FlowCellect™ Annexin Red Kit

100 Tests

Cat. No. FCCH100108

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Application

Apoptosis, or programmed cell death, is an important and active regulatory pathway of cell growth and proliferation and is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Altered level of apoptosis is a factor in many neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. Understanding the mechanistic machinery of apoptosis at molecular levels provides for a greater understanding of compound mode of action and disease processes (10).

The process of apoptosis is accompanied by multiple morphological, biochemical and physiological changes in response to specific induction signals. Among these are externalization of phosphatidylserine (PS) to the cell surface, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, and loss of membrane integrity (in late stages).(1-5) The translocation of phosphatidylserine on the external surface of apoptotic cells is considered a critical marker of apoptosis. Early in the apoptotic process, the membrane phospholipid phosphatidylserine is translocated from the inner to the outer layer of the plasma membrane, thus exposing phosphatidylserine to the external environment of the cells. Annexin V is a 35-36kD calcium-dependent, phospholipid-binding protein with high affinity and specificity for phosphatidylserine and when fluorescently labeled can be used as a sensitive probe for the detection of apoptosis (6-7). Since phosphatidylserine is expressed in the inner membrane of cells, late stage of apoptotic or necrotic cells with compromised membrane integrity can also exhibit staining with Annexin V in junction with a vital dye. Hence, Millipore's FlowCellect™ Annexin Red Assay kit includes 7-AAD (8), a membrane impermeable dead cell dye to clearly distinguish early apoptotic cells from late stage apoptotic and necrotic cells.

Evaluation of annexin V expression on surface of apoptotic cells is finding increased importance in compound screening and mechanistic pathways, optimization of cell culture conditions, in multiple disease states and in kinetic and dose response studies (11). The FlowCellect™ Annexin Red kit also provides researchers increased multiplexing flexibility to pair apoptotic markers with their cell type specific/other markers(9) which fluoresce in the green and yellow channels and in the analysis of GFP expressing cell lines (12).

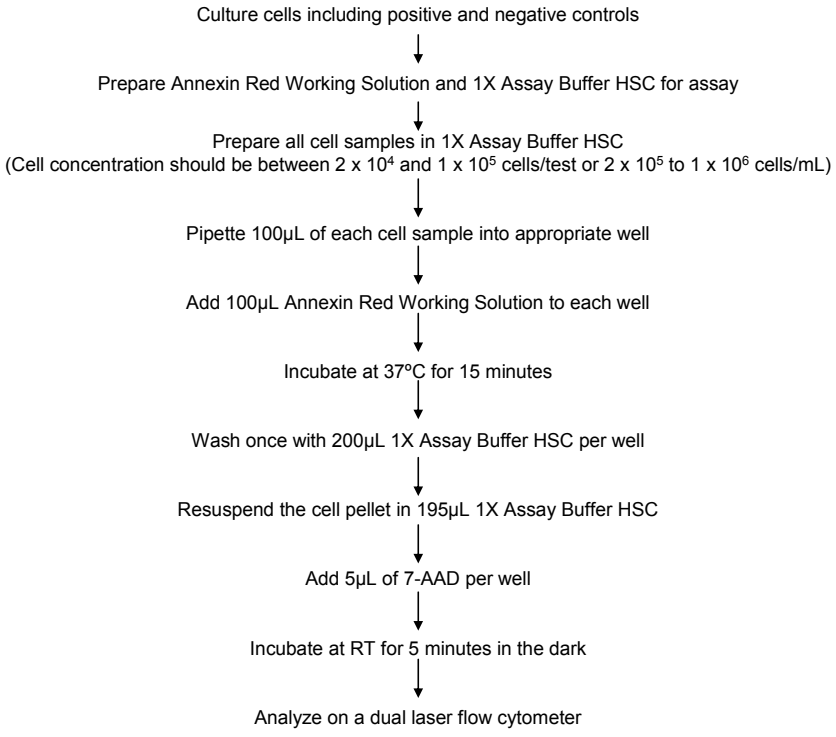
Test Principle

Millipore's FlowCellect™ Annexin Red Kit provides a rapid and convenient assay to monitor early and late apotosis. The kit includes (1) recombinant Annexin V conjugated to a red sensitive dye CF647 to provide maximum sensitivity and (2) 7-Aminoactinomycin (7-AAD) a membrane impermeant dead cell dye, which is an indicator of cell membrane structural integrity and (3) an assay buffer. The simultaneous use of these reagents allows researchers to obtain information on early and late apoptotic cells in one simple assay. Annexin V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidyl serine expressed on the cell membrane. Annexin V, CF647 is excitable by a red laser, fluoresces maximally at 670 nm and can be detected in the Red2 channel of dual laser cytometers. Live cells demonstrate little or no Annexin V, CF 647 binding and demonstrate minimal Red2 fluorescence. In apoptotic cells, due to the translocation and exposure of phosphatidylserine on the external cell surface, use of Annexin V, CF647 allows the detection of apoptotic cells and these cells demonstrate increased Red2 fluorescence. Late apoptotic and necrotic cells also demonstrate increased Annexin V CF 647 binding and increased Red2 fluorescence but can be distinguished from early apoptotic cells due to the inclusion of 7-AAD in the kit. 7-Aminoactinomycin (7-AAD) a cell impermeant DNA intercalator is excitable by a 488 nm laser and can be detected in the Red channel. 7-AAD is excluded from live, healthy cells as well as early apoptotic cells and these cells have low red fluorescence. After staining cells with Flowcellect Annexin Red Kit, three populations of cells can be identified in this assay:

- Non-apoptotic cells: Annexin V(-) and 7-AAD(-)
- Early apoptotic cells: Annexin V(+) and 7-AAD(-)
- Late stage apoptotic or dead cells: Annexin V(+) and 7-AAD(+)

The kit thus permits users to pair the provided reagents with other reagents that fluoresce in the green and yellow channels of the flow cytometer. Sufficient reagents are provided for 100 tests. The kit includes all optimized fluorescently labeled reagents and buffers necessary for cell preparation and analysis.

Figure 1. FlowCellect™ Annexin Red Assay Workflow



Kit Components

- Annexin V, CF647 Reagent (Part No. 4300-0325) One vial containing 500 μL Annexin V, CF647.
- 7-AAD Reagent (Part No. 4000-0290) One vial containing 500 μL of 7-AAD.
- 10X Assay Buffer HSC (Part No. 4200-0131) One bottle containing 10 mL of Assay Buffer HSC

Materials Not Supplied

1. guava HT System (guava® easyCyte 8HT or easyCyte 6HT-2L) with guavaSoft™ Software or equivalent flow cytometry system with ability to detect Red2 and Red fluorescence
2. ViaCount™ reagent (Catalog No. 4000-0041)
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, flasks, detachment reagents, etc.)
6. Polypropylene tubes for sample and buffer preparation and storage.
7. Microplates
8. Pipettors with corresponding tips capable of accurately measuring 1 – 1000 μL
9. Tabletop centrifuge.
10. Vortex mixer
11. Milli-Q™ Distilled Water or deionized water.
12. Guava ICF Instrument Cleaning Fluid (Catalog Number 4200-0140), optional
13. guava easyCheck Kit (Catalog 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 reagents 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 the expiration date.

Storage

Upon receipt, all reagents and buffer in the kit should be stored at 2-8°C.

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

Preparation of Reagents

1. Preparation of 1X Assay Buffer: 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 test.
    a. Allow 10X Assay Buffer HSC to come to room temperature.
    b. Mix 1 part of 10X Assay Buffer HSC with 9 parts of deionized water. Mix thoroughly.
    c. **Note:** Prepared 1X Assay Buffer HSC is stable up to one month if stored at 2-8ºC,

2. Preparation of Annexin Red Working Solution: Prepare Annexin Red working solution by diluting the Annexin V, CF647 stock solution 1:20 in 1X Assay Buffer HSC. Each sample to be tested requires 100 µL of the Annexin Red Working Solution. Annexin Red Working Solution must be made fresh each day of use.
    a. Dilute the stock solution with 1X Assay Buffer as suggested in Table 1. Vortex immediately after mixing the reagents.
    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 Annexin Red Working Solution

	1 Test	10 Tests	25 Tests	100 Tests
Annexin V.CF647 Stock Reagent	5 µL	50 µL	125 µL	500 µL
1X Assay Buffer HSC	95 µL	950 µL	2375 µL	9500 µL

    b. The Annexin 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 of early and late apoptotic cell populations induced in culture and plated in 96-well microplates. For optimal performance, final cell concentrations should be between 2 x 10^4 and 1 x 10^5 cells/well (or 2 x 10^5 to 1 x 10^6cells/mL). Care should be taken to keep cell concentrations as constant as possible in all samples of an experiment.

FlowCellect™ Annexin Red is a live cell assay and cell samples 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 may have more limited stability. 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, 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. Upon induction of apoptosis, different cell types vary in the amount of PS exposed on the cell surface. You may need to adjust the amount of Annexin V, CF647 Reagent or incubation time 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™ Annexin Red Reagent and acquiring data on Guava System usually takes approximately 1 - 2 hours. However, preparing cells for testing requires periodic maintenance and cultivation of cells several days in advance. Once you cultivate the proper number of cells for your experiment, it takes an additional 2 to 48 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™ Annexin Red Assay, see Appendix A on page 13.

Example Cell Staining Protocol

1. Prepare 1X Assay Buffer and Annexin Red 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 on page 13.
3. Resuspend cells at 5 x 10^5 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 on page 13.
5. For every cell sample (treated and untreated), add 100 µL of Annexin V, CF647 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 xg for 5 minutes at RT. Aspirate off supernatant.
   Note: In several cases, analysis can also be performed without any washes and proceeding to Step 10 directly. However, improved separation of populations is obtained when wash steps in 7 & 8 are performed.
8. Wash 1 more time with 200 µL of 1x Assay Buffer HSC per well and centrifuge cells at 300 xg for 5 minutes at RT. Aspirate off supernatant.
9. Resuspend cells in each well with 195 µL of 1x Assay Buffer HSC.
10. Add 5 µL of 7AAD reagent to each well and mix by repeat pipetting.
11. Incubate the samples in the dark at RT for 5 minutes.
12. Samples are now ready for acquisition on a flow cytometer equipped with a blue and red lasers.

Sample Data

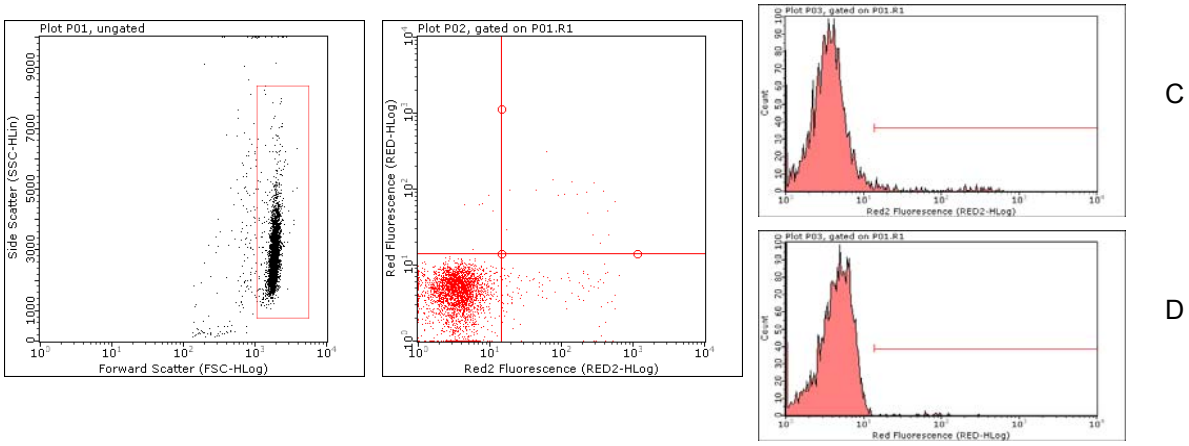


Figure 2: Display of Plots for Sample Acquisition: Set up of plots for data acquisition for samples treated with the FlowCellect™ Annexin Red Kit**.** Plot A provides the plot of FSC (log) vs. SSC which is typically used to gate and count cells of the desired population (3000 events are typically counted). Plot B is a dot plot of Annexin V, CF647 (x-axis, Red2 channel) vs. 7-AAD (y-axis, Red Channel) Fluorescence.. If histogram analysis is desired by the user, Annexin V, CF647 (Red2 channel) histogram (Plots C) and 7-AAD (Red) histogram (Plot D) can also be set up during data acquisition. Adjust settings using the negative control (uninduced) sample such that the gated cellular population in Plot B has low Red2 and low Red fluorescence as shown above.

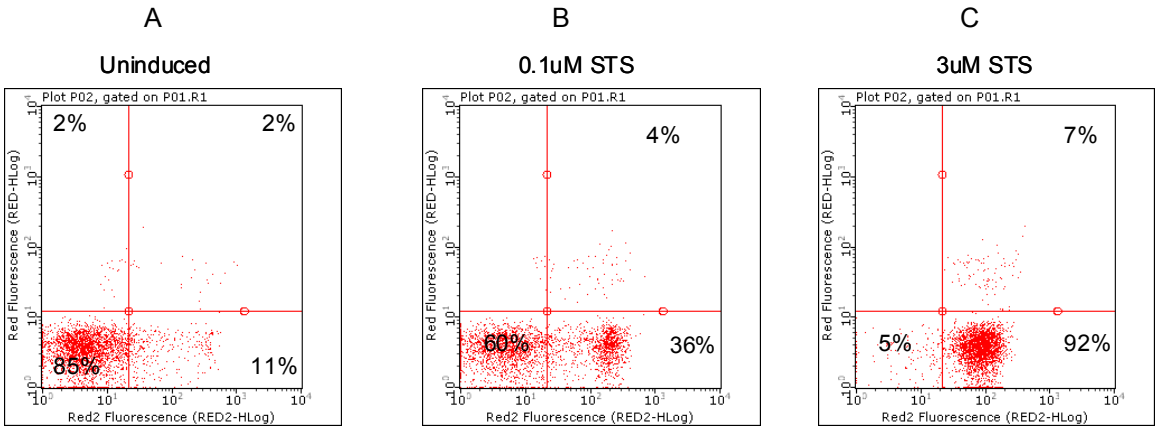


Figure 3 Analyzed Dual Parameter Data: Dot plots depicting Jurkat cells treated with multiple concentration of inducers and stained using Annexin Red Kit**.** Jurkat cells were untreated (Plot A), or treated with 0.1 μM (Plot B) or with 3 μM Staurosporine (Plot C) for 3 h and then stained using FlowCelllect Annexin Red Kit.

Technical Hints

- All kit reagents, Annexin V, CF647 reagent, 10 X Assay Buffer 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 cells have good viability prior to use.
- For certain cell cultures cell pellets may become loose following the induction, making it difficult to see. When handling limited number of cells, plate-based staining allows better sample recovery and handling.
- The guava System and FlowCellect™ Annexin Red Reagent 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	• Check first for the lack of sufficient sample volume in your tube or well. If using an automated system, you must first Pause the acquisition and eject the tray. If the sample volume is less than 50 µL (in a well) or 35 µL (in a tube), then there is insufficient sample. Either add additional buffer to bring the sample volume up to greater 50µL, or proceed to the next sample.
Instrument clogging	• If the sample volume is more than 50 µL, then the lack of events acquired is probably due to blockage of the flow system which can be caused by cell aggregates, cell debris, salt crystals, or other particulates. Perform a Backflush step to flush out the system, this step reverses fluid into the W1 tube which should contain a small amount of 20% bleach for disinfection. Then perform a Quick Clean cleaning cycle to remove bleach residue. You may then continue with your acquisitions. If this procedure does not alleviate the problem, consult the appropriate Guava System User's Guide or contact technical service for additional help. • If your instrument clogs frequently, the samples may contain significant amounts of cellular debris that might build up in the flow system. It is recommended to perform a Clean & Rinse cycle at the end of every 12 or 24 samples. This option should be selected from the WorkEdit program, refers to the guava HT System user's guide.
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.
Too high cell concentration during acquisition	• If the concentration of the stained cell sample for acquisition is high (>500cells/ µL), the accuracy of data will most likely be compromised. Dilute the sample further with 1X Assay Buffer HSC to have the cell concentration below 500cells/ µL. For best results, it is recommended that the cell concentration is in the range of 200 - 300cells/ µL.
Background and/or non-specific staining of cells	• If cells have high background staining, the cells may be damaged as dead cells tend to aggregate and nonspecifically adsorb fluorescent reagent. Avoid damaging cells when handling them in culture. • Non-specific staining and background may indicate that less fluorescent reagent 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.
No or Low level of Annexin V positive staining	• 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, CF647 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. • The reagents provided in the FlowCellect™ Annexin Red Kit have been optimized for optimal reactivity utilizing many different cell types. Other cell types may show different patterns of reactivity that require adjustment to reagent concentration and incubation time.
Poor resolution	• Poor resolution of uninduced and induced cells may indicate staining of too many cells or too short of an incubation time or lower temperature used during staining. Make sure to follow the recommended protocol and conditions specified. For best results, preliminary testing of cells to determine the titer of each reagent is recommended.
Variability in day to day experiments	• If the FlowCellect™ Annexin Red Assay results are inconsistent, check that the samples were well mixed prior to acquisition. If

	using a guava 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 Guava Instrument is working properly. Run the guava easyCheck Procedure using the easyCheck Kit (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™ MitoPotential Red Kit (Catalog No. FCCH100105)
2. FlowCellect™ MitoDamage Kit (Catalog No. FCCH100106)
3. FlowCellect™ MitoLive Kit (Catalog No. FCCH100107)
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)

Appendix A

Cell Sample Preparation

Preparing Controls

Regardless of the type of cells (adherent or non-adherent) 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 undergo apoptosis. The stained negative control sample should be run at the beginning of the experiment, and used to adjust the instrument settings for baseline fluorescence.
- Positive control sample: The positive control should be a sample of apoptotic and dead cells from your cell culture treated with a known apoptosis induction method.

Preparing non-Adherent and Adherent Cells

The following protocols describe how to harvest non-adherent or adherent cells cultured in 96-well plates, or in flask or in other tissue culture vessels. Each culture condition requires different protocol 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 concentration of 2 x 10^4 to 1 x 10^5 cells in 100 µL of serum- or albumin containing medium (2 x 10^5 to 1 x 10^6 cells/mL).
2. 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 100 µL of 1X Assay Buffer HSC (2 x 10^5 to 1x 10^6 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 96-well plates

For harvesting adherent cells, we suggest using Guava ViaCount Cell Dispersal Reagent (Cat. No. 4700-0050) instead of other enzymatic treatment. 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 concentration of 2 x 10^4 to 1 x 10^5 cells in 100 µL (2 x 10^5 to 1x 10^6 cells/mL).
2. Centrifuge cells at 300 xg for 5 to 7 minutes.
   - Adherent cells may detach from the plate as they undergo into apoptosis. Centrifugation of cells prior to detaching ensures that all cells are at the bottom of the well before aspirating off the medium.
3. Aspirate off the culture medium and rinse each well once with 50 µL of PBS.
4. Pipette the PBS wash, which may contain detached apoptotic cells, into a new 96-well plate.
5. Dilute the ViaCount Cell Dispersal Reagent 1:3 with PBS.
6. 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).

7. Add 100 µL of medium (containing at least 5% BSA or serum) to each well and pipette repeatedly to detach cells from the well bottom.
8. Add the 50 µL of PBS from step 5 back into each appropriate well.
9. Centrifuge cells at 300 xg for 5 to 7 minutes.
10. Aspirate the culture medium, being careful not to disturb the cell pellet.
11. Resuspend the cell pellet with 100 µL of 1X Assay Buffer HSC per well.
12. 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 Cel 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 cell and dispersing cell clumps over other enzymatic treatments, such as trypsin.

Alternately, some cells can be dislodged mechanically or by using an EDTA solution.

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

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

   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 neutralize the activity of the enzymes in the ViaCount Cell Dispersal Reagent.
7. Transfer detached cells to the 50-mL conical screw cap tube used in step 1.
8. Centrifuge cells at 300 xg for 5 to 7 minutes.
9. Aspirate off 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 cells in suspension using either Guava ViaCount Reagent or Guava ViaCount Flex Reagent. Refer to the ViaCount or ViaCount Flex package insert for suggested protocol.
12. Adjust the cell concentration with serum- or albumin-containing medium so cells are between 2 $x10^5$ to 1 x 10^6 cells/mL.
13. Transfer 2 x 10^4 to 1 x 10^5 cells in 100 µL of serum- or albumin-containing medium (2 $x10^5$ to 1 x 10^6 cells/mL) to each well in a 96-well round bottom microplate or into a 1.5-mL microcentrifuge tube.
13. Proceed to Cell Staining Procedure on page 6.

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