enables the cell to drive the synthesis of ATP. Loss of the mitochondrial inner transmembrane potential is often [1-7], but not always [8,9], observed to be associated with the early stages of apoptosis. Collapse of this potential is believed to coincide with the opening of the mitochondrial permeability transition pores, leading to the release of cytochrome C into the cytosol, which then triggers the downstream events in the apoptotic cascade. Mitochondrial membrane potential changes have been implicated in apoptosis, necrotic cell death and caspase independent cell death processes. Depolarization of the inner mitochondrial membrane potential is thus a reliable indicator of mitochondrial dysfunction and cellular health, which has become increasingly important in the study of apoptosis, drug toxicity and multiple disease states.

Fluorescence-based assays designed to evaluate the functional status of mitochondria are emerging as useful tools to elucidate the role of mitochondrial activity in the apoptosis cascade and other cellular processes. The FlowCellect MitoPotential Red Kit allows for the simultaneous measurement of 2 important cell health parameters; change in mitochondrial potential, considered an early hallmark of apoptosis and cellular plasma membrane permeabilization or cell death. The simultaneous measurements of these parameters minimizes assay workflow and time to results, utilizes less sample and ensures more precise measurements. Multiparametric evaluation of mitochondrial membrane potential changes along with cell death is of great utility in detecting apoptosis, drug toxicity and compound screening studies, understanding mechanistic machinery and as a reflection of disease states.

Test Principle

Millipore's FlowCellect™MitoPotential Red Kit is a dual parameter assay kit that 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) 7-AAD a membrane impermeant dead cell dye. The simultaneous use of the reagents allows researchers to obtain information on mitochondrial dysfunction and cell death 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. 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 [10]. The kit can thus distinguish 3 populations: 1) Live cells with intact mitochondrial membrane 2) Early apoptotic cells with dissipated membrane potential but no 7AAD staining 3) Late Apoptotic Cells or dead cells that have dissipated membrane potential and compromised membranes demonstrate an increase in red fluorescence. The entire assay can be performed in approximately 30 min once cellular samples are ready as shown in the flow chart (Figure 1). Sufficient reagents are provided for 100 tests. The kit includes optimized preformulated dyes and buffers necessary for cell preparation and analysis on a flow cytometer equipped with a blue and red laser. The FlowCellect™MitoPotential Red Kit involves simple and fairly rapid assay protocols, requires no compensation and allows for easy to interpretation of test results.

Figure 1. FlowCellect™ MitoPotential Red Assay Workflow



Kit Components

- MitoSense Red Dye (Part No.4300-0315) One vial containing 200 uL of MitoSense Red Dye.
- 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
15. 0.5-mL microcentrifuge tubes (VWR Cat. No. 16466-036 or equivalent) for sample acquisition
16. 1.5-mL microcentrifuge tubes (VWR Cat. No. 16466-030 or equivalent) for cleaning
17. 96-well microplate plates, round bottom (Falcon Cat. Nos. 353910 or 353918) or flat bottom (Falcon Cat. No. 353075 or 353915), or equivalent. Refer to the appropriate Guava System user's guide for other compatible microplates.

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 fluorescent dyes and included buffers should be stored at 2-8°C.

***Caution:**. Any deviation in temperature for long periods of time may compromise the performance of the reagents.*

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

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 HSC is required per sample to be stained.
    a. Mix 1 part of Assay buffer Buffer (10X) with 9 parts of deionized water. Mix thoroughly.
    Note: Prepared 1X Assay Buffer HSC is stable up to one month if stored at 4ºC

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

Preparation of MitoSense Red Dye Working Solution

	1 Test	10 Tests	25 Tests	100 Tests
MitoSense Red Dye	2 uL	20 uL	50 uL	200 uL
1X Assay Buffer HSC	98 uL	9980 uL	2450 uL	9800 uL

    b. The MitoPotential Red 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 and count of cells with depolarized membrane potential 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.

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 hours. This time variability is a consequence of using live, unfixed cells. The user is advised to determine the stability of results for your own cells.

Time considerations: The process of staining cells with the FlowCellect™ MitoPotential Red 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™ MitoPotential Red Kit, see Appendix A.

Example Cell Staining Protocol

1. Prepare FlowCellect™MitoPotential Red Kit Working Solution and 1X Assay Buffer HSC as described under Preparation of Reagents.
2. Culture cells including positive and negative controls by desired method. 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 HSC
4. Add 100 µL of cells in suspension to each well or tube.
5. For every cell sample add 100 uL of MitoPotential Red 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 HSC and centrifuge cells at 300 x g 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 equipped with a 488 nm and a 633 nm laser.

Sample Data

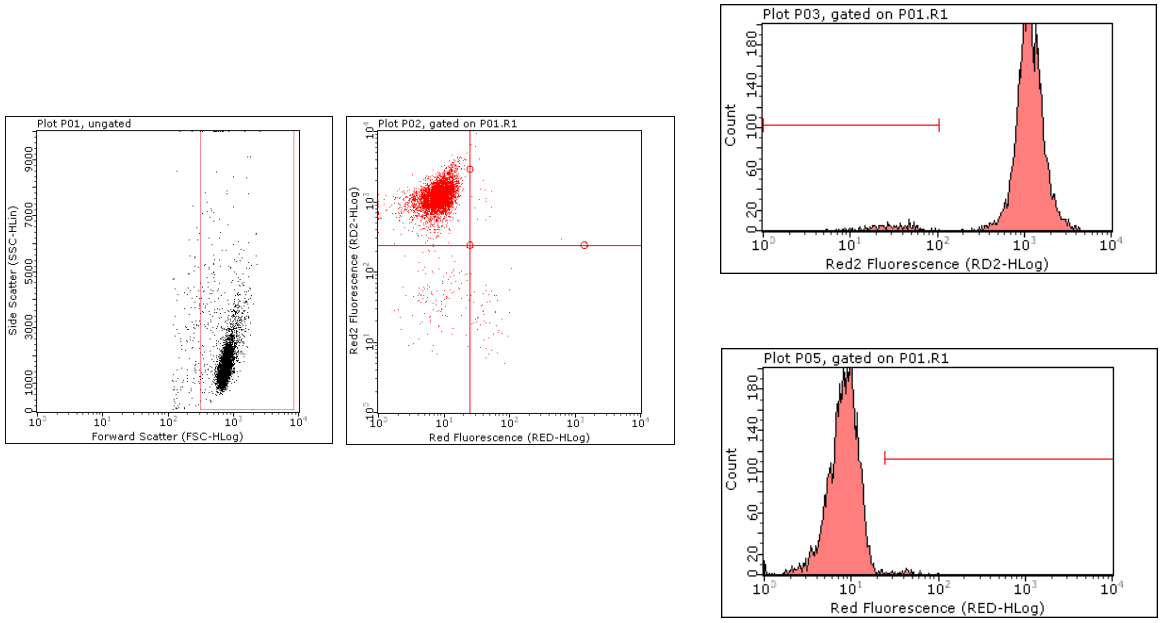


Figure 2. Display of Plots for Sample Acquisition: Set up of plots for data acquisition for samples treated with the MitoPotential Red Kit. Plot 1 provides the plot of FSC (log) vs. SSC which is typically used to gate and count cells (typically 3000 events are counted). Plot 2 provides comparison of MitoSense Red (y-axis, Red2 channel) vs. 7-AAD (Red channel); Plot 3 provides a histogram of MitoSense Red (y-axis, Red2 channel) and Plot 4 provides a histogram of 7-AAD (Red Channel). Use the uninduced sample to adjust settings. Adjust settings such that the Red2 fluorescent population for the uninduced population such that it is centered around 10^3. Adjust Red settings such that the population is centered around 10^1

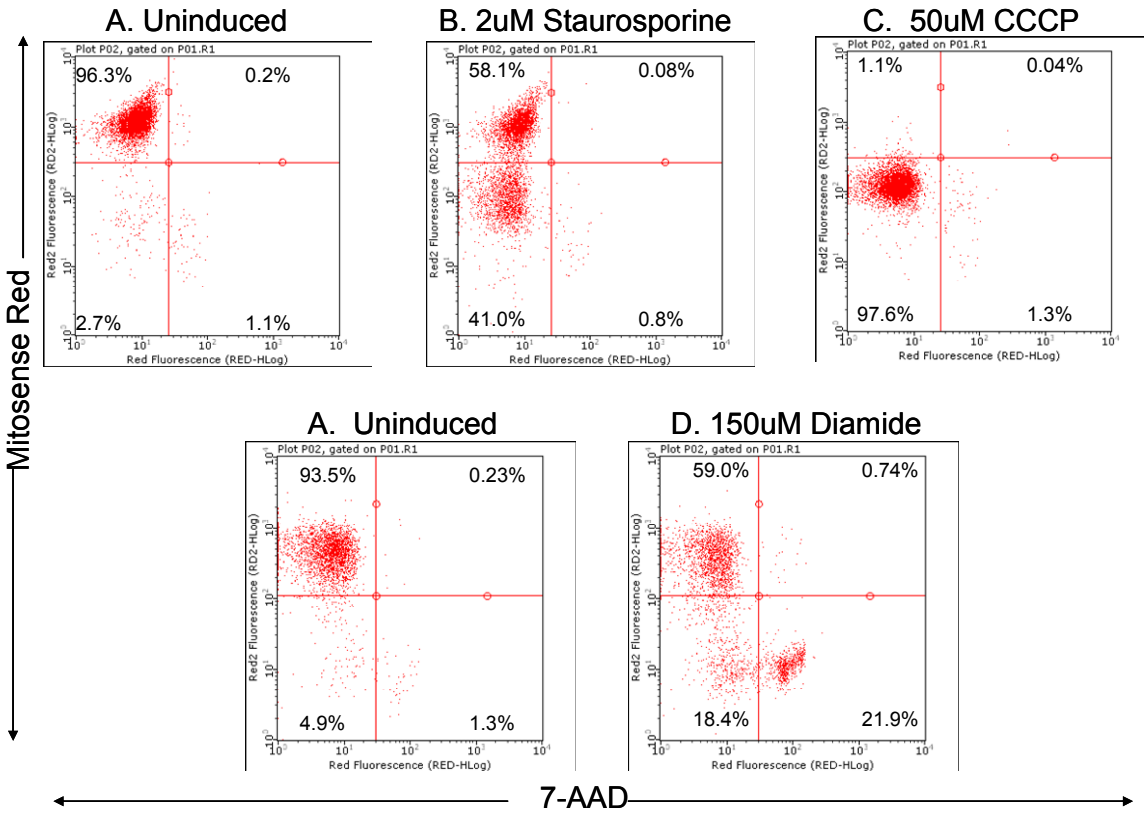


Figure 3. Analyzed Dual Parameter Data: Dot plots depicting Jurkat cells stained using MitoPotential Red Kit. Jurkat cells were treated with 0 (Plot A), 2 uM staurosporine for 3 hrs, (Plot B) , 50uM CCCP for 15 minutes (Plot C), 150uM Diamide for 2 hours (Plot D) and then stained using the MitoPotential Red Kit. Quadrant gates were set up on the uniduced cells and applied to the treated cells. Samples which undergo a change in mitochondrial potential will show a downward shift in fluorescence and samples which demonstrate cell death will show an increase in Red fluorescence.

Technical Hints

- All kit reagents, MitoSense Red Dye, 10 X Assay Buffer HSC and 7AAD Reagent should be brought to room temperature prior to staining and washing.
- After removing the desired amount of MitoSense Red Dye, the reagent should be desiccated and placed back at 2-8ºC.
- 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™MitoPotential Red Kit yield optimal results when the stained cell sample used for acquisition is between 2 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.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	• Cell concentration may be too high. Decrease the number of cells per microliter by diluting sample to 300 – 500 cells/uL. The Guava EasyCyte™ Plus or guava easyCyte HT systems gives the most accurate data when the flow rate is less 500 cells/uL. • 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.
No positive cells	• Cells may not have undergone a change in membrane potential. Positive controls should be included for each experiment to ensure accurate staining protocol. Treatments to induce a change in membrane potential in various cell lines include CCCP, valinomycin, and staurosporine.
Dim or Low level of staining of MitoSense Red	• Possible reagent degradation. Verify that the reagent has been stored desiccated and is not past its expiry date. • Live/uninduced control samples are recommended for each experiment. • 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.
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 indcubator.
Variability in day to day experiments	• If the FlowCellect™MitoPotential Red 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

References

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Related Kits

1. FlowCellect™ MitoDamage Kit (Catalog No. FCCH100106)
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 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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