



Muse™ Annexin V & Dead Cell Kit

User's Guide

Catalog No. MCH100105 (100 Tests)

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

Application

The Muse™ Annexin V & Dead Cell Assay allows for the quantitative analysis of live, early and late apoptosis, and cell death on both adherent and suspension cell lines on the Muse™ Cell Analyzer. Minimal sample preparation is required in this no-wash, mix-and-read assay to obtain accurate and precise results. The software provides:

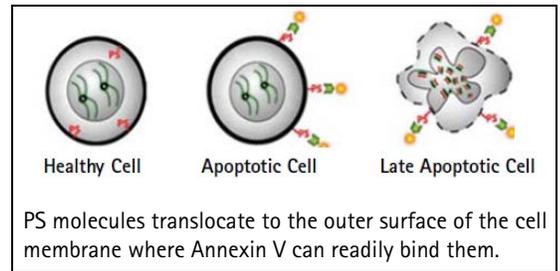
- Concentrations (cells/mL) for live, early apoptotic, late apoptotic, total apoptotic, and dead cells,
- Percentage of live, early apoptotic, late apoptotic, total apoptotic, and dead cells

The Muse Annexin V & Dead Cell Assay is for use with the Muse™ Cell Analyzer. The Muse™ System makes sophisticated fluorescent-based analysis fast, easy, convenient, and affordable. Sample preparation is minimal, and after loading samples onto Muse™ Cell Analyzer, intuitive software provides detailed or summary analysis of your cell sample in a few short steps.

Sufficient reagent is provided for the preparation and analysis of 100 tests.

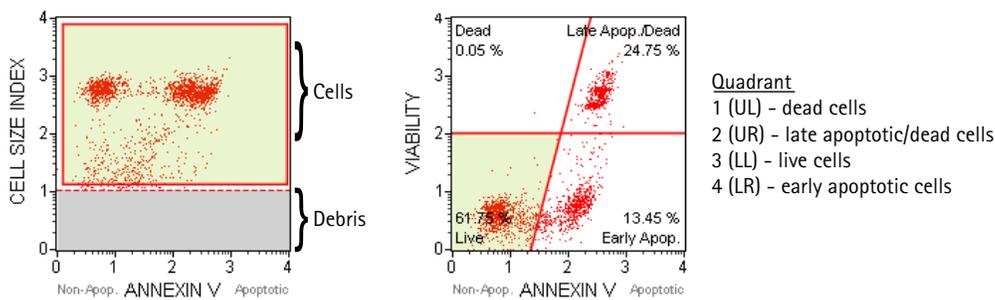
Test Principle

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 a high affinity for PS, a membrane component normally localized to the internal face of the cell membrane.⁶⁻⁸ 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



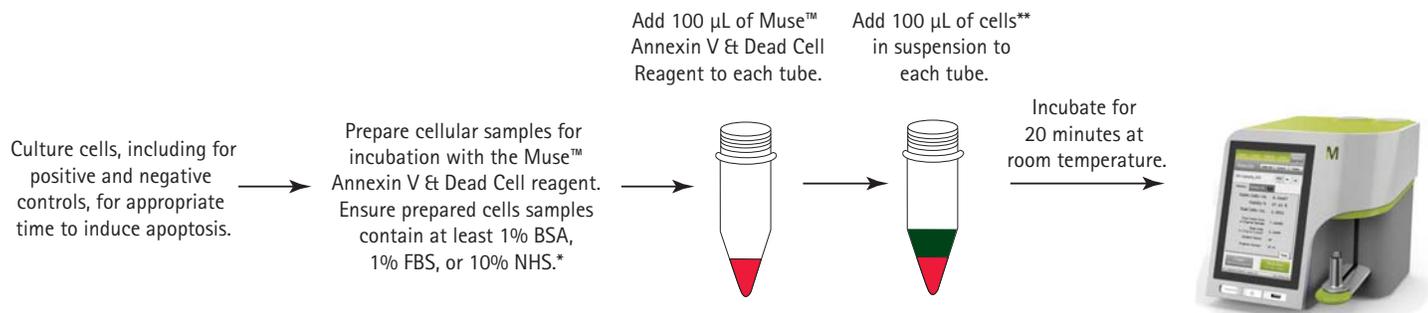
The Muse™ Annexin V & Dead Cell Assay utilizes Annexin V to detect PS on the external membrane of apoptotic cells. A dead cell marker is also used as an indicator of cell membrane structural integrity.¹⁵ It is excluded from live, healthy cells, as well as early apoptotic cells. Four populations of cells 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 (+)
- mostly nuclear debris: Annexin V (-) and 7-AAD (+)



Each plot has moveable markers. The first plot has a threshold marker, allowing you to eliminate debris based on size, as well as a gate, allowing you to gate on cells. The second plot has quadrant markers, allowing you to obtain statistics on four cell populations—live, early apoptotic, late apoptotic, and dead.

Summary of Protocol



* Cells not resuspended in 1% BSA, 1% FBS, or 10% NHS should be resuspended in 1X Assay Buffer HSC (Catalog No. 4700-1325).

** Adherent cells have been validated for this assay. For information on preparing adherent cells, see "Appendix A: Cell Sample Preparation" on page 13.

Kit Components

Muse™ Annexin V & Dead Cell Reagent (Part No. 4700-1485, 100 tests/bottle)

Materials Required but Not Supplied

- Muse™ Cell Analyzer
- Cell suspensions untreated and treated to undergo apoptosis
- Micropipettors
- Disposable micropipettor tips
- Microcentrifuge tubes with screw caps, 1.5 mL (VWR, Catalog No. 16466-030, or equivalent)
- Vortex mixer
- Disposable gloves
- 20% bleach solution
- Deionized water
- Centrifuge
- Muse™ Cell Dispersal Reagent (Catalog No. MCH100107), optional
- Guava® ICF Instrument Cleaning Fluid (Catalog No. 4200-0140), optional
- Muse™ System Check Kit (Catalog No. MCH100101), optional

Precautions

- The Muse™ Annexin V & Dead Cell Reagent is intended for research use only.
- Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling this reagent.
- The Muse™ Annexin V & Dead Cell Reagent contains dyes that may be carcinogenic and/or mutagenic. Exercise standard precautions when obtaining, handling, and disposing of potentially carcinogenic and mutagenic reagents. Refer to the MSDS for specific information on hazardous materials.
- The Annexin V & Dead Cell Reagent contains sodium azide, which is toxic if ingested. Reagents containing sodium azide should be considered a poison. If products containing sodium azide are swallowed, seek medical

advice immediately and show product container or label. (Refer to NIOSH, National Institute for Occupational Safety and Health; CAS#: 2628-22-8; and also to GHS, The Globally Harmonized System of Classification and Labeling of Chemicals.) Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas. Any reagents containing sodium azide should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent build-up in plumbing. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be regulated as hazardous.

- Avoid microbial contamination of the solution, which may cause erroneous results.
- All biological specimens and materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.
- Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
- The fluorescent dyes in this reagent are light sensitive. Store in the dark and shield from excessive exposure to light.
- 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.
- During storage and shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For maximum recovery of the product, centrifuge the vial briefly prior to removing the cap.
- Do not use the reagent beyond the expiration date.
- Material Safety Data Sheets (MSDS) for kit reagents are available from our website (www.millipore.com/muse), by contacting Millipore Technical Support, or from the Millipore technical library at www.millipore.com/techlibrary.

Storage

- Store the Muse™ Annexin V & Dead Cell Reagent refrigerated at 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 Muse™ Annexin V & Dead Cell Reagent contains light-sensitive dyes. Shield from excessive exposure to light.

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 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 remains constant. However, if the cell concentration exceeds 5×10^5 cells/mL, the Annexin V 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 is 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 the dead cell marker, 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 Muse™ Annexin V & Dead Cell 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: Staining cells with the Muse™ Annexin V & Dead Cell Reagent takes 20 minutes. Acquiring data on the Muse™ Cell Analyzer takes approximately 2 minutes per sample. 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, see "Appendix A: Cell Sample Preparation" on page 13.

Always run a System Check prior to performing the assay. For details refer to the *Muse™ Cell Analyzer User's Guide*.

Staining Protocol

1. Allow the Muse™ Annexin V & Dead Cell Reagent to warm to room temperature.
2. Add 100 µL of cells in suspension to each tube. For instructions on preparing cell suspensions, see "Appendix A: Cell Sample Preparation" on page 13.

NOTE: You must have cells in suspension with at least 1% BSA, 1% FBS, or 10% filtered NHS when performing this assay. Make sure to stain positive and negative controls.

3. Add 100 µL of the Muse™ Annexin V & Dead Cell Reagent to each tube.

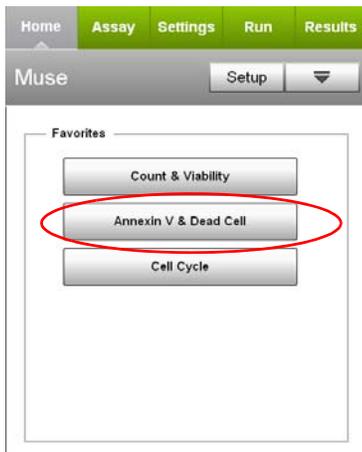
NOTE: Should your induced cells express large amounts of PS, it may be necessary to add more reagent. You can add up to 150 µL of the Annexin V & Dead Cell Reagent to each tube. If you need to use more reagent for optimal staining, then it is better to decrease the volume of medium that the cells are in from 100 to 50 µL and add between 150 to 175 µL (up to 200 µL) of the reagent.

4. Mix thoroughly by pipetting up and down or vortexing at a medium speed for 3 to 5 seconds.
5. Stain samples for 20 minutes at room temperature in the dark.

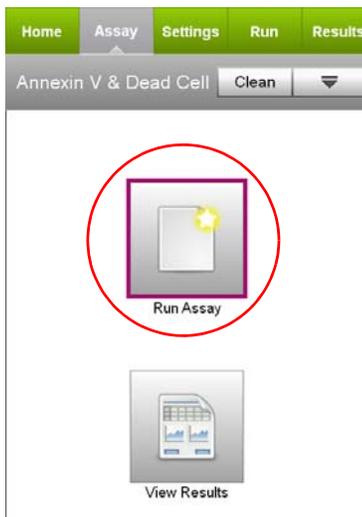
Setup and Acquisition on the Muse™ Cell Analyzer

Run a System Check prior to performing the assay. For information on Muse™ System Check, refer to the *Muse™ Cell Analyzer User's Guide*.

1. Select **Annexin V & Dead Cell** from the main menu.



2. Select **Run Assay**.



3. Adjust the instrument settings.

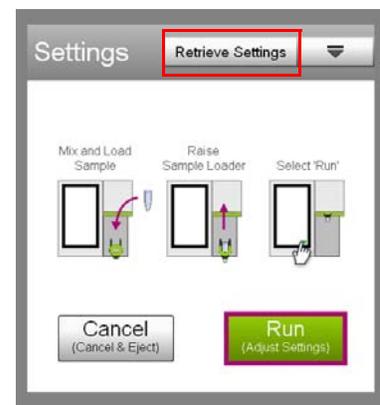
- Load the sample for adjusting the settings and select **Run**.

NOTE: Perform the adjust settings step using a negative control, then verify the settings using a positive control.

- Or, to retrieve previously saved instrument settings, select **Retrieve Settings**. For more information on retrieving settings, see the *Muse™ Cell Analyzer User's Guide*.

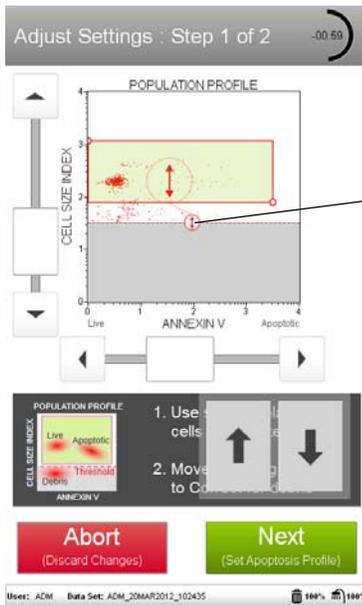
4. Fine tune the settings for the ANNEXIN V vs CELL SIZE INDEX plot, if necessary.

- Adjust the CELL SIZE INDEX slider to the left of the plot and the ANNEXIN V slider below the plot to move the cellular populations into the green region.
- Drag the threshold up or down to exclude debris. Drag to make large changes. Touch the arrow buttons located below the plot to make small changes. The arrow buttons appear after you touch the threshold.



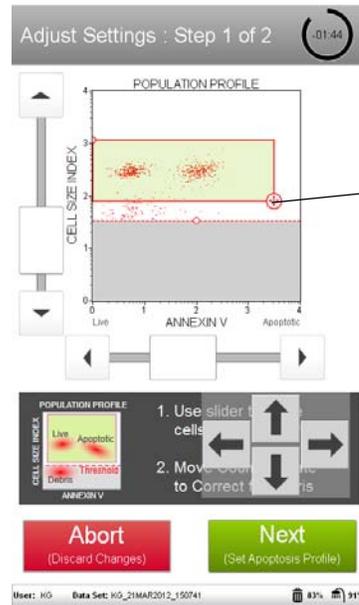
- Touch the upper-left and/or lower-right corners of the gate and drag to adjust the gate size.

NOTE: If the acquisition times out (after 2 minutes), you can select **Back** to restart the adjust settings step or **Next** to accept the settings and continue to the next step.



Touch threshold to move up/down.

These examples show typical gate and threshold settings.

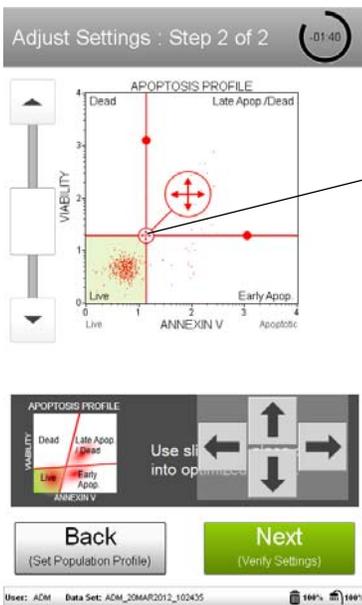


Touch and drag upper-left or lower-right corner to adjust the gate.

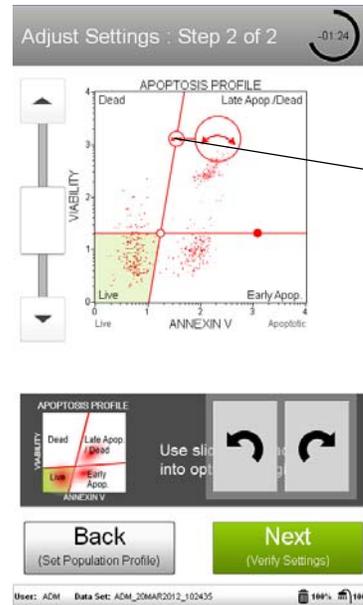
Negative control sample

Positive control sample

5. Select **Next** when you've completed the adjustments.
6. Fine tune the settings for the ANNEXIN V vs VIABILITY plot, if necessary.
 - Adjust the VIABILITY slider to place all populations (live, dead, and apoptotic) on scale.
 - Adjust the quadrant markers. You can move the marker intersection in any direction, as well as adjust the angle of each line. To move the markers as they are, touch the open circle at the intersection and drag the markers to make large changes, or touch the arrow buttons below the plot to make small changes. To adjust the angle of either line, touch the solid circle and drag in an arc, or touch the arrow buttons below the plot.



Touch center circle to move the fixed quadrant markers in any direction.



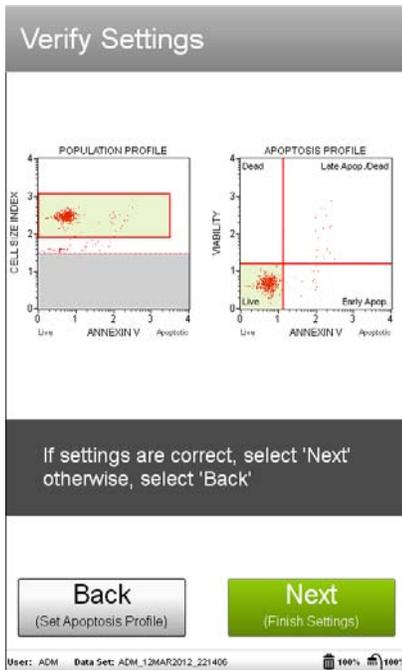
Touch a solid circle on either line (horizontal or vertical) to adjust the angle of the line.

Negative control sample

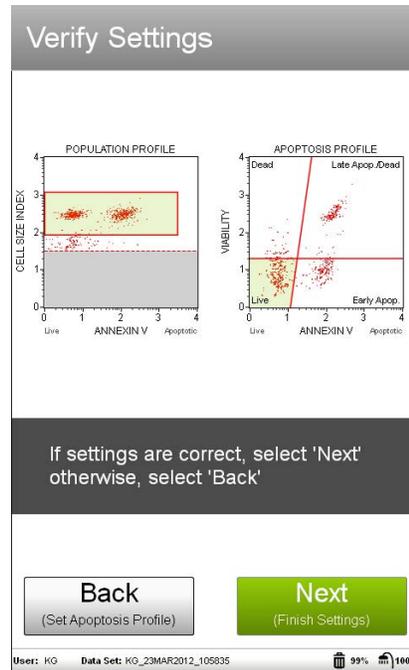
Positive control sample

7. Select **Next** when the adjustments are complete.

8. Verify the settings for the negative control. Then select **Back** and repeat steps 4 through 7 using a positive control. When the settings are correct, select **Next**.



Negative control sample



Positive control sample

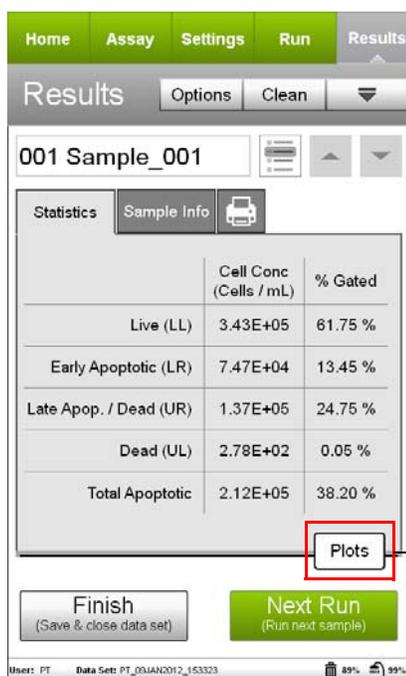
9. Enter the sample ID by touching the field, then using the keypad to input the ID. Touch **Done** when you've finished entering the ID. If necessary, change the Events to Acquire and/or Dilution Factor by touching the field, then selecting the value from the pop-up menu. Select **Next**.

10. Mix the first sample and load it on the instrument. Select **Run** to run the sample.

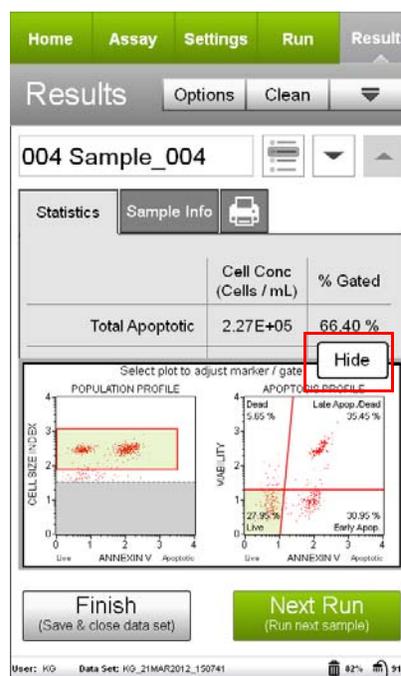


11. When acquisition is complete, the results are displayed. If desired, select **Plots** to display dot plots for the sample.

You can view or change the sample ID and dilution factor, as well as add annotations for the current sample by selecting the Sample Info tab. To print the results for the current sample select the printer tab.



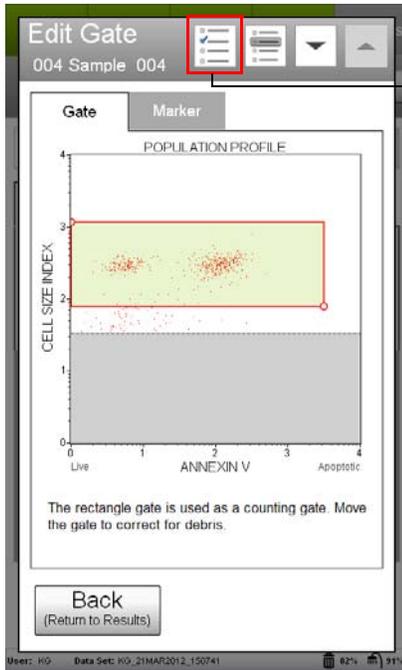
Select to display plots.



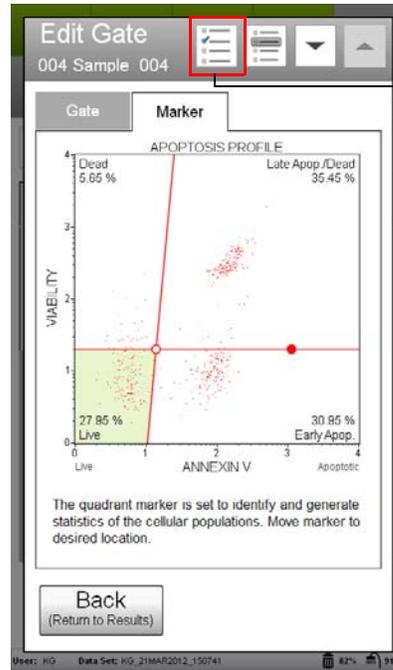
Select to hide plots.

12. (Optional) If changes are needed to the gate or markers, touch a plot to enlarge it, then adjust the cell size gate or markers, as described in steps 4 and 6, respectively. You cannot adjust the cell size threshold after the sample has been acquired.

If you adjust the gate or marker and wish to apply the changes to other samples that you already acquired, select the Apply Changes button () in the title bar. Select the samples you want to apply the changes to or choose **Select All**, then select **Apply**. The sample you originally made changes to must be selected.



Apply changes



Select to apply changes to other samples.

13. If no adjustments are needed, select **Next Run** and repeat steps 9 through 12 for the remaining samples.

NOTE: During the run, a message may appear prompting you to load a tube of DI for a Quick Clean. Load the water then select **Clean** to perform the Quick Clean. Select **Next** to continue with the run. The frequency of Quick Cleans was set by your system administrator. Your administrator may also have chosen to allow you to skip the Quick Clean when the prompt appears. You can choose to perform additional Quick Cleans at any time during a run by selecting **Clean** in the title bar, then **Quick Clean** from the menu.

14. When you have acquired the last sample, select **Finish**.

15. (Optional) Select **Options** in the title bar to rename the data set, export the data set, save the current instrument settings, or view the event log. Refer to the *Muse™ Cell Analyzer User's Guide* for more information.



Results

Results from each run are stored in a data file, as well as its corresponding spreadsheet (CSV) file. The data file and spreadsheet file contain the following statistics.

Events in each of the four quadrants are as follows:

- sample number
- sample ID
- concentration (cells/mL) of cells in each quadrant

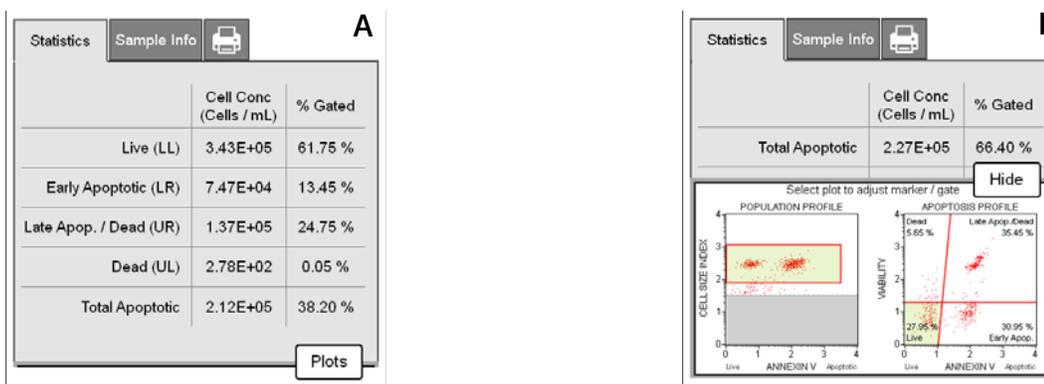
lower-left: viable cells, not undergoing detectable apoptosis [Annexin V-PE (-) and dead cell marker (-)]

lower-right: cells in the early stages of apoptosis [Annexin V-PE (+) and dead cell marker (-)]

upper-right: cells in the late stages of apoptosis or dead by apoptotic mechanisms [Annexin V-PE (+) and dead cell marker (+)]

upper-left: cells that have died via necrosis but not through the apoptotic pathway [Annexin V-PE (-) and dead cell marker (+)]

- percentage of gated cells in each quadrant
- concentration and percentage of total apoptotic cells (cells in upper-right and lower-right quadrants)
- dilution factor (input value)
- fluorescence intensity values for live and apoptotic populations



Figures A and B. Healthy Jurkat cells were treated with staurosporine to induce apoptosis, then stained with the Muse™ Annexin V & Dead Cell Reagent, and acquired on the Muse™ Cell Analyzer. Figure A shows summary data, while Figure B shows results displayed with optional dot plots. The statistics show the concentration (cells/mL) for the events in each quadrant and the percentage of gated cells in each quadrant, as well as the concentration and percentage of total apoptotic cells. The first plot in Figure B shows Annexin V vs Cell Size and the second plot shows Annexin V vs Viability.

Technical Tips

1. Mix each cell sample thoroughly on a mixer before acquiring samples for consistent and accurate results. However, avoid vigorous mixing, which can cause cellular breakdown and splashing, resulting in volume loss and erroneous results.
2. Multiple acquisitions of a cell sample minimizes sampling error. Statistically, multiple acquisitions yield more accurate cell counts and viability results.
3. The default number of events to acquire is 2000. You may select a different number; however, your statistical error will increase as you decrease the number of acquisition events.
4. If results deviate from expected values, prepare a freshly stained sample and reacquire the data.

5. If the cell count results deviate from expected values, check that the correct values were entered for dilution factor of the cell suspension. The Annexin V & Dead Cell application can be used to recalculate cell counts. Open the data file corresponding to the mistaken entry. Reenter the correct dilution factor and the cell count values will be recalculated automatically.
6. Periodically run Quick Clean using a tube of DI water (after every 20 sample acquisitions) to prevent a buildup from cellular debris in the system. If your samples contain significant amounts of cellular debris, run the Quick Clean cycle more often to prevent clogs or blockage.
7. If you are acquiring data from a sample but the progress bar is not moving, there is probably either insufficient volume to continue to acquire the sample or a blockage of the flow system. First check to ensure that there is at least 100 μL of sample in the tube. If not, add additional buffer to bring the volume up to 100 μL or proceed to the next sample. If the sample volume is greater than 100 μL , then the lack of events is probably due to a clog. A clog or blockage can be caused by cell aggregates, cell debris, bleach crystals, or other particulates. Perform a Backflush to flush out the clog into a tube containing 20% bleach. Then run Quick Clean to remove bleach residue. If this procedure does not alleviate the problem, refer to the *Muse™ Cell Analyzer User's Guide* for additional troubleshooting tips, or contact Millipore Technical Support for help.
8. Annexin V & Dead Cell works best with samples in a homogeneous, single cell suspension. Cell aggregates may clog or be excluded from the flow cell, affecting the accuracy of your results. If you want to use the Muse™ Annexin V & Dead Cell assay with a "clumpy" cell line, such as Chinese Hamster Ovary (CHO) cells, we recommend that you order Muse™ Cell Dispersal Reagent (Catalog No. MCH100107) to disaggregate the cells. Contact Customer Service or visit our website at www.millipore.com/muse for detailed information on the Muse™ Cell Dispersal Reagent and assay method. For more troubleshooting tips, refer to the Muse™ Cell Analyzer User's Guide.

For more information, contact the Millipore office nearest you. In the US, call 1-800-MILLIPORE (1-800-645-5476). Outside the US, visit our website at www.millipore.com/offices for up-to-date worldwide contact information. You can also view the tech service page on our web site at www.millipore.com/techservice.

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition taking longer than expected or progress bar stops during acquisition	Ensure that the System Check procedure was run and passed. If the progress bar stops during acquisition, the fluid system may be clogged. Run a Quick Clean procedure.
Instrument clogging Too many cells	Run a Quick Clean to clean out capillary. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.
Low Cell Concentration warning during acquisition	The sample concentration may be too low. The assay instructions are optimized to give you a range of cells between 100–500 cells/ μL in the final sample volume so accurate population count results are obtained. Repeat sample preparation with a lower dilution factor to allow for adequate cell numbers. A substantial decrease in cell numbers can lead to difficulty in adjusting settings.
High Cell Concentration warning during acquisition	If the concentration of the stained cell sample for acquisition is high (>500 cells/ μL), the accuracy of data will most likely be compromised. Dilute the sample further with 1X Assay Buffer HSC to adjust the cell concentration below 500 cells/ μL . For best results, it is recommended that the cell concentration is in the range of 200 to 300 cells/ μL .

Potential Problem	Experimental Suggestions
Background staining and/or non-specific staining of cells	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-PE and/or dead cell marker positive cells that are 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 dead cell marker reactivity.
Low level of staining	<ul style="list-style-type: none"> • Although the assay procedure has been optimized to function utilizing multiple cell types, every cell line behaves differently. A lack of signal may indicate that excess dilution factors may need to be altered to obtain accurate results. • 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 to achieve the best results for Annexin V-PE staining. • If there are no Annexin V-positive cells, your cells may not have induced or the Annexin V-PE 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 known to induce apoptosis in your cell line.
Poor separation of live and apoptotic populations	If the separation between populations is poor, the Annexin V concentration may be too low. Muse™ Annexin V & Dead Cell 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 the amount of reagent used. For best results, titer the Annexin V & Dead Cell reagent to determine the amount for maximal staining of cells.
Precipitate forms during incubation of the cells with the Annexin V & Dead Cell Reagent	Repeat the experiment with freshly induced cells, and increase the percentage of BSA, FBS, or NHS used during the staining procedure.
Variability in day-to-day experiments	<ul style="list-style-type: none"> • If the results are inconsistent, check that the samples were well mixed prior to acquisition. 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 Muse™ Cell Analyzer is working properly. Run the Muse™ System Check Procedure (Part No. MCH100101) to verify proper instrument function and accuracy.

Limitations

- The results of the assay are dependent upon proper handling of samples, reagents, and instruments.
- 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} This assay does not detect early apoptosis in cell types that do not translocate PS to the cell surface upon induction of apoptosis.
- The Muse™ Annexin V & Dead Cell Reagent is designed for use on unfixed cells. Fixing cells yields inaccurate results.
- The Muse Cell Analyzer and Muse™ Annexin V & Dead Cell 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, Millipore recommends using between 1×10^5 to 5×10^5 cells/mL when possible.

Appendix A: Cell Sample Preparation

Preparing Non-Adherent and Adherent Cells

The following protocols describe how to harvest non-adherent or adherent cells cultured in 96-well plates, as well as non-adherent or adherent cells cultured in flasks or other tissue culture vessels. Each of the culturing conditions requires different protocols to harvest the cells.

Preparing non-adherent cells

1. Set up initial culture conditions, such that after culture and treatment, cells are at a concentration of 1×10^5 to 1×10^7 cells/mL in serum- or albumin-containing medium.
2. Proceed to "Staining Protocol" 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 Muse™ Cell Dispersal Reagent (Catalog No. MCH100107) may be used to dissociate clumps.

1. Using your preferred method for dissociation, detach the cells from their culture vessel.
2. Add fresh serum- or albumin-containing medium to each well so final concentration is between 1×10^5 to 1×10^7 cells/mL.
3. Proceed to "Staining Protocol" on page 4.

References

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