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# Guava Nexin® Reagent

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# Overview

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 physiological changes. 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]

Annexin V is a calcium-dependent phospholipid binding protein with high affinity for PS,[6-8] a membrane component normally localized to the internal face of the cell membrane. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V can readily bind them.[9-14] The Guava Nexin® Assay utilizes Annexin V-PE to detect PS on the external membrane of apoptotic cells. The cell impermeant dye, 7-AAD, is also used in the Guava Nexin Assay as an indicator of cell membrane structural integrity.[15] 7-AAD is excluded from live, healthy cells as well as early apoptotic cells. Three populations of cells can be distinguished in this assay:

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

Fewer than 2 x $10^4$ cells in 100 µL original volume are required for the mix-and-read Guava Nexin Assay. The cells are stained directly in the 96-well microplate or tube with Guava Nexin Reagent, a pre-made cocktail containing Annexin V-PE and 7-AAD in buffer, in a 200 µL final volume. After a 20-minute incubation at room temperature, the samples are ready to be acquired on a Guava® System.

Acquired data results are displayed on the computer screen in a dot-plot format with a user-controlled quadrant marker. The results include the count and percentage of cells in each of the quadrant-defined populations, as well as the mean fluorescence intensity of Annexin V and 7-AAD for each population. A summary table presents the values for the Annexin V positive and 7-AAD positive populations. All data are automatically saved to allow post-acquisition analysis. The results are exported into a spreadsheet for future reference.

# Guava Nexin® Workflow

Culture cells, including positive and negative controls,
for appropriate time to induce apoptosis.

↓

Allow Guava Nexin Reagent to warm to room temperature.

↓

Prepare all cell samples for incubation with Guava Nexin Reagent.

↓

Pipette 100 µL of each cell sample into appropriate tube/well.
(Cell concentration should be between 2 x $10^4$ and 1 x $10^5$ cells/sample
or 2 x $10^5$ to 1 x $10^6$ cells/mL)

↓

Add 100 µL Guava Nexin Reagent to each tube/well.

↓

Incubate for 20 minutes at room temperature in the dark.

↓

Acquire samples on a Guava System.

# Safety

- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this product.
- The Guava Nexin® Reagent contains dyes that may be carcinogenic and/or mutagenic. Exercise standard precautions when obtaining, handling, and disposing of potentially carcinogenic and mutagenic reagents.
- The Guava Nexin Reagent contains sodium azide, which is toxic.
- 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 results.
- Safety Data Sheets (SDSs) for kit reagents are available from our website (www.luminexcorp.com) or by contacting Luminex Technical Support.

# Required Materials

## Handling and Storage

1. The Guava Nexin® Reagent should be stored refrigerated (2 to 8°C). Do not freeze. Refer to the expiration date on the package label. Do not use the reagent after the expiration date.
2. The Guava Nexin Reagent contains light-sensitive dyes. Shield from excessive exposure to light.

## Kit Materials

Guava Nexin® Reagent includes one of the following:

| Reagent Kit Part Number | Contents |
|---|---|
| 4500-0450 Guava Nexin® Reagent 100 tests | One 10 mL vial of Guava Nexin® Reagent (Part No. 4700-1140) |
| 4500-0455 Guava Nexin® Reagent 500 tests | Five 10 mL vials of Guava Nexin® Reagent (Part No. 4700-1140) |

## User Supplied Equipment and Materials

- Guava system with the Guava Nexin® software module (or equivalent flow cytometry system with ability to detect PE and 7-AAD fluorescence)
- Cell suspensions, untreated and treated to undergo apoptosis
- Micropipettors
- Disposable micropipettor tips
- Vortex mixer

- Disposable gloves
- Centrifuge
- 37°C $CO_2$ incubator
- Deionized water
- Guava® Instrument Cleaning Fluid (ICF) [Cat. No. 4200-0140], optional
- 20% bleach solution

### For High-Throughput (HT) Guava® Systems

- 0.5-mL microcentrifuge tubes (VWR Cat. No. 16466-036 or equivalent) for sample acquisition
- 1.5-mL microcentrifuge tubes (VWR Cat. No. 16466-030 or equivalent) for cleaning
- 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.
- Reagent reservoirs, 50 mL (VWR Cat. No. 82026-354 or equivalent)

### For Single-Loader (SL) Guava® Systems

- 1.5-mL microcentrifuge tubes (VWR Cat. No. 16466-030 or equivalent), or 1.2-mL titer tubes (E&K Scientific, Cat. No. 604508-RC or equivalent) for sample acquisition

### Additional User Supplied Materials

- Guava® easyCheck™ Kit (4500-0025)
- Guava ViaCount™ Cell Dispersal Reagent (Cat. No. 4700-0050) for adherent cells, optional

**For High-Throughput (HT) Guava® Systems**

- Guava® ViaCount™ Flex Reagent (Cat. No. 4700-0060), optional

**For Single-Loader (SL) Guava® Systems**

- Guava® ViaCount™ Reagent (Cat. No. 4000-0040), optional

# Protocol

## 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. The mean fluorescent intensity of Annexin V-PE bound to early and late apoptotic cells can vary significantly with a two-fold change in cell concentration, although the percentage of cells bound by Annexin V-PE remains constant. However, if the cell concentration exceeds 5 x $10^5$ cells/mL, the Annexin V-PE reagent may be in limiting concentration and will therefore bind to fewer cells, resulting in lower percentages for both early and late apoptotic cells.

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.[16-18] Upon induction of apoptosis, different cell types vary in the amount of PS exposed on the cell surface.[11, 19] You may need to adjust the amount of Guava Nexin® Reagent 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 Nexin Reagent and acquiring data on your Guava® System usually takes approximately 1 hour. 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 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 Guava Nexin Assay, see *Appendix A: Cell Sample Preparation* on page 8.

## Cell Staining Procedure

1. Allow Guava Nexin® Reagent to warm to room temperature.
2. 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 8.
3. Add 100 µL of Guava Nexin Reagent to each well or tube.

   **NOTE:** Should your induced cells express large amounts of PS, it may be necessary to add more Guava Nexin Reagent. You can add up to 125 µL of Guava Nexin Reagent to each well, or up to 150 µL to each tube. If you need to use more Guava Nexin Reagent for optimal staining, then it is better to decrease the volume of medium that the cells are in from 100 to 50 µL and add between 150 to 175 µL (up to 200 µL if using tubes) of the reagent.
4. Stain samples for 20 minutes at room temperature in the dark.
5. Samples are now ready to be acquired on a Guava® System.

# Expected Results

The Guava Nexin® Software Module performs calculations automatically. The results are displayed on the computer screen after each sample is acquired. Acquired data are displayed in dot-plot format with a user-controlled quadrant marker for instantaneous on-screen presentation of the results. The display includes the count and percentage of cells in each of the quadrant-defined populations, as well as the mean fluorescence intensity for the Annexin V-PE and 7-AAD parameters of each population.

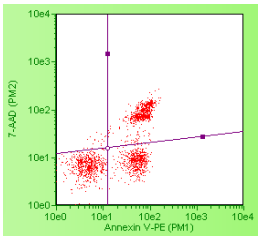
Figure 1 shows an example of results obtained using the Guava Nexin Reagent to stain Jurkat cells treated with staurosporine to induce apoptosis.

Events in each of the four quadrants are as follows:

- Lower-left quadrant: viable cells, not undergoing detectable apoptosis [Annexin V-PE (-) and 7-AAD (-)]
- Lower- right quadrant: cells in the early stages of apoptosis [Annexin V-PE (+) and 7-AAD (-)]
- Upper-right quadrant: cells in the late stages of apoptosis or dead (by necrotic or apoptotic mechanisms) [Annexin V-PE (+) and 7-AAD (+)]
- Upper-left quadrant: mostly nuclear debris [Annexin V-PE (-) and 7-AAD (+)]

In Figure 1, Jurkat cells were induced to undergo apoptosis by incubation with 1 µM staurosporine for 3 hours at 37°C. After incubation, cells were mixed with heat-killed Jurkats, stained with Guava Nexin Reagent, and acquired on a Guava System. The results show 36% early apoptotic cells (lower right) and 29% late apoptotic/dead cells (upper right) in this culture.

**Figure 1**




**Apoptosis Quadrant Analysis Results**

| | Count | % Gated | MFI-x | MFI-y |
|---|---|---|---|---|
| Upper left | 0 | 0.0% | 0 | 0 |
| Upper right | 527 | 29.4% | 102 | 78 |
| Lower left | 628 | 35.1% | 5 | 5 |
| Lower right | 635 | 35.5% | 45 | 6 |
| Annexin V+ | 1162 | 64.9% | 71 | 39 |
| 7-AAD+ | 527 | 29.4% | 102 | 78 |

# Troubleshooting Tips

1. If the Guava Nexin® Assay results are inconsistent, check that the samples were well mixed prior to acquisition. If using a single-loader system, thoroughly vortex or otherwise resuspend the cells. If using an HT system, be sure that the mixing option has been selected in the Worklist file used to collect data in the Guava Nexin Software Module. Cells may quickly settle in your samples and your results will be inaccurate unless the cells are mixed just prior to acquisition.
2. If adherent cells have high background staining, the cells may be damaged. Avoid damaging adherent cells when removing them from their substrate.
3. If the percent apoptotic and/or dead increases greatly over time, your samples may have taken too long to acquire. The post-staining stability of early and late apoptotic cells can vary from cell line to cell line.
4. If there are low levels of Annexin V-PE staining, it is possible that your cells may not be fully induced. Translocation of phosphatidylserine (PS) to the cell surface is an early event in apoptosis, which can precede DNA fragmentation by several hours, and can be reversed in some cases.[20] The Annexin V-PE staining results can vary over time as apoptosis progresses. To determine optimal apoptotic induction, conduct a time-course study in order to achieve the best results for Annexin V-PE staining.
5. If there are no Annexin V positive cells, your cells may not have induced or the Annexin V may 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. Use a cell line previously characterized as inducible for apoptosis. Treatments used to induce apoptosis in various cell lines include 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 cell line.
6. If all samples appear to be induced even when low levels of induction are expected, your cultures may be compromised. It is important to run negative control samples for each experiment. The negative control

should be a sample from your cell culture, not treated to induce apoptosis. Typically, negative control samples show a low level of Annexin V and/or 7AAD positive cells that is distinct from that of induced cells because healthy cell cultures contain a small number of apoptotic and/or dead cells. However, sub-optimal culture conditions may stress cells in culture, causing them to undergo apoptosis in the absence of experimental induction treatment. The negative control from a stressed culture often shows increased Annexin V and/or 7AAD reactivity.

7. If the separation is poor between the live population and apoptotic populations, the Annexin V-PE concentration may be too low. Guava Nexin Reagent has been formulated for optimal performance using Jurkat, CHO, HeLa, PC3, HB, and Daudi cells. Other cells may show different patterns of reactivity that require adjustments to amount of reagent used. For best results, titer the Guava Nexin Reagent to determine the amount for maximal staining of cells.
8. 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 Guava easyCheck Kit (4500-0025) to verify proper instrument function and accuracy.
9. If you are acquiring data from a sample but the Cell Count number is not increasing and the Events to Acquire bar is not moving, there is probably either insufficient volume to continue to acquire sample, or a blockage of the flow system. Check first for the lack of sufficient sample volume in your tube or well. If using an HT 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. You must dilute the sample with a 1:2 mixture of Guava Nexin Reagent in your medium before trying to acquire the sample again. If the sample volume is more than 50 µL, then the lack of events acquired is probably due to a clog. A clog or blockage of the flow system can be caused by cell aggregates, cell debris, salt crystals, or other particulates. Perform a Backflush to flush out the clog into a tube containing 20% bleach. 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.
10. If your instrument clogs frequently, the samples may contain significant amounts of cellular debris that might build up in the flow system. If using an SL system perform Quick Clean cleaning cycles frequently, depending on the quality of your samples. If using an HT system, a Quick Clean cycle will be performed at the end of every worklist, but you should select more frequent Quick Clean cycles from the WorkEdit program.

For more troubleshooting tips, refer to the appropriate Guava System user's guide or contact Luminex Technical Support.

# Limitations

1. The results of the assay are dependent upon proper handling of samples, reagents and instruments.
2. Cell types vary in the PS content of their cell membrane.[16-18] The amount of PS exposed on the cell surface varies among cell types after apoptosis is induced.[11, 19] The Guava Nexin® Assay does not detect early apoptosis in cell types that do not translocate PS to the cell surface upon induction of apoptosis.
3. The Guava Nexin Reagent is designed for use on unfixed cells. Fixing cells yields inaccurate results.
4. The Guava System and Guava Nexin Reagent yield optimal results when the stained cell sample used for acquisition is between $1 \times 10^4$ to $5 \times 10^5$ cells/mL. To obtain the most accurate results, adjust the cell concen-

trations to within the recommended range. However, to optimize throughput, Luminex recommends using between 1 x $10^5$ to 5 x $10^5$ cells/mL when possible.

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# 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, 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 $1 \times 10^5$ to $1 \times 10^7$ cells/mL in serum- or albumin containing medium.
2. Proceed to *Cell Staining Procedure* on page 4.

### 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 Guava® Cell Dispersal Reagent (Cat No. 4700-0050) 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 $1 \times 10^5$ to $1 \times 10^7$ cells/mL.
3. Proceed to *Cell Staining Procedure* on page 4.

# Trademarks



## Order Information

For ordering information or technical support contact Luminex Technical Support.

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