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Guava® MitoDamage Kit User's Guide

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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 Guava® MitoDamage Kit allows for the simultaneous measurement of three 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 three 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

The Guava® MitoDamage Kit includes:

- 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
- Annexin V–conjugated to a green sensitive dye CF™488A which binds to phosphatidylserine on surface of apoptotic cells
- 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 CF™488A, 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:

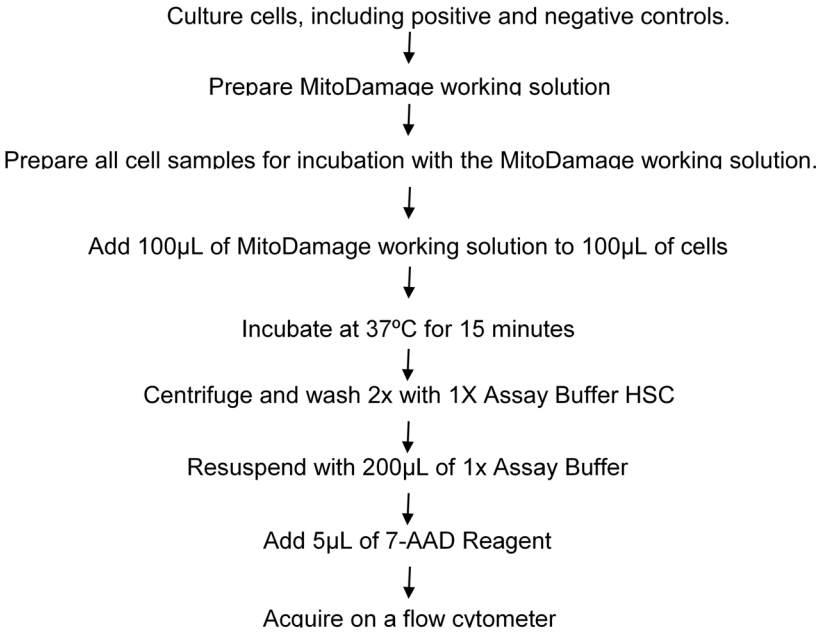
- Live cells with intact mitochondrial membrane

- Cells with dissipated membrane potential but no Annexin V or 7-AAD staining
- Early apoptotic cells with dissipated membrane potential and Annexin V binding
- Late Apoptotic Cells or dead cells
- Cells with dissipated membrane potentials

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 minutes 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 antibodies, dyes, and buffers necessary for cell preparation and analysis.

Figure 1: Guava MitoDamage Assay Workflow



Kit Components

- MitoSense Red Dye (Part No. 4300-0315) – One vial containing 200 µL of MitoSense Red Dye
- Annexin V, CF™488A Reagent (Part No. 4300-0320) – One vial containing 500 µL Annexin V, CF™488A
- 7-AAD Reagent (Part No. 4000-0290) – One vial containing 500 µL of 7-AAD
- 10X Assay Buffer HSC (Part No. 4700-1325) – One bottle containing 10 mL of Assay Buffer

Materials Not Supplied

- 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
- ViaCount™ reagent (Catalog No. 4000-0041) or ViaCount Flex reagent (Catalog No. 4700-0060)
- Cell line of interest
- Media for cell line of interest
- Tissue culture instruments and supplies (including 37 C incubator, growth media, plates, detachment buffer, etc.)
- Polypropylene tubes and or bottles for sample and buffer preparation and storage.
- Pipettors with corresponding tips capable of accurately measuring 1–1000 µL
- Tabletop centrifuge capable of exceeding x300G.
- Vortex mixer
- Milli-Q™ Distilled Water or DI water.
- Reagent reservoirs, optional
- Guava® Instrument Cleaning Fluid (ICF) (Cat. No. 4200-0140), optional
- Guava easyCheck Kit (Cat. No. 4500-0025), optional
- 20% bleach solution

Precautions

- The Guava® MitoDamage Kit is intended for research use only.
- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling this reagent.
- The Guava MitoDamage Kit contains sodium azide, which is toxic if ingested. Reagents containing sodium azide should be considered a poison. If products containing sodium azide are swallowed, seek medical advice immediately and show product container or label. (Refer to NIOSH, National Institute for Occupational Safety and Health; CAS#: 2628-22-8; and also to GHS, The Globally Harmonized System of Classification and Labeling of chemicals.) Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic as. Any reagents containing sodium azide should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent build-up in plumbing. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be regulated as hazardous.
- Avoid microbial contamination of the solution, which may cause erroneous results.
- All biological specimens and materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.
- Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous result.
- 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.

- The fluorescent dyes in this reagent are light sensitive. Store in the dark and shield from excessive exposure to light.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of the product, centrifuge the vial briefly prior to removing the cap.
- Do not use the reagent beyond the expiration date.
- Safety Data Sheets(SDSs) for our kits are available from our web site (www.luminexcorp.com) or by contacting Luminex Technical Support.

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.

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, CF™488A 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 Guava® 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 Guava MitoDamage Kit, see "Appendix A: Cell Sample Preparation" on page 11.

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.

      NOTE: Prepared 1X Assay Buffer is stable up to one month if stored at 4°C.

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 µL	20 µL	50 µL	200 µL
Annexin V, CF™488A	5 µL	50 µL	125 µL	500 µL
1X Assay Buffer HSC	93 µL	930 µL	2325 µL	9300 µL

   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.

Example Cell Staining Protocol

1. Prepare Guava® MitoDamage Kit Working Solution as described under "Preparation of Reagents" on page 5.
2. Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction. For instructions on making cell suspensions, see "Appendix A: Cell Sample Preparation" on page 11.
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: Cell Sample Preparation" on page 11.
5. For every cell sample (treated and untreated), add 100 µL 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 two 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 µL 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 is typically used to gate and count cells. Plot 2 provides comparison of MitoSense Red (y-axis, Red2 channel) vs. Annexin V, CF™488A (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, CF™488A (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.

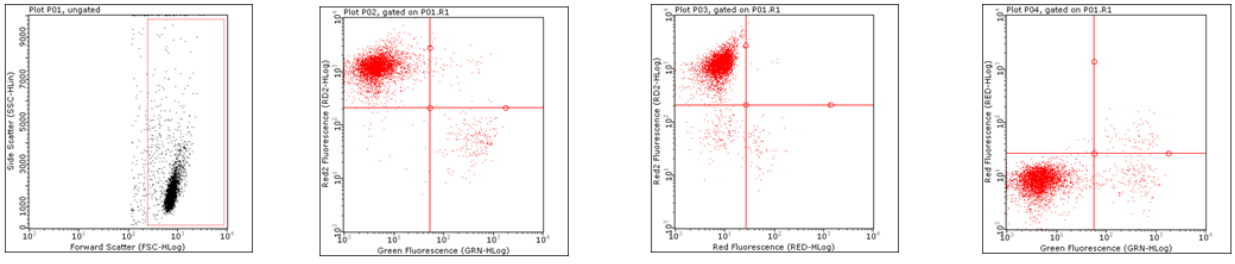
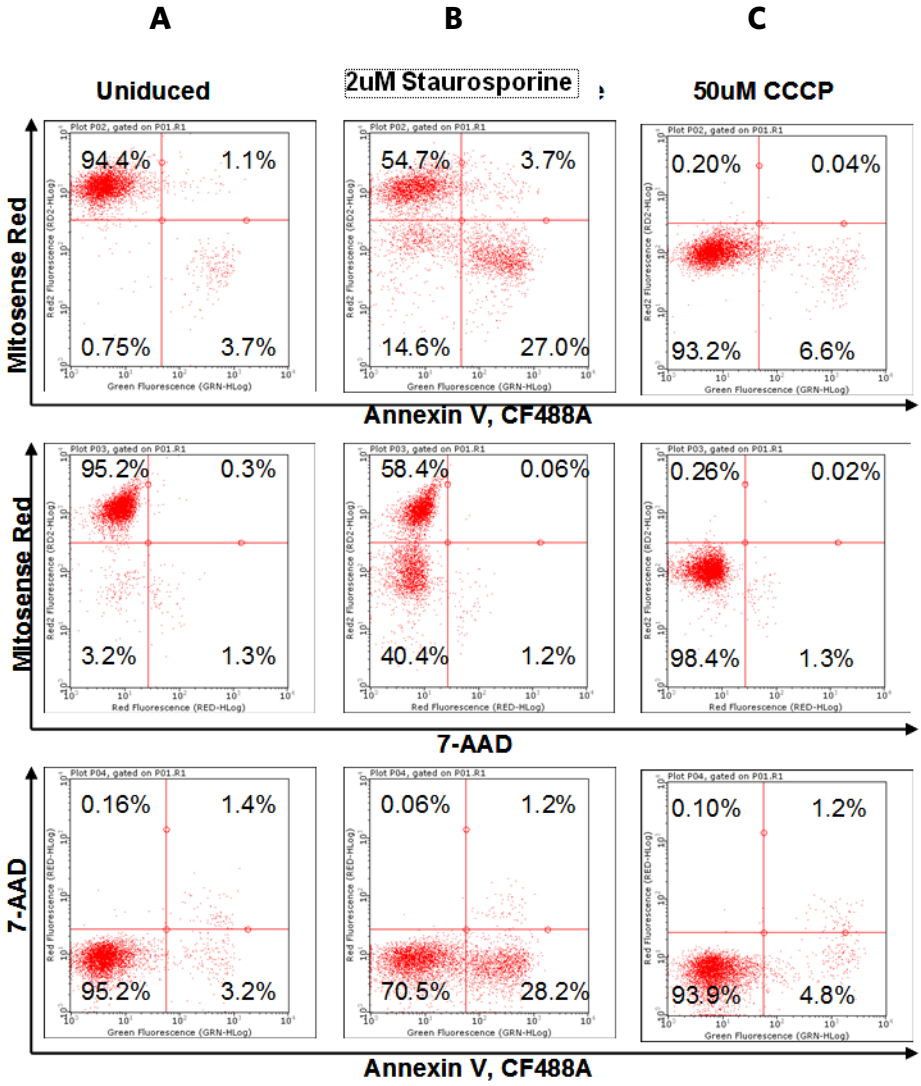


Figure 3: Analyzed Dual Parameter Data: Dot plots depicting Jurkat cells treated with multiple inducers and stained using MitoDamage Kit. Jurkat cells were treated with 0 (Plot A), 2 µM Staurosporine (Plot B) and CCCP

(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, CF™488A, 10X Assay Buffer HSC and 7-AAD 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 Guava® 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, Luminex 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.
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.

Potential Problem	Experimental Suggestions
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, CF™488A 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 7-AAD 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 7-AAD 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 Guava 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.

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Related Kits

- Guava® Annexin Red Kit (Catalog No. FCCH100108)
- Guava® Cytochrome c Kit (Catalog No. FCCH100110)
- Guava Nexin® Reagent (Catalog No. 4500-0450, 4500-0455)

Appendix A: Cell Sample Preparation

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.

Preparing Non-adherent Cells

1. Set up initial culture conditions, such that after culture and treatment, cells are at a concentration of 1 x 10^5 to 1 x 10^7 cells/mL in serum- or albumin containing medium.
2. Proceed to "Example Cell Staining Protocol" on page 6.

Preparing Adherent Cells

For harvesting adherent cells, use your method of removal. Reagents such as EDTA or trypsin can be used to dissociate the cells from the flask and should create single-cell suspensions. If using mechanical means to dislodge the cells, additional reagents such as Muse® Cell Dispersal Reagent (Catalog No. MCH100107) may be used to dissociate clumps.

1. Using your preferred method for dissociation, detach the cells from their culture vessel.
2. Add fresh serum- or albumin-containing medium to each well so final concentration is between 1 x 10^5 to 1 x 10^7 cells/mL.
3. Proceed to "Example Cell Staining Protocol" on page 6.

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