



Guava Nexin[®] Reagent Package Insert

RUO For Research Use Only. Not for use
in diagnostic procedures.
4600-2620, Rev H
Catalog No. 4500-0450 (100 tests)
Catalog No. 4500-0455 (500 tests)
06/2020

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Description

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

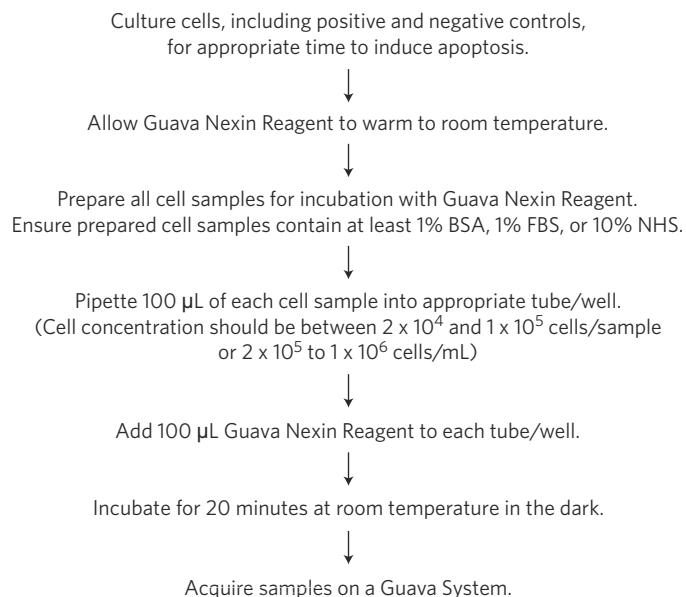
Annexin V is a calcium-dependent phospholipid binding protein with high affinity for PS,⁶⁻⁸ a membrane component normally localized to the internal face of the cell membrane. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V can 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 2×10^4 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.

Summary of Protocol



Materials Provided

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)

Materials Required but Not Provided

- 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 CO₂ incubator
- Deionized water
- Guava® Instrument Cleaning Fluid (ICF) [Cat. No. 4200-0140], optional
- 100% Bleach solution containing between 5-6% hypochlorite

For High-throughput (HT) Guava® Systems

- 0.5-mL microcentrifuge tubes. Refer to the appropriate Guava® easyCyte™ System User's Guide for compatible tubes.
- 1.5-mL microcentrifuge tubes. Refer to the appropriate Guava® easyCyte™ System User's Guide for compatible tubes.
- 96-well microplate plates, round bottom. Refer to the appropriate Guava® easyCyte™ System User's Guide for compatible tubes.
- Reagent reservoirs, 50 mL (VWR Cat. No. 82026-354 or equivalent)

For Single-loader (SL) Guava® Systems

- 1.5-mL microcentrifuge tubes. Refer to the appropriate Guava® easyCyte™ System User's Guide for compatible tubes.

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

For Single-Loader (SL) Guava® Systems

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

Warnings and Precautions

1. For Research Use Only. Not for use in diagnostic procedures.
2. Wear appropriate personal protective equipment (PPE), including a lab coat and disposable gloves, when performing procedures. Wash your hands thoroughly after performing the test.
3. 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.
4. The Guava Nexin Reagent contains sodium azide, which is toxic.
5. Avoid microbial contamination of the solution, which may cause erroneous results.

6. Handle all samples as if infectious using safe laboratory procedures such as those outlined in CDC/ NIH *Bio-safety in Microbiological and Biomedical Laboratories, and in the CLSI Document M29 Protection of Laboratory Workers from Occupationally Acquired Infections*.
7. Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
8. Do not pipette by mouth.
9. Perform the procedure given in this package insert as described. Any deviation from the outlined protocols may result in assay failure or cause erroneous results.
10. Do not use the kit or any kit components past the expiration date indicated on the kit carton label. Do not interchange kit components from different kit lots. Lot numbers are identified on the kit label.
11. Follow your institution's safety procedures for working with chemicals and handling biological samples.
12. In the event of damage to the protective packaging, consult the Safety Data Sheet (SDS) for instructions.
13. Safety Data Sheets (SDS) are available by contacting Luminex Corporation or visiting our website at www.luminexcorp.com

Reagent Storage, Handling, and Stability

- 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.
- The Guava Nexin Reagent contains light-sensitive dyes. Shield from excessive exposure to light.

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 2×10^4 and 1×10^5 cells/well (or 1×10^5 to 5×10^5 cells/mL), although apoptosis can be detected in cultures with as few as 2×10^3 cells/well (or 1×10^4 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 5×10^5 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 *Cell Sample Preparation* on page 25.

Prepare and Stain Samples

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 *Cell Sample Preparation* on page 25.

NOTE: You must have cells in suspension with at least 1% BSA or FBS, or at least 10% filtered NHS when performing the Guava Nexin Assay.

3. Add 100 µL of Guava Nexin Reagent to each well or tube.

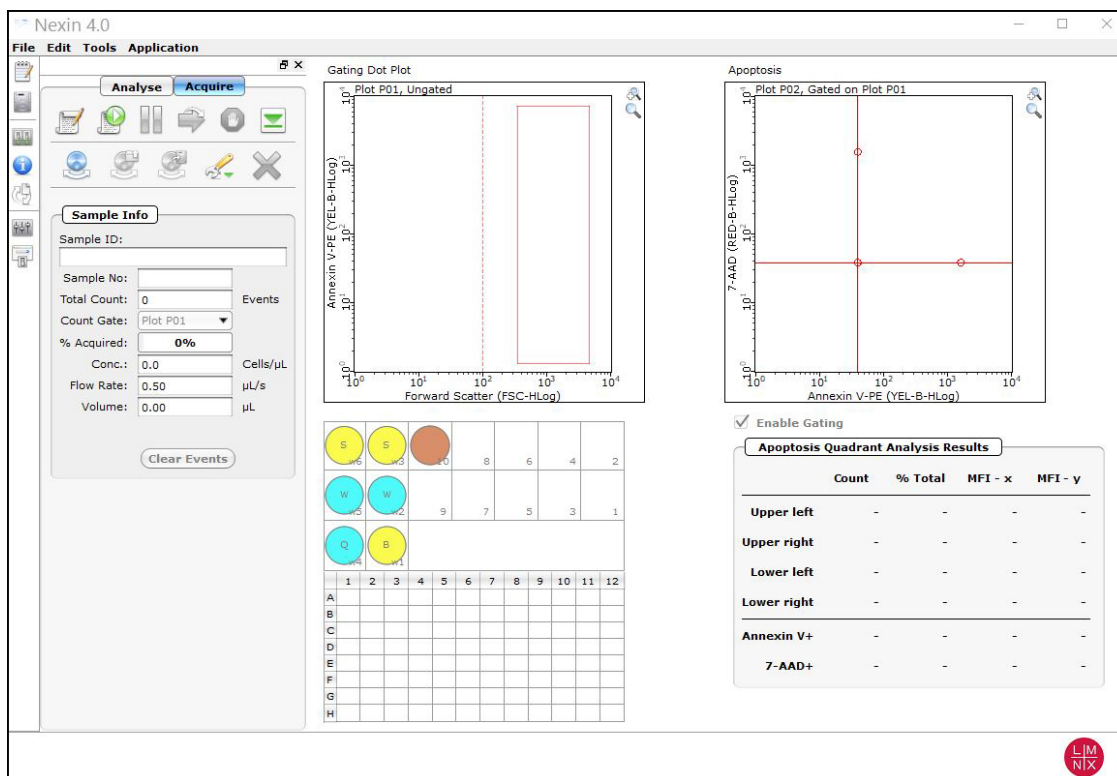
NOTE: Should your induced cells express large amounts of PS, it may be necessary to add more Guava Nexin Reagent. You can add up to 125 µL of Guava Nexin Reagent to each well, or up to 150 µL to each tube. If you need to use more Guava Nexin Reagent for optimal staining, then it is better to decrease 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.

Run the Assay on a High-throughput HT System

For details on software screen buttons and control panels, see the appropriate Guava® easyCyte™ User's Guide.

1. Open GuavaSoft™ Software and click **Nexin** from the Favorites list. If you do not see the assay listed in the Favorites list, click the **Program Search** drop-down menu, choose the assay, and click **Launch**,



2. Prepare samples for analysis in a microplate or sample tubes.
3. Click the **Edit Worklist** button, the first button in the Button panel, or select **Application > Worklist Editor** to open Worklist Editor and define the worklist parameters.

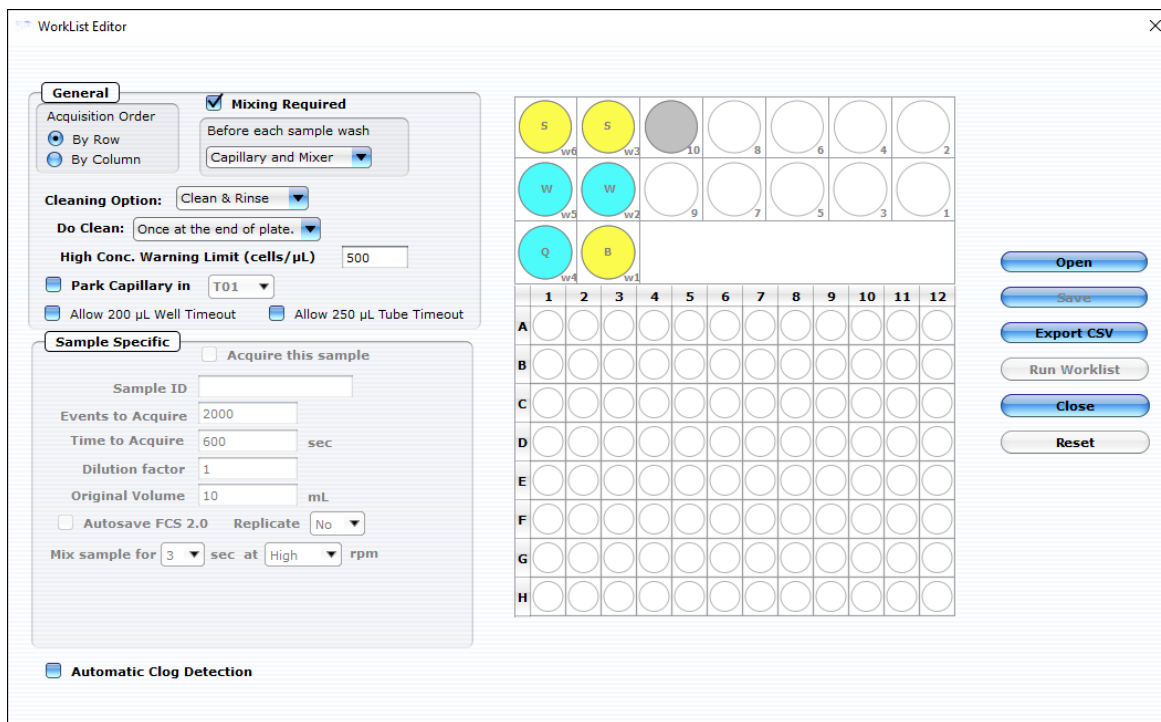
NOTE: You can also open an existing worklist.

- a. To open an existing worklist, click the **Start Worklist** button from the Button panel. The **Open WorkList File** dialog box displays.
- b. Choose between opening a saved worklist or running the currently loaded worklist. Worklists are .xml files.



- c. Or, from the WorkList Editor, click **Open**.

- d. Choose the WorkList file and click **Run Worklist**. A dialog box displays prompting you to enter a data set file name.



4. Define the worklist parameters in WorkList Editor software and click **Run Worklist**. The **Prepare For Acquisition** dialog box displays allowing you to select a data file name and location and instrument settings.



- a. Click **Select Output FCS file** to save the file. The **Create New Session File** dialog box displays. Navigate to the location you want to save the file to, and click **Save**.

NOTE: The default storage location is in My Documents. The default file name is the date and time.

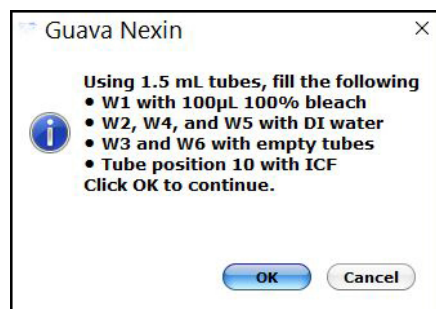
- b. For **Settings**, to retrieve a setting file, click **Choose**. The Read Settings from dialog box displays. Navigate to the .gst or .fcs file you want to open, and click **Open**.

NOTE: The FCS or instrument settings file must be from InCyte, version 3.0, or later.

NOTE: Always save GuavaSoft data files directly to the system's hard drive during acquisition. Saving data to a network or location other than the computer's hard drive may result in data loss. You may copy the files to another location when acquisition is complete.

5. Click **Adjust Settings**, the sample tray ejects. If you retrieved instrument settings and you want to skip the adjust settings step, click **Acquire** to begin acquiring the first sample.

NOTE: Whether you are ready to acquire or adjust settings, a dialog box displays prompting you to load your samples.

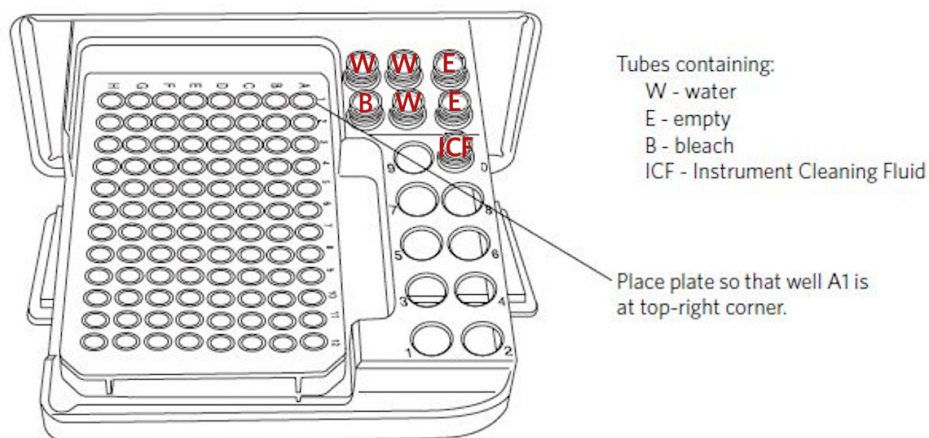


6. Place the microplate or sample tubes, as well as the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:


- Load tubes containing 1.5 mL of water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).

NOTE: Ensure that the tube in position w4 (for Quick Clean) is filled with water.

- Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
- Load a tube containing 100 µL of 100% bleach in position w1 (for a backflush) to disinfect material deposited from a backflush.
- Load a tube containing 1.5 mL of ICF in position 10 for Clean & Rinse.
- Load a tube containing 1.5 mL of water for parking the capillary.



- Click **OK** in the dialog box after you are finished loading samples and cleaning tubes to load the sample tray.

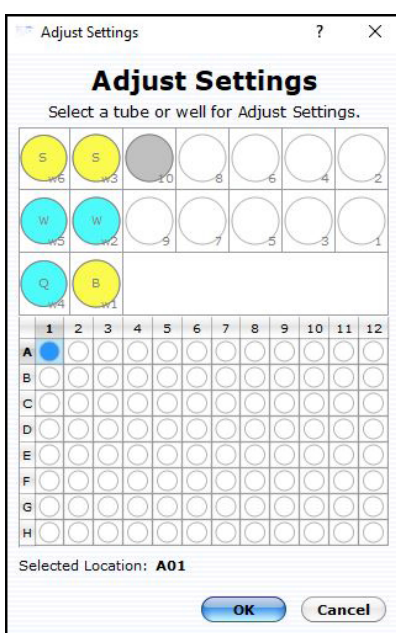


Keep the area clear as the tray loads.

Always use the **Eject Tray** button in the Nexin software module to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

Adjust the Instrument Settings and Acquire Data

- A dialog box displays prompting you to select the sample for adjusting settings. Click to select the well or tube (1-9) used to adjust settings, then click **OK**. Luminex recommends using a stained negative or positive control sample for the initial adjustments. You can choose to skip the adjust settings step even if you did not retrieve settings, however Luminex recommends always performing the adjust settings step.



- Check the **Conc** (Cells/ μ L) value in the Sample Info pane and ensure that it is less than or equal to 500.

NOTE: If the value is greater than the high limit for the corresponding flow rate, dilute the sample with the appropriate buffer to lower the concentration and minimize the risk of coincident events. For optimal performance, Luminex recommends a concentration of 250 cells/ μ L or lower.

3. To fine tune the settings, you can make the following adjustments using the Gain Controls. Open each instrument adjustment window using the Tools menu or the icons in the tool bar (left edge of the application window). If necessary, click the window's title bar to drag the window to a new location.



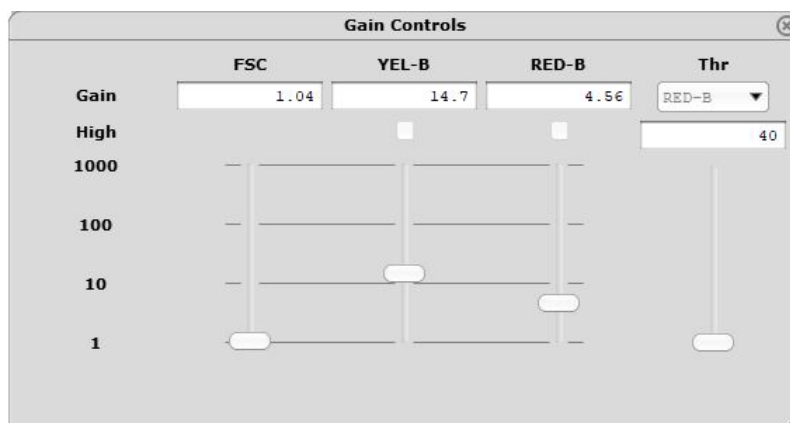
a. Click the **Gain Controls** icon. The **Gain Controls** dialog box will display.

- Set the **Refresh Rate** to the maximum number of events you want to display.
- Set the **Flow Rate** to Low (0.24 $\mu\text{L/s}$) or Medium (0.59 $\mu\text{L/s}$). The recommended flow rate is Medium.
- Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the cells are visible and on scale.
- To adjust the FSC threshold, click and drag the threshold marker up or down the FSC axis of the FSC vs. YEL-B dot plot until the desired amount of debris is eliminated below the threshold.

NOTE: When setting the threshold, allow for a possible shift in the apoptotic cell population. Any debris that gets acquired can be excluded during analysis with the optional dot plot gate.

- Adjust the voltages (using the YEL-B and RED-B sliders or the arrow keys on the keyboard) so that the live cell population is positioned in the lower-left corner of the YEL-B vs. RED-B dot plot. Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.

NOTE: Use the **Clear Events** button in the Sample Info panel to clear the display.



4. When you are finished adjusting settings, click the **Next Step** button.
- If necessary, you can repeat the adjust settings step to ensure that other samples (such as another positive control) are on scale and appropriately positioned, by clicking **Adjust Settings**, loading the sample, and clicking **OK**.
 - If you want to save the instrument settings, click the **Save Settings** icon in the control panel. Enter a file name and click **Save** to save a .gst file.

NOTE: Once the worklist is complete, you can no longer save the instrument settings.

5. Click the **Resume Worklist** button. The system acquires the first sample.

NOTE: You may click the **Pause Worklist** button at any time during the run to select **Eject Tray** or **Capillary Cleaning Tools** then select **Backflush, Clean & Rinse,** or **Quick Clean.** The system will complete the current step before pausing. Click **Resume Worklist** to continue.

NOTE: If you want to adjust the instrument settings during the run, click **Pause Worklist,** then **Adjust Settings.** When the settings are set, click **Next Step,** then **Resume Worklist.**

NOTE: The % Acquired progress bar provides an estimate of the target event count during the acquisition period.

NOTE: The plate map in the control panel provides a visual status of acquisition. The well currently being acquired appears with an open blue circle. Wells acquired appear as a solid blue circle.

The system automatically performs a Quick Clean or a Clean & Rinse at the end of the assay. At the completion of the worklist, a copy of the files are automatically loaded into the Analysis control panel. Guava Nexin saves the data for all samples as a single FCS 3.0 file to the specified location.

The FCS file contains:

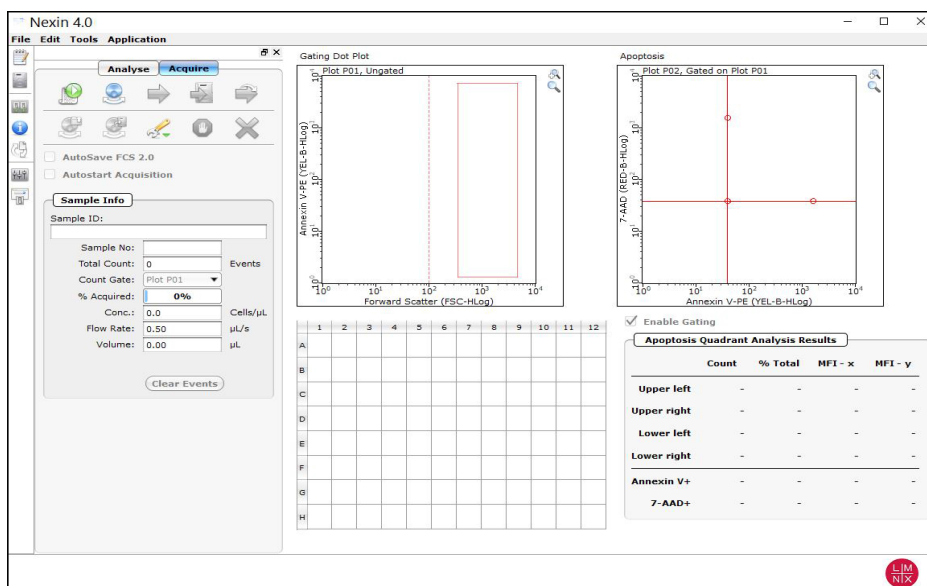
- the acquired data for all wells or tubes in the run
- instrument settings (gates, miscellaneous settings)

6. If necessary, back up data files, to free up hard disc space.

Run the Assay on a Single-loader (SL) System

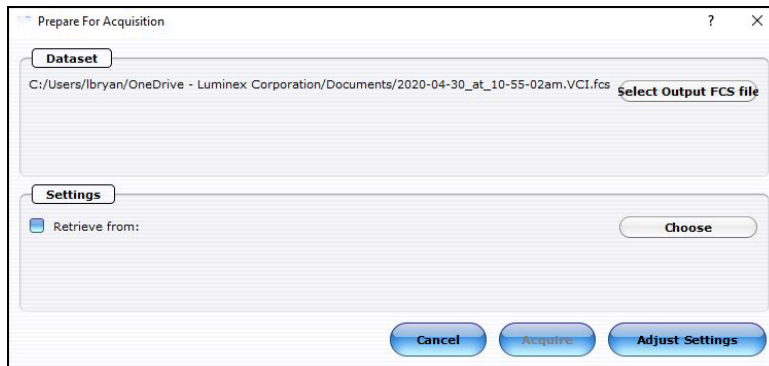
NOTE: The Guava® Nexin® screen opens in Acquisition mode, if the easyCyte™ System is turned on.

1. Open GuavaSoft™ Software and click **Nexin** from the Favorites list. If you do not see the assay listed in the Favorites list, click the **Program Search** drop-down menu, choose the assay, and click **Launch,** .



2. Prepare samples for acquisition in 1.5 mL sample tubes.

- Click the **Start New Session File** icon. A **Prepare For Acquisition** dialog box displays allowing you to choose a data file name and location and instrument settings.



- Click **Select Output FCS** file to save the file. The **Create New Session File** dialog box displays. Navigate to the location you want to save the file to, and click **Save**.

NOTE: The default storage location is in My Documents. The default file name is the date and time.

- For **Settings**, to retrieve a settings file, click **Choose**. The **Read Settings** from dialog box displays. Navigate to the .gst or .fcs file you want to open, and click **Open**.

NOTE: The FCS or instrument settings file must be from InCyte, version 3.0, or later.

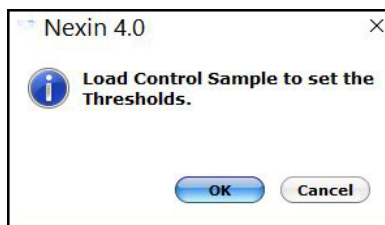
NOTE: Always save GuavaSoft Software data files directly to the laptop's hard drive during acquisition. Saving data files to a network or location other than the laptop's hard drive may result in data loss. You may copy the file(s) to another location when acquisition is complete.

NOTE: Your system administrator may have configured GuavaSoft Software to disable the retrieval of Methods and instrument settings from Method files and individual instrument settings files. If the Settings retrieval options are disabled, you will not be able to adjust instrument settings. However, you can retrieve settings and Methods from a single FCS file.

- Click **Adjust Settings**.

NOTE: If you have already retrieved instrument settings and you want to skip the Adjust Settings step, click **Acquire** in the **Prepare for Acquisition** dialog box to proceed to acquisition. A dialog box displays prompting you to load the control sample into the instrument.

- Mix a stained cell sample and load the sample tube onto the sample loader of the Guava easyCyte System. Luminex recommends using a stained negative or positive control sample for the initial adjustments.
- Click **OK** in the Nexin 4.0 dialog box.



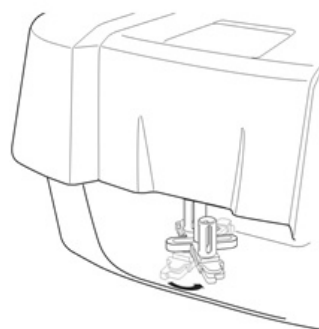
- Adjust the appropriate settings for your experiment and once you are satisfied and ready to acquire data, click **Next Step**.

Adjust the Instrument Settings

1. Mix a stained cell sample and load the sample tube on the sample loader of the Guava® easyCyte™ System. Luminex recommends using a stained negative or positive control sample for the initial adjustments.
2. From the Guava Nexin Acquisition screen, click the **Adjust Setting** icon from the Button panel.
 - a. To load sample tubes, pinch the release lever with your thumb and forefinger and gently lower the arm assembly (1st image).
 - b. Once lowered, rotate the assembly to change the sample tube (2nd image), then push up on the assembly until you hear a click.
 - c. If prompted, click **OK** in the **Messages** dialog box.



1. Pinch release lever and gently lower sample holder.



2. Rotate loader assembly to change tubes.

3. Check the **Conc** (Cells/ μ L) value in the **Sample Info** pane and ensure that it is less than or equal to 500.

NOTE: If the value is greater than the high limit for the corresponding flow rate, dilute the sample with the appropriate buffer to lower the concentration and minimize the risk of coincident events. For optimal performance, Luminex recommends a concentration of 250 cells/ μ L or lower.

4. To fine tune the settings, you can make the following adjustments using the Gain Controls. Open each instrument adjustment window using the Tools menu or the icons in the tool bar (left edge of the application window). If necessary, click the window's title bar to drag the window to a new location.



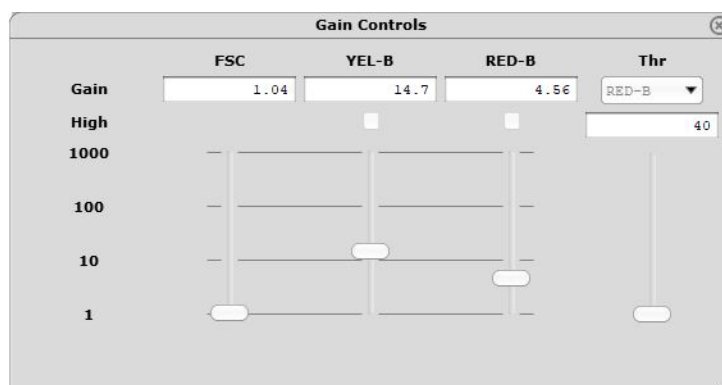
- a. Click the **Gain Controls** icon. The **Gain Controls** dialog box will display.

- Set the **Refresh Rate** to the maximum number of events you want to display.
- Set the **Flow Rate** to Low (0.24 μ L/s) or Medium (0.59 μ L/s). The recommended flow rate is Medium.
- Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the cells are visible and on scale.
- To adjust the FSC threshold, click and drag the threshold marker up or down the FSC axis of the FSC vs. YEL-B dot plot until the desired amount of debris is eliminated below the threshold.

NOTE: When setting the threshold, allow for a possible shift in the apoptotic cell population. Any debris that gets acquired can be excluded during analysis with the optional dot plot gate.

- Adjust the voltages (using the YEL-B and RED-B sliders or the arrow keys on the keyboard) so that the live cell population is positioned in the lower-left corner of the YEL-B vs. RED-B dot plot. Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.

NOTE: Use the **Clear Events** button in the Sample Info panel to clear the display..



- When you are finished adjusting settings, click the **Next Step** button.
 - If necessary, you can repeat the adjust settings step to ensure that other samples (such as another positive control) are on scale and appropriately positioned, by clicking **Adjust Settings**, loading the sample, and clicking **OK**.
 - If you want to save the instrument settings, click the **Save Settings** icon in the control panel. Enter a file name and click **Save** to save a .gst file.

NOTE: Once the worklist is complete, you can no longer save the instrument settings.

- Vortex the first sample and load it on the instrument.
- Enter the number of events to acquire in the Sample Info panel. The default number of events to acquire is 2000.
- If you want to identify individual samples or sets of samples, enter an optional ID in the Sample ID field. The sample ID may be any text up to 40 characters long. If you do not enter a sample ID, the default sample number will be used as the sample ID, for example, Sample #1, Sample #2, etc.

NOTE: If you want to enter additional sample characteristics, you can do so with the Change Custom Field Labels feature and create up to 2 user defined labels. These fields are editable similar to the standard Sample ID. The Change Custom Field Labels feature is located in the Application menu.

NOTE: If you select **Autostart Acquisition**, acquisition automatically starts when you load each tube. You do not need to click **Acquire Next Sample**. Autostart Acquisition automatically turns off if you click any of the following: **Adjust Settings**, **Retrieve Settings**, **Quick Clean**, or **Backflush**. You must recheck the box to continue using the feature. If you click **Autostart Acquisition** and want to enter a sample ID, type the ID into the Sample ID field after acquisition is complete and before loading the next sample.

- Click the **Acquire Next Sample** button. The system acquires the first sample.

NOTE: The progress bar provides an estimate of the target event count during the acquisition period.

NOTE: If a clog or total loss of signal is detected, we recommend removing the clog by running a Quick Clean with water, then a Quick Clean with ICF, followed by Backflush into an empty tube, then another Quick Clean with ICF, and a final Quick Clean with water.

NOTE: If the acquisition rate appears to slow dramatically, the fluid pathway may be blocked. Click the **Abort** button, then click **Capillary Cleaning Tools** and select **Backflush**. Load a tube of 20% bleach and click **OK**. When the backflush is complete, select **Quick Clean**. Load a tube of DI water and click **OK**. Click **Acquire Next Sample** to continue.

10. Click **Save and Close Current Sample**. You may still enter or change the Sample ID for the current sample before clicking **Save and Close Current Sample**.
11. Repeat the steps above for the remaining samples.
12. Click the **Stop and Close Session File** after the last sample.
13. When you are finished, click the **Capillary Cleaning Tools** icon in the Button panel and select **Quick Clean**. Load a tube of deionized water and click **OK**.

NOTE: If you ran blood samples, run two Quick Clean cycles with water, followed by one cycle using ICF, and finally one cycle using water to rinse.

At the completion of the run, a copy of the Data, Method, and AnalysedGroup are automatically loaded into the Analysis control panel. Guava Nexin saves the data for all samples as a single FCS 3.0 file to the specified location.

The FCS file contains:

- the acquired data for all tubes in the run
- instrument settings (gains, miscellaneous settings)

Guava® Nexin Analysis

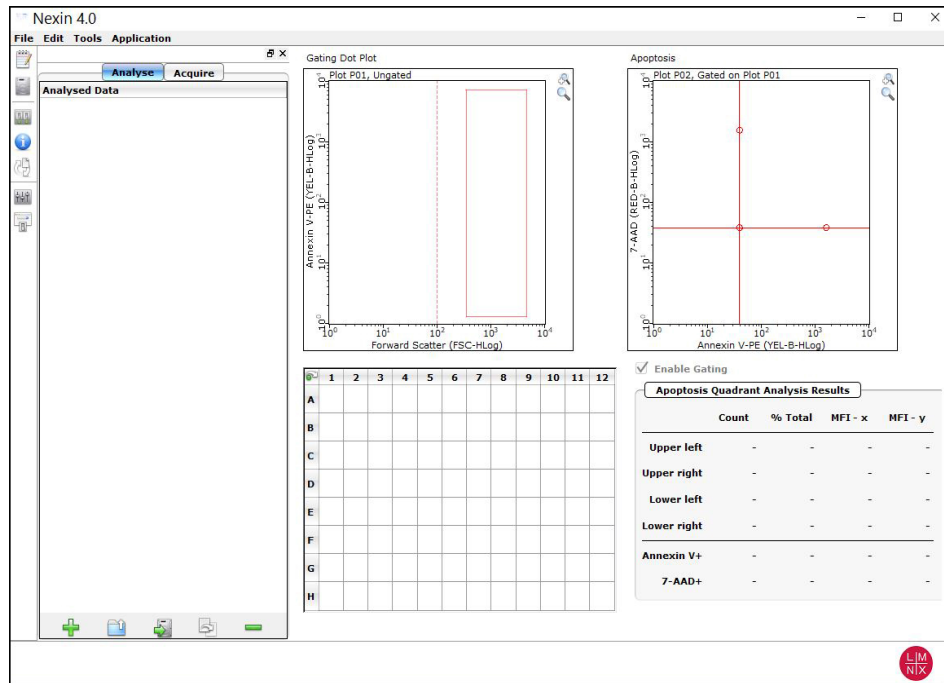
Use the Analysis screen to analyze samples, print results, log comments, or view the event log from a data set that was saved previously. You can also export data to FCS 2.0 files or a spreadsheet file.

You can save changes made to the sample ID, gate, or markers within Analysis by overwriting the existing file or saving a new file.

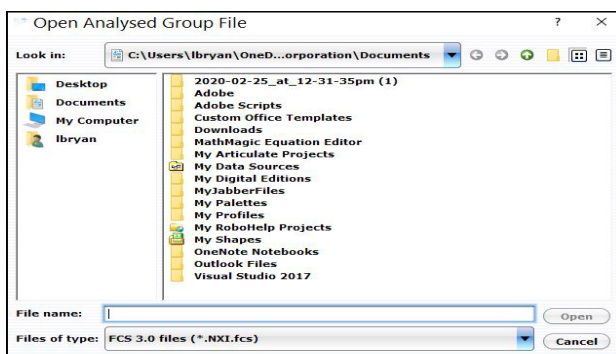
NOTE: If your system administrator has configured GuavaSoft™ software to disable overwriting files, you must save any changes to a separate file with a new name.

1. Open the GuavaSoft Software and click **Nexin** from the Favorites list. If you do not see the assay listed in the Favorites list, click the **Program Search** drop-down menu, choose the assay, and click **Launch**,

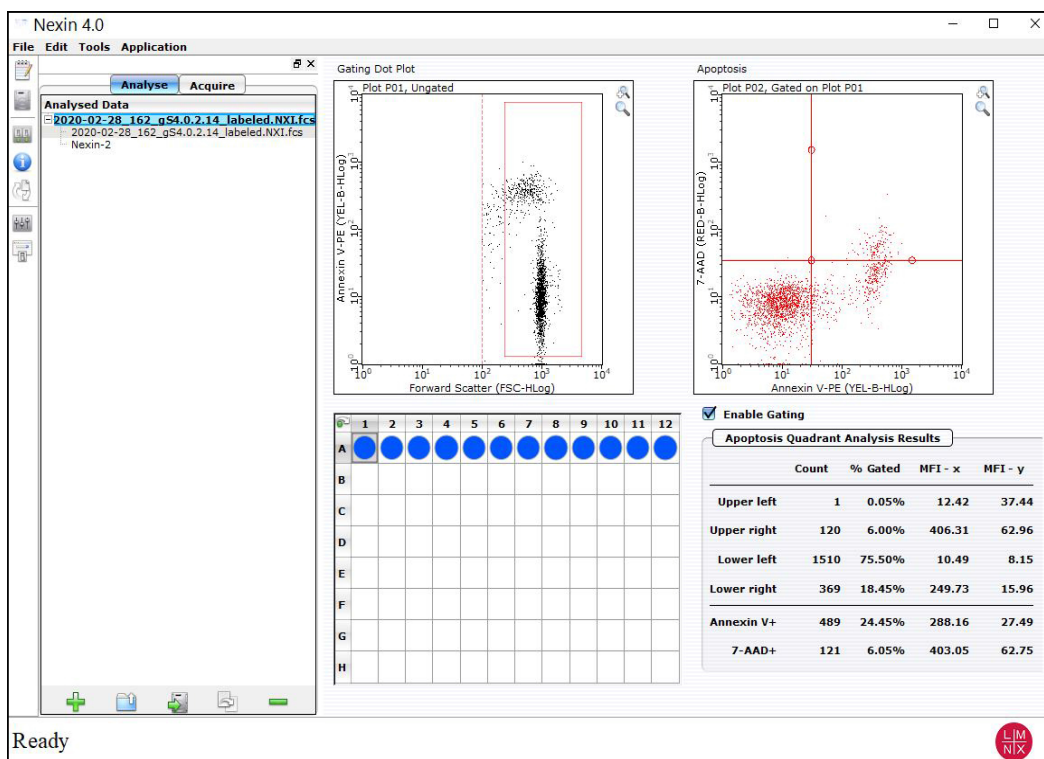
2. Click the **Analyse** button to switch from the Acquisition screen to the Analyse screen.



3. Click **Open Analysed Group**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker setting appears as it was when the sample was acquired. Check the positive control to ensure the gate and quadrant markers are set appropriately.

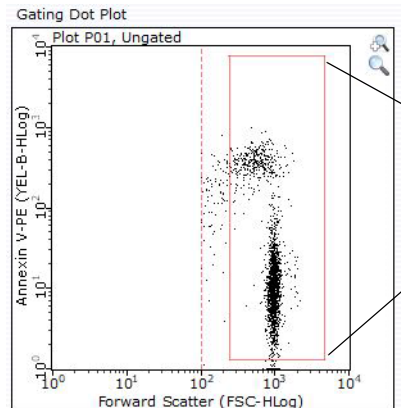


Set the Dot Plot Gate

To analyze the data for the stained negative control, set the quadrant markers on the Annexin V-PE vs. 7-AAD dot plot. If the FSC vs YEL-B dot plot shows excessive debris, you can set an optional gate first to exclude the debris from the analysis.

1. Click **Enable Gating** to turn on the gate.
2. To set a gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle and appear in red are included in the gate.

NOTE: If using the stained negative control to set the gate, be sure to extend the gate high enough up the y-axis (as shown) so that YEL-B-positive events in subsequent samples are included in the gate.



Click and drag handles to manually set gate. A label displays handle's current x and y coordinates.

FSC vs. YEL-B dot plot gate identifying Annexin V-positive (apoptotic) and -negative (live) cells.

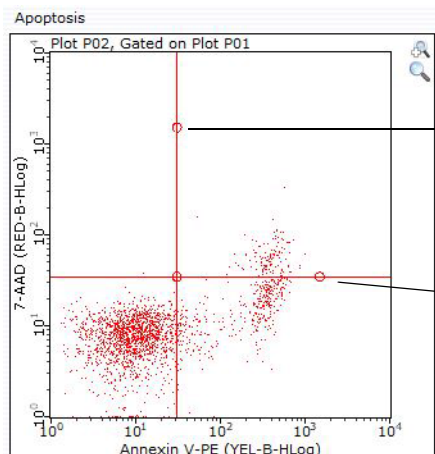
Adjust the Quadrant Markers

Adjust quadrant markers on the Annexin V-PE vs. 7-AAD dot plot to generate statistics on the selected populations.

1. To set the markers, position the cursor over the handle at the intersection, then click and drag to the desired location.

The Apoptosis Quadrant Analysis Results are automatically updated each time you move the markers.

2. If necessary, you can adjust the angle of the markers $\pm 44^\circ$ from their original locations. Click and drag the handle (solid square) towards the end of the marker and tilt it to the desired location.



Click and drag to set marker angle. A label displays markers' x and y coordinates and angle.

Click and drag to set markers. A label displays markers' x and y coordinates.

Annexin V-PE vs. 7-AAD quadrant markers differentiating Annexin V(+) cells into 7-AAD(+) and 7-AAD(-).

3. Click the next sample in the acquired sample plate map.
4. When you have finished analyzing the samples in the current file, you can save any analysis changes you made by clicking **Save Analysed Group** icon. A dialog box displays prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name. Results are automatically exported to a CSV file that is given the same name as the FCS file.

NOTE: Exporting to the CSV file may take several minutes, especially with a large number of samples.

5. If you want to view the event log, click **Event Log**.

Guava Nexin® Quadrant Results

The statistics for the data within each quadrant are displayed. Results include the count, % of Total (all data in the dot plot) or % Gated if a gate was enabled, and the mean fluorescence intensity (MFI) for each parameter (YEL-B and RED-B). Additionally, statistics for Annexin V(+) cells (upper-right and lower-right quadrants) and 7-AAD(+) cells (upper-left and upper-right quadrants) are included.

Apoptosis Quadrant Analysis Results				
	Count	% Gated	MFI - x	MFI - y
Upper left	1	0.05%	12.42	37.44
Upper right	120	6.00%	406.31	62.96
Lower left	1510	75.50%	10.49	8.15
Lower right	369	18.45%	249.73	15.96
Annexin V+	489	24.45%	288.16	27.49
7-AAD+	121	6.05%	403.05	62.75

Statistics for each quadrant as well as Annexin V(+) (upper-right and lower-right quadrants) and 7-AAD(+) (upper-left and upper-right quadrants) are displayed.

Results for each quadrant are displayed as a count and as a percentage of all data in the dot plot. If a gate was set, the values represent data within the gate.

Guava Nexin® apoptosis quadrant statistics.

The summary of each quadrant is outlined in the table below.

Quadrant	Staining	Population
upper left	Annexin V(-), 7-AAD(+)	nuclear debris
upper right	Annexin V(+), 7-AAD(+)	late apoptotic cells
lower left	Annexin V(-), 7-AAD(-)	live, healthy cells
lower right	Annexin V(+), 7-AAD(-)	early apoptotic cells

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.

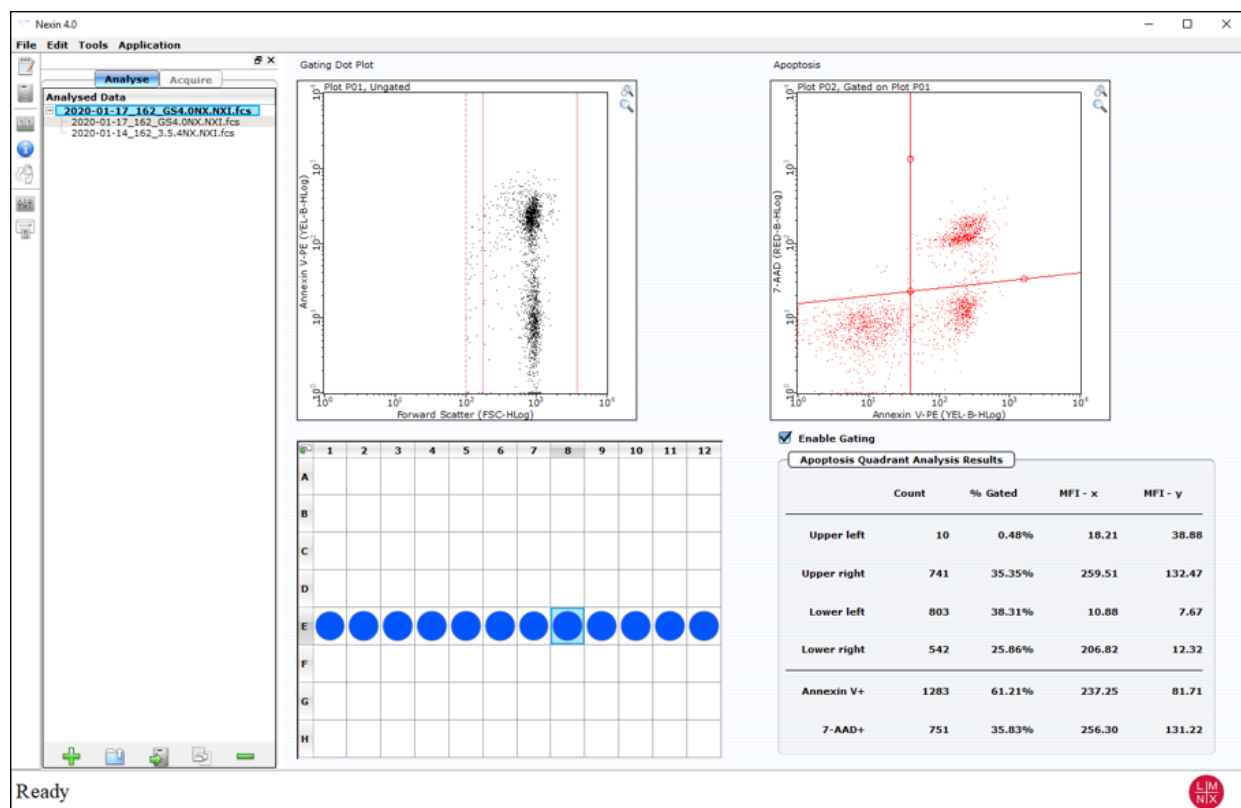
Events in each of the four quadrants are as follows:

- 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 μ M staurosporine for 3 hours at 37°C. After incubation, cells were mixed with live and 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



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 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
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 a precipitate forming during the incubation of the cells with the Guava Nexin Reagent, repeat the experiment with freshly induced cells, and increase the percentage of BSA, FBS, or NHS used during the Cell Staining Procedure.
9. 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.
10. 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.
11. 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.

Guava Nexin® Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save Guava Nexin® spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save Guava Nexin FCS file to another directory or give it a new name.
Guava Nexin Software Module starts in Analysis mode. Acquisition mode is not available.	<ol style="list-style-type: none"> 1. Communication problem between instrument and laptop. 2. A registration code was not entered or was entered incorrectly. 	<ol style="list-style-type: none"> 1. Ensure USB cable is connected between instrument and laptop. 2. Enter the registration code. The code is case sensitive.
Message: <i>Less than 10 particles/µL. Sample is too dilute. Accuracy may be compromised</i> Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes for HT systems, and 90 µL for single-loader systems. 3. Ensure sample mixing option was selected in WorkEdit Software, or click Abort, remove sample, mix, and reload.

Problem	Possible Cause	Solutions
<p>Message: <i>Less than 10 particles/μL. Sample is too dilute. Accuracy may be compromised.</i></p> <p>No events, as indicated in Particle Count section of Sample Information control panel.</p>	<ol style="list-style-type: none"> Sample tube or plate not loaded. Insufficient sample volume. Clogged flow cell. Broken flow cell. Sample pump not working. Laser not operational. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> Ensure tube or plate is in place and tray is loaded. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes for HT systems, and 90 μL for single-loader systems. Perform a Backflush. Follow with Quick Clean. Remove flow cell and inspect for damage. Replace if necessary. Run Quick Clean and watch for fluid in waste vial. Contact Luminex Technical Support. Ensure tubing connector is secure.
<p>Message: <i>More than 500 particles/μL. Sample is too concentrated. Please dilute or accuracy may be compromised.</i></p>	Sample is too concentrated.	Dilute sample with appropriate buffer. Recommended concentration range for accurate counting is 1×10^5 to 1×10^6 cells/mL.
Unexpected events appearing in YEL-B vs RED-B dot plot.	Threshold set too low or debris included in gate.	If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs. YEL-B dot plot but not in YEL-B vs. RED-B dot plot.	FSC vs. YEL-B gate excludes events.	Ensure FSC vs. YEL-B gate is set to include population of interest.
Events appear off scale in dot plots.	YEL-B and /or RED-B voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease YEL-B and/or RED-B voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample.

Problem	Possible Cause	Solutions
Poor resolution between PE+ and PE- populations.	<ol style="list-style-type: none"> 1. YEL-B voltage too low to detect PE signal. 2. Incomplete staining with Annexin V-PE. 3. Incomplete induction of apoptosis in test suspension. 4. Uninduced population reacting with Annexin V because cells have been stressed. 5. Apoptotic bodies included in analysis. 6. Annexin V expired, or not stored properly. 7. Background noise too high. 8. Fluorescence background too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase YEL-B signal. 2. Refer to package insert for proper staining instructions. 3. Check induction control. 4. Stabilize unstained cells by resuspending in 10-20% FBS for 30 min and restain. 5. Adjust FSC threshold or set gate to remove debris. 6. Refer to package insert for proper storage instructions. Do not use expired reagent. Do not expose reagent to light. 7. Adjust settings to increase FSC threshold to remove debris. 8. Washing cells may remove residual reagent.
Poor resolution between 7-AAD+ and PE+ populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with 7-AAD. 3. Too much Annexin V-PE in staining tube. 4. Fluorescence background too high. 5. YEL-B signal too high causing PE to bleed into RED-B. 6. RED-B voltage too low to optimally detect 7-AAD signal. 7. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check 7-AAD expiration date. 3. Washing cells may remove residual reagent. 4. Washing cells may remove residual reagent. 5. Adjust settings to reduce YEL-B voltage. 6. Adjust settings to increase RED-B voltage. 7. Adjust settings to increase FSC threshold to remove debris.

Cell Sample Preparation

Prepare 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.

Prepare 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.

Prepare Non-Adherent cells

1. Set up initial culture conditions, such that after culture and treatment, cells are at a concentration of 1×10^5 to 1×10^6 cells/mL in serum- or albumin containing medium.
2. Proceed to *Prepare and Stain Samples* on page 5.

Prepare 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 1×10^5 to 1×10^6 cells/mL.
3. Proceed to *Prepare and Stain Samples* on page 5.

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 1×10^4 to 5×10^5 cells/mL. To obtain the most accurate results, adjust the cell concentrations to within the recommended range. However, to optimize throughput, Luminex recommends using between 1×10^5 to 5×10^5 cells/mL when possible.

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