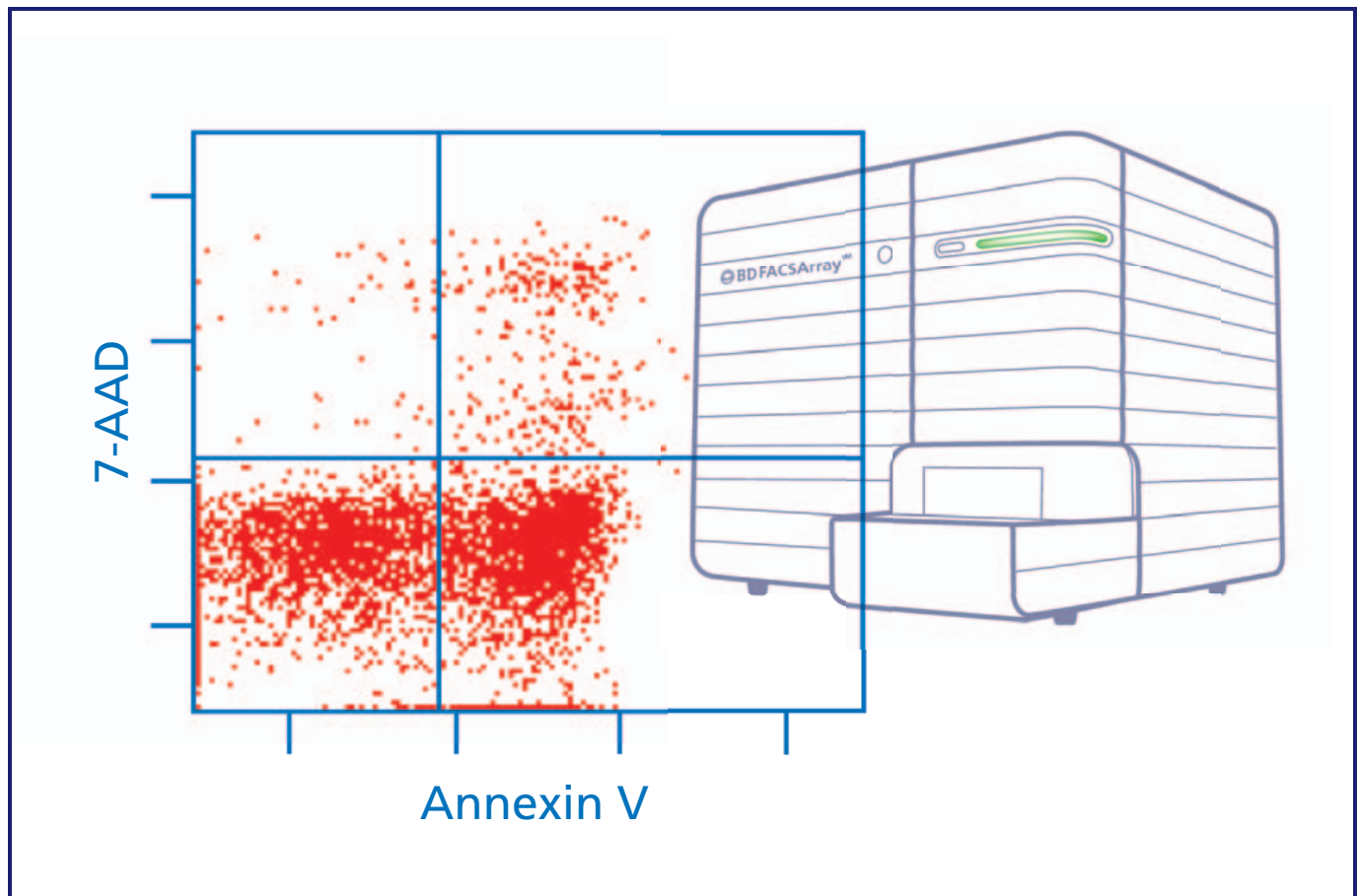


BD FACSArry™ Bioanalyzer

Analysis of Apoptotic Cells using the BD FACSArry™ Bioanalyzer



This application note describes a method for using the BD FACSArry™ Bioanalyzer to quantitate apoptotic cells with Annexin V and the vital dye 7-AAD after apoptosis induction.

Introduction

Apoptosis is a normal physiologic process which occurs during embryonic development as well as in maintenance of tissue homeostasis. The apoptotic program is characterized by certain morphologic features. These include changes in the plasma membrane such as loss of membrane asymmetry and attachment, a condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Changes in the plasma membrane are one of the earliest of these features. In early apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to

the external cellular environment. Annexin V is a 35 – 36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS¹. PS binding protein in the presence of Ca^{2+} can be used to detect externalized PS. Annexin V staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with Annexin V is often used in conjunction with a vital dye, such as 7-Amino-actinomycin D (7-AAD) to allow the discrimination of early apoptotic cells. Viable cells are Annexin V and 7-AAD negative. Cells that are in early apoptosis are Annexin V positive and 7-AAD negative.

Cells that are in late apoptosis or are already dead are positive for both Annexin V and 7-AAD. Using this method, a single observation is unable to distinguish cells that have undergone apoptotic death from those that have died as a result of a necrotic pathway. However, when apoptosis is measured over time and/or an apoptosis inducing agent is used, cells can often be tracked from Annexin V and 7-AAD negative (viable, or no measurable apoptosis), to Annexin V positive and 7-AAD negative (early apoptosis, membrane integrity is present) and finally to Annexin V and 7-AAD positive (end stage apoptosis and death).

The number of techniques designed to identify, quantify, and characterize apoptosis is escalating as we learn more about the complex mechanisms which underlie the process. This application note gives a concise method for the induction of apoptosis in Jurkat cells using camptothecin, followed by staining with Annexin V and 7-AAD, a vital dye, to simultaneously distinguish live, early apoptotic and late apoptotic/dead cells using the BD FACSAarray bioanalyzer.

Fluorochromes to Use with the BD FACSAarray™ Bioanalyzer

The BD FACSAarray™ bioanalyzer has a green laser at 532 nm wavelength and a red laser at 635 nm wavelength. Fluorescent emissions from fluorescent dyes or fluorochromes excited by the green laser are detected with the Yellow and the Far Red detectors, while fluorescent emissions excited by the red laser are detected by the Red and NIR detectors.

Table 1. BD FACSAarray™ Bioanalyzer Instrument Configuration for this Experiment

FLUORESCENT PARAMETER	FLUOROCHROME OR DYE	LASER
Yellow	-	Green
Far Red	7-AAD	Green
Red	Annexin V-APC	Red
NIR	-	Red

Table 2. Materials

MATERIALS	CAT. NO.
Jurkat Cell Line (Clone: E6-1)	ATCC TIB-152
Camptothecin*	Sigma C9911
Dimethyl Sulfoxide	Sigma D8779
50 mL Conical Centrifuge Tube	BD Falcon 352098
Cell Culture Flask (25 cm ²)	BD Falcon 353109
BD Pharmingen Stain Buffer (BSA)	554657
96-Well U Bottom Plate	BD Falcon 351190 or BD Falcon 353910
BD Pharmingen Annexin V Binding Buffer, 10X Concentrate	556454
BD Pharmingen APC conjugated Annexin V	550474
BD Pharmingen 7-AAD Staining Solution	559925

* This chemical is known to be toxic. Refer to product documentation for appropriate cautions.

Methods And Protocols

Protocol for Cell Staining with Annexin V and 7-AAD for the Analysis of Apoptosis using the BD FACSAarray™ bioanalyzer.

The protocol in this application note uses Jurkat cells incubated with camptothecin, an inhibitor of Topoisomerase I, in order to induce apoptosis. Treated and control cells are stained with APC conjugated Annexin V and the vital dye 7-AAD. The cells are then acquired and analyzed on the BD FACSAarray bioanalyzer in order to distinguish three different populations: live, early apoptotic and late apoptotic/dead cells.

Outline of Sample Preparation Steps

1. Reagent Preparation
2. Induction of Apoptosis
3. Sample Preparation
4. Plate Setup and Staining Procedure
5. Acquisition and Analysis with the BD FACSAarray bioanalyzer

Reagent Preparation

Before performing the following assay, verify that the following dilutions and stock solutions are available.

1. Prepare a 20 mM stock solution of camptothecin using Dimethyl Sulfoxide.
2. Dilute 10× BD Pharmingen™ Annexin V Binding Buffer to 1× using deionized water.

Induction of Apoptosis

The steps below should be performed in a tissue culture hood. Once the induction part of the procedure has been completed, aseptic conditions are no longer required. Always follow universal precautions.

Although the staining procedure and acquisition portions of this note require only 4.0×10^6 cells (8 wells with 0.5×10^6 cells/well), the recommended cell number for the induction procedure is 10×10^6 cells, or greater. This allows for larger incubation volumes, simplifying cell manipulation and allowing extra wells to be run if necessary.

1. Obtain a log phase culture of Jurkat cells and perform a cell count. Isolate at least 10×10^6 cells and transfer to a sterile 50 mL conical tube.
2. Centrifuge the cell suspension at $300 \times g$ for 5 minutes and remove the supernatant.
3. Suspend the cells in fresh cell culture media at a concentration of 1.0×10^6 cells/mL.
4. Divide the cell suspension into two equal volumes and place each in a tissue culture flask. Label one group as the control cells and the other as the treated cells.
5. Add camptothecin to the treated cells to achieve a final concentration of 4 – 12 μM .
 - If your stock solution of camptothecin is 20mM, add 2 – 6 μl for every 10 μl of media to adjust final concentration.
 - The experiment shown used 4 μl of a 20 mM stock solution per 10 mL of media to achieve a final concentration of 8 mM camptothecin.
6. Incubate both flasks at 37°C in a 5%CO₂ humidified incubator for 2.5 hours.

Note: The level of apoptosis induction is dependent on both incubation time and camptothecin concentration. If necessary, an incubation time course and/or a titration of camptothecin can be performed.

Sample Preparation

1. Remove flasks containing the treated and untreated cells from the incubator and transfer the cells to separate conical centrifuge tubes.
2. Centrifuge for 5 min at $300 \times g$.
3. Aspirate the supernatant and suspend each pellet in 10 mL of BD Pharmingen™ Stain Buffer.
4. Centrifuge for 5 min at $300 \times g$.
5. Repeat steps 3 and 4.
6. After the second wash with staining buffer, aspirate the supernatant and suspend the cell pellet in $1 \times$ BD Pharmingen™ Annexin V Binding Buffer to achieve a final concentration of 10.0×10^6 cells/mL.

Plate Setup and Staining Procedure

The BD FACSArray™ bioanalyzer is capable of performing automated spillover calculations (compensation). To perform these calculations, unstained and single-color setup wells are necessary. When creating the plate layout, the BD FACSArray bioanalyzer will automatically place these wells before any sample wells that will be acquired. Therefore, it is important to include these setup wells in the correct position in the experiment itself. To ensure that the spillover calculations are optimized for all of the cells in your experiment, use cells representing the brightest staining expected in each of the channels. In this experiment the camptothecin treated cells should be used to setup and optimize the instrument.

WELL	CELLS USED	STAIN USED	PURPOSE
A1	Treated	None	BD FACSArray Setup
A2	Treated	7-AAD	BD FACSArray Setup
A3	Treated	Annexin V-APC	BD FACSArray Setup
A4	Untreated	None	Experimental Control
A5	Untreated	7-AAD	Experimental Control
A6	Untreated	Annexin V-APC	Experimental Control
A7	Untreated	7-AAD and Annexin V-APC	Untreated Investigation Well
A8	Treated	7-AAD and Annexin V-APC	Treated Investigation Well

1. Referring to the layout above, add 50 μ L of either treated or untreated Jurkat cells in $1 \times$ BD Pharmingen™ Annexin V Binding Buffer to the appropriate wells. This will give approximately 0.5×10^6 cells per well.
 - Add camptothecin treated cells to Wells A1, A2, A3 and A8.
 - Add untreated cells to Wells A4, A5, A6 and A7.
2. For this assay, the recommended volume of BD Pharmingen™ Annexin V-APC is 5 μ L/test. Dilute the Annexin V-APC in Annexin V Binding Buffer so that 5 μ L BD Pharmingen™ Annexin V-APC is present in each 50 μ L of the final dilution.
 - In a 1.5 mL microcentrifuge tube, add 25 μ L Annexin V-APC to 425 μ L BD Pharmingen™ Annexin V Binding Buffer.
 - Vortex well.

3. Following the plate layout above, add 50 μ L of the diluted Annexin V-APC to the appropriate wells. Add 50 μ L Annexin V Binding Buffer (no Annexin V-APC) to the remaining wells.
 - Add Annexin V-APC in Annexin V Binding Buffer to Wells A3, A6, A7 and A8.
 - Add Annexin V Binding Buffer to Wells A1, A2, A4 and A5.
4. Gently vortex the plate and incubate the cells for 15 minutes at room temperature. Protect from light.
5. After incubation, add 100 μ L of $1 \times$ Annexin V Binding Buffer to each well.
6. Centrifuge for 5 min at $300 \times g$.
7. Discard supernatant.
8. Add 200 μ L of $1 \times$ Annexin V Binding Buffer to each well. Dislodge pellets by gentle agitation of the plate.
9. Centrifuge for 5 min at $300 \times g$.
10. Discard supernatant.
11. The recommended volume of 7-AAD is 5 μ L/test. Dilute the 7-AAD so that 5 μ L is present in each 250 μ L of the final dilution.
 - In a 1.5 mL microcentrifuge tube, add 25 μ L of 7-AAD to 1.225 mL Annexin V Binding buffer.
 - Vortex well.
12. Following the plate layout above, add 250 μ L of the diluted 7-AAD to the appropriate wells. Add 250 μ L of Annexin V Binding Buffer (no 7-AAD) to the remaining wells.
 - Add 7-AAD in Annexin V Binding Buffer to Wells A2, A5, A7 and A8.
 - Add Annexin V Binding Buffer to Wells A1, A3 A4 and A6.
13. Dislodge pellets by gentle agitation of the plate.
14. Incubate the plate for 10 minutes at room temperature. Protect from light.
15. Do not wash the samples after the incubation. Samples are now ready to be acquired on the BD FACSArray bioanalyzer.

Acquisition and analysis with the BD FACSArray™ Bioanalyzer

The BD FACSArray™ bioanalyzer aspirates cell samples from a 96-well plate during flow cytometric analysis; the sample volume that can be aspirated at one time cannot exceed 100 μ L per well. The maximum volume of 250 μ L per well should be added to each well to allow the user to aspirate sample more than once for instrument optimization.

Before using the unstained and single color controls to optimize instrument settings and acquire data to calculate optical spillover, you will create a new experiment through the Experiment Wizard.


Choosing a Wizard Session

The Experiment Wizard allows you to create an experiment by answering a series of questions. Each wizard session can be saved and re-opened for a similar experimental setup at a later time. The following information is saved in each Experiment Wizard session:

- sample and well information
- template used
- fluorophore labels
- custom keywords (if any selected)

1. Click the Experiment Wizard tool



() in the application toolbar (*Figure 1*). The Experiment Wizard Welcome dialog appears. The view remains for about 2 seconds and then automatically advances to the next view (*Figure 2*).

2. Choose the Default Wizard session when creating an experiment session for the first time; click Next.

- The Template view appears (*Figure 3*).

Choosing a Template

The Template view in the wizard session allows you to choose one of the predefined templates for cellular applications. The templates contain default loader and instrument settings that serve as a starting point for sample setup and optimization for the apoptosis application. Each predefined template contains a specific number of plots for acquisition and analysis of data.

1. Choose the 2 Color template from the Template drop-down menu; click Next.

- The Instrument setup and Optical Spillover view appears (*Figure 4*).

Entering Sample Information

The software will automatically calculate optical spillover, if setup wells are included. A setup well will be added for each color of this experiment. The names used within this experiment are examples. Customize the names for your own particular experiment.

1. Click the Yes radio button to enable the color selection check boxes.
2. Click in each check box for the colors to be used.

Note: When adding setup wells to your experiment, an unstained well is also added. The unstained well is run before the other setup wells. For this two-color experiment, a total of three setup wells will be added.



Experiment Wizard

Figure 1. Application toolbar

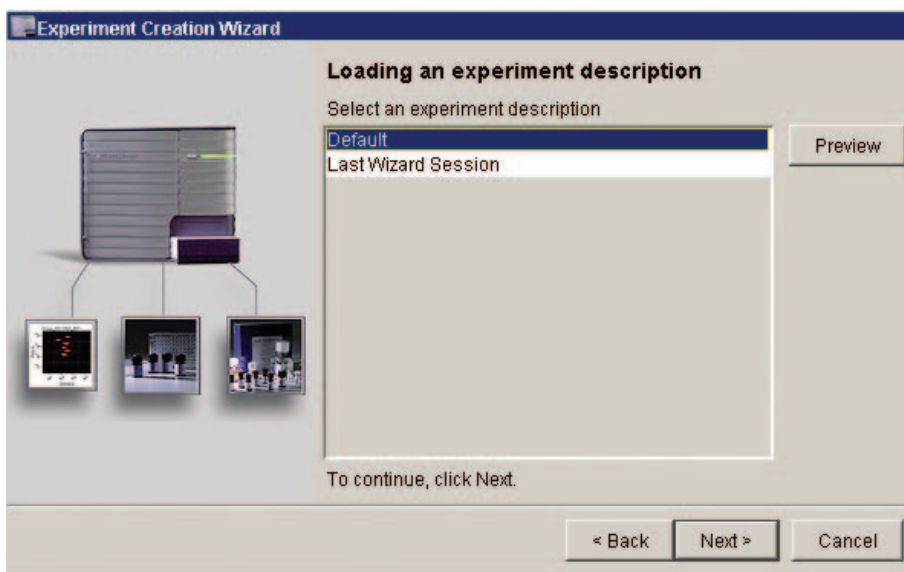


Figure 2

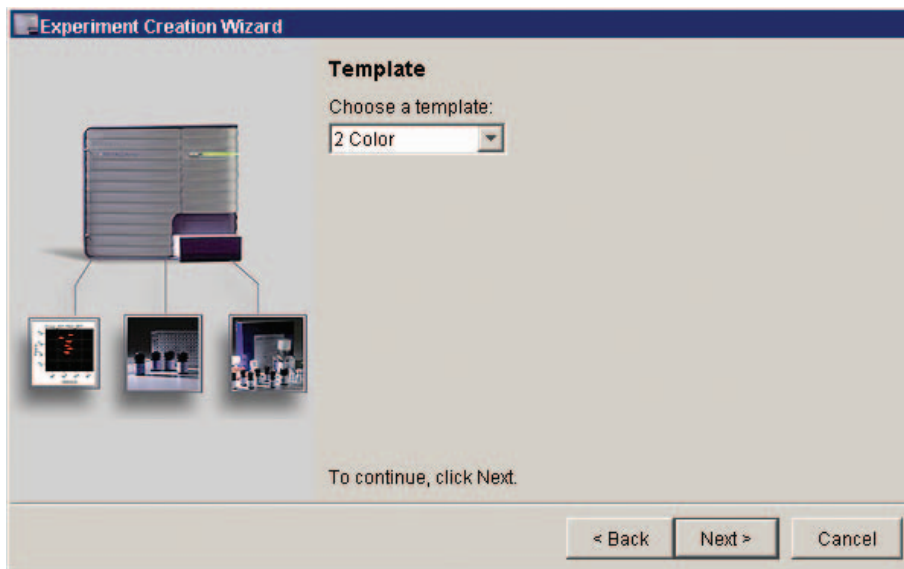


Figure 3

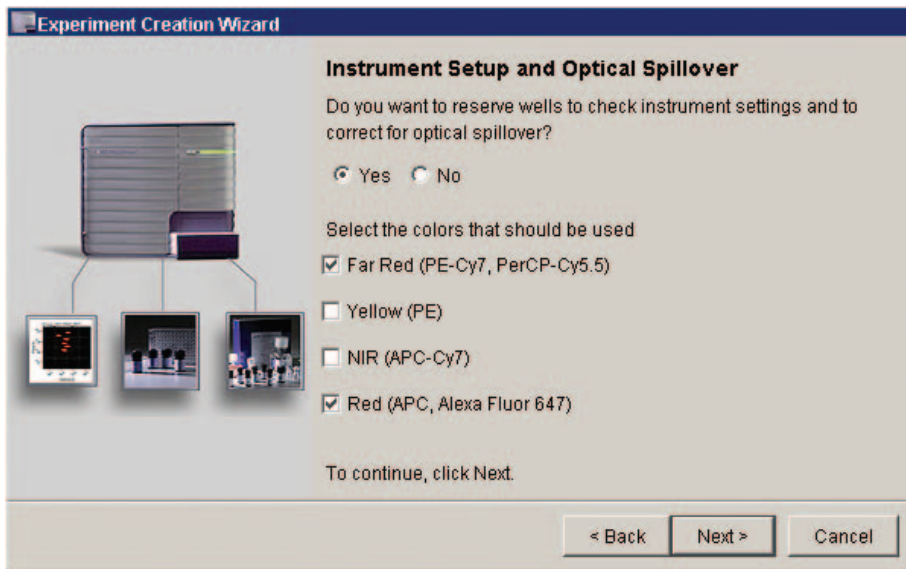


Figure 4

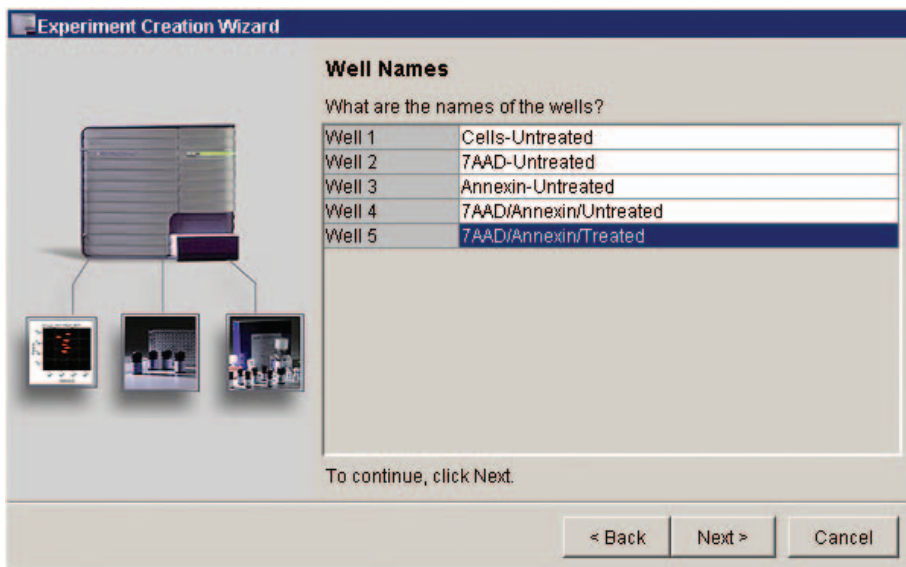


Figure 5

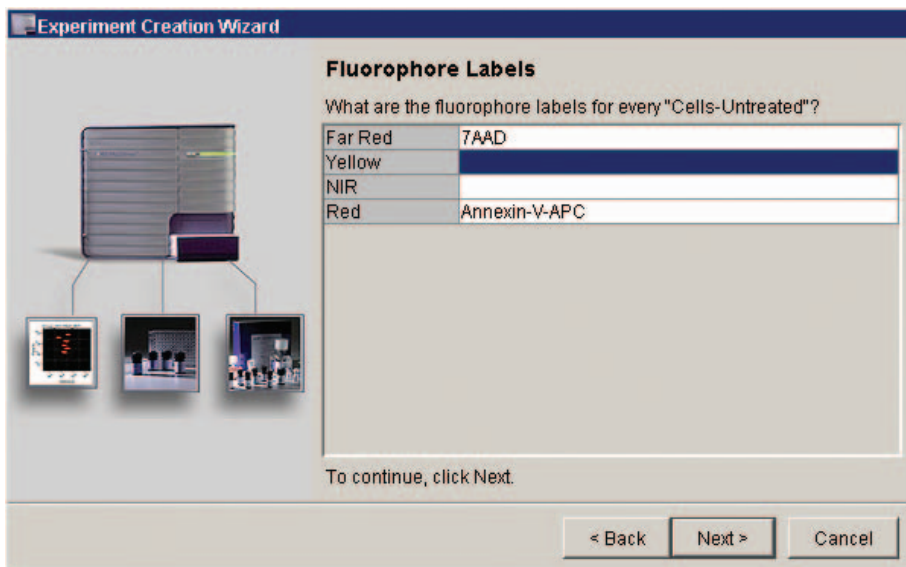


Figure 6

3. Click Next.
 - The Number of Samples view appears.
4. Enter the number 1 for the one sample that will be analyzed for this experiment; click Next.
 - The Sample Identification view appears.
5. Leave the default name for the sample name, Sample_001, in the Sample Identification view.

Entering Well Information

1. Click Next.
 - The Number of Wells per Sample view appears.
2. Change the number to 5 wells per sample; click Next.
 - The Well Names View appears (Figure 5).
3. Enter the following names for each well.

Well 1: Cells-Untreated
 Well 2: 7AAD-Untreated
 Well 3: Annexin-Untreated
 Well 4: 7AAD/Annexin/Untreated
 Well 5: 7AAD/Annexin/Treated

- To enter text in the text field:
 - Double-click in the white area of the text field to highlight the existing entry.
 - Enter the new name.
 - Press the Enter key on your computer keyboard.

Tip: Click the tab button to easily move between text fields.

4. Click Next.

The Fluorophore Labels view appears (Figure 6). The wizard will allow you to enter the fluorophore labels (axis labels) for each of the wells within a given sample. The labels will appear in the plots displayed in the Template view.

5. Enter the following names for the "Cells-Untreated" sample well:
 - 7AAD (for Far Red).
 - Annexin V-APC (for Red).
6. Press Enter. The same window remains in view but the sample has advanced to the next well.
7. Leave the fluorophore labels for each well the same, pressing Enter to advance through all of the wells.

Choosing Additional Options

1. Click Next.

The Custom Keywords view appears. Do not add keywords in this Wizard session.

2. Click Next.

The Adjusting Plate Layout view appears (*Figure 7*). In this view, you can choose how to lay out samples on the plate.

3. For this experiment, choose Contiguous Horizontal from the drop-down menu, and 0 as the number of separators.

Specifying Loader and Acquisition Settings

1. Click Next.

The Loader Settings view appears (*Figure 8*).

2. Leave the default loader settings; click Next.

The Acquisition Settings view appears where you can choose how many events per well to acquire and whether to use a stopping gate.

3. Leave the default acquisition settings for the Events to Acquire at 5000 events and the Stopping Gate on All Events.

4. Click Next.

The Experiment Name view appears (*Figure 9*).

Saving Experiment and Wizard Session

1. Type in “Apoptosis Experiment” as the name of the experiment.

2. Click Next.

- The Saving Wizard Session view appears where you can choose to name and save the Wizard session. Saving the Wizard session allows you to reuse the session for other similar experiments, but still allows you to modify any of the existing settings.

3. Click the Yes radio button and enter “Apoptosis Experiment Wizard” for this wizard session; click Next.

- The Completing the Experiment Wizard summary view appears.

4. Review the choices that were made.

5. Click Finish.

The experiment that has just been created opens in the Prepare workspace.

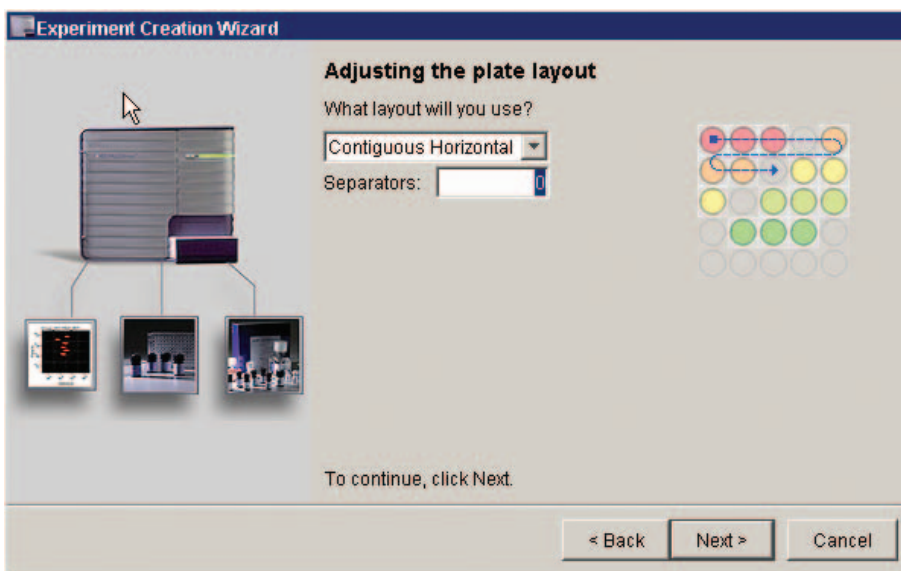


Figure 7

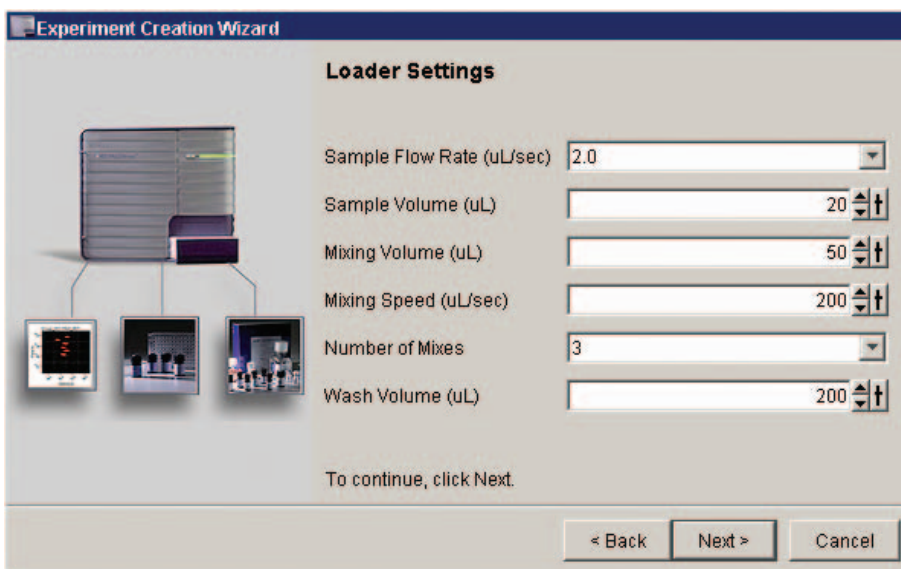


Figure 8

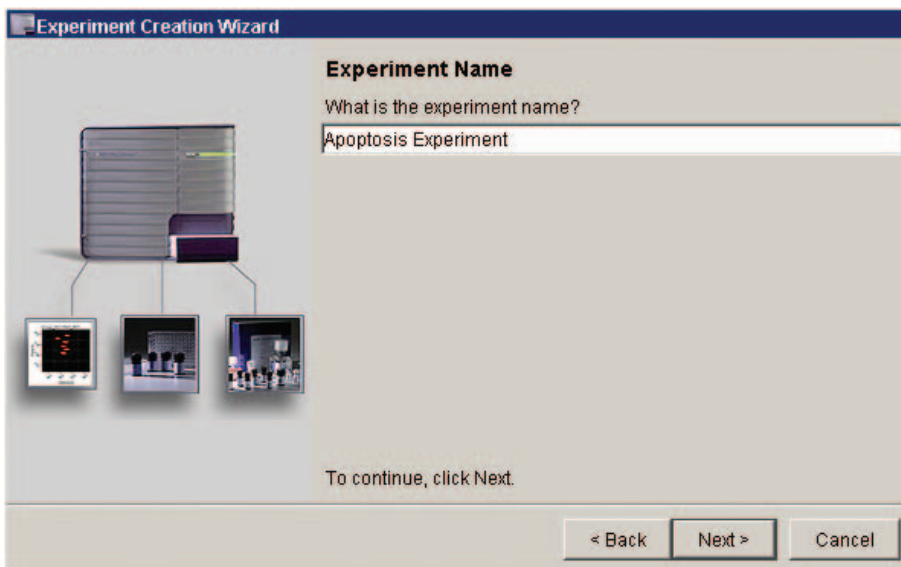


Figure 9

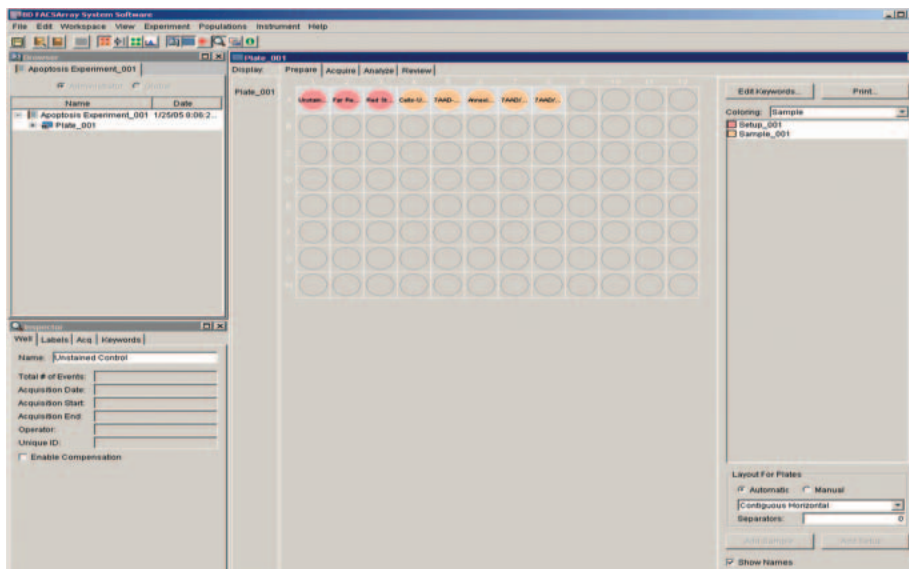


Figure 10

Review the Experiment in the Prepare Workspace

1. In the Prepare workspace, review your plate layout. Click the prepare



tool () in the tool bar to choose the Prepare Workspace (Figure 10).

Note: The first three wells in the plate view have been designated as setup wells. These wells will be used to optimize instrument settings and calculate automatic optical spillover correction by the software.

2. Click well A1.
3. In the Inspector, choose the Acq. Tab, and perform the following:

- Decrease the sample rate from 2.0 $\mu\text{L}/\text{sec}$ to 0.5 $\mu\text{L}/\text{sec}$ (the minimum value); press Enter.
- Increase the sample volume from 20 μL to 100 μL (the maximum value); press Enter.

Tip: Decreasing the sample rate and increasing the sample volume for the setup wells allows you to view live events for a longer period of time while optimizing instrument settings.

4. Repeat step 3 for the remaining setup wells (A2 and A3).

5. Click the Save button () to save the work.



Preparing for Instrument Optimization

Preparing the Plate

1. Print the plate view of the experiment by clicking Print in the upper right corner of the Prepare workspace.
2. Ensure there is approximately 250 μL of the prepared samples in of the following wells:

A1: Cells-Treated
 A2: 7-AAD-Treated
 A3: Annexin-Treated
 A4: Cells-Untreated
 A5: 7-AAD-Untreated
 A6: Annexin-Untreated
 A7: Annexin/7-AAD/Untreated
 A8: Annexin/7-AAD/Treated

Loading the Plate

1. Click the Setup tool in the application toolbar to open the Setup workspace.



Setup Tool

- The plate view shows that the setup wells have been selected for acquisition, and they are numbered in the acquisition order (Figure 11).

2. Place the plate containing the stained samples on the plate loader.
 - Position the plate so that well A1 is over the A1 mark on the plate loader.
3. Click the Load button in the Plate Control group.
 - If no lid is detected on the plate, the plate is completely retracted into the bioanalyzer.
 - The Status box should display the Instrument Ready Plate Loaded message.

Optimizing Instrument Settings

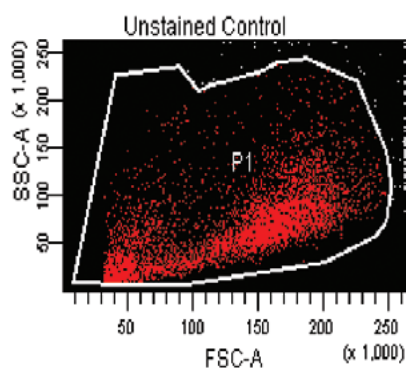
Next, you will perform the following steps to optimize settings for the Annexin V Experiment.

- Adjust Forward Scatter (FCS), Side Scatter (SSC), and threshold.
- Gate the population of interest.
- Adjust parameter voltages.
- Save data for the setup wells.
- Calculate optical spillover.

Optimize FSC, SSC, and Threshold Settings

To perform this set of optimization steps, you need to view data only from the first well.

1. In the Select Control group, click None to deselect the wells, and then click well A1.
2. Click the Setup button in the Acquisition Control group.
After a short pause, events appear in the plots, but data is not being saved in Setup mode.
3. Adjust the FSC and SSC voltages to place the cell population on scale and above the noise level in the FSC vs SSC plot, if needed.



- Adjust the signal for events displayed in plots by changing voltage settings.
4. Click the Parameters tab in the Instrument frame to display the Parameters pane (Figure 12).
 - Click in the Voltage field to edit this setting. Voltages can be adjusted from 1 – 1,000 by using the up or down arrows, or the slider bar, or by entering a value in the field.
 - Press Enter on your computer keyboard to save the changes.
 5. Adjust the threshold to eliminate debris at the lower end of the forward scatter (FSC) scale.
 - Click the Threshold tab in the Instrument frame to display the Threshold pane (Figure 13).
 - Click in the Value field to edit the settings.
- Events below the threshold value are excluded.
- Press Enter on your computer to save the changes.
 - Press the Setup button to stop acquisition of sample from the A1 well.

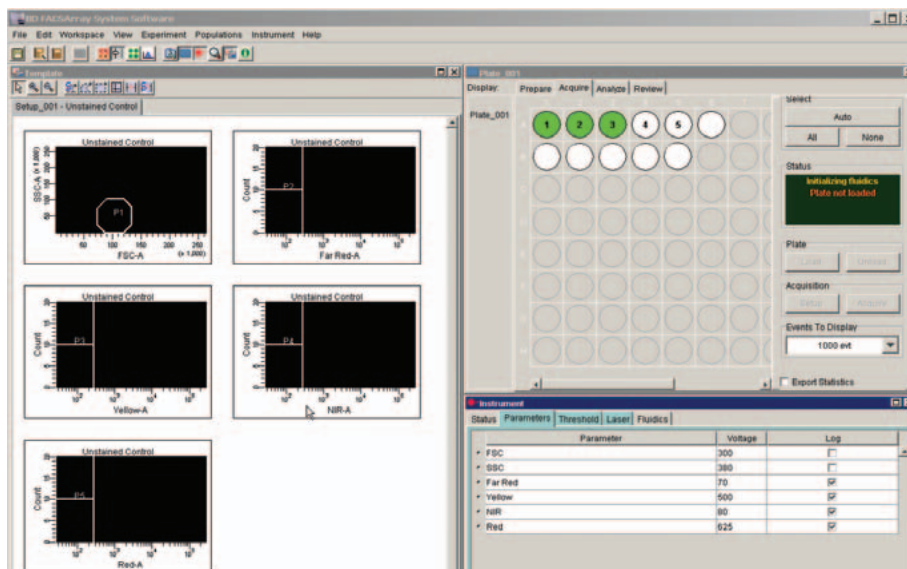


Figure 11

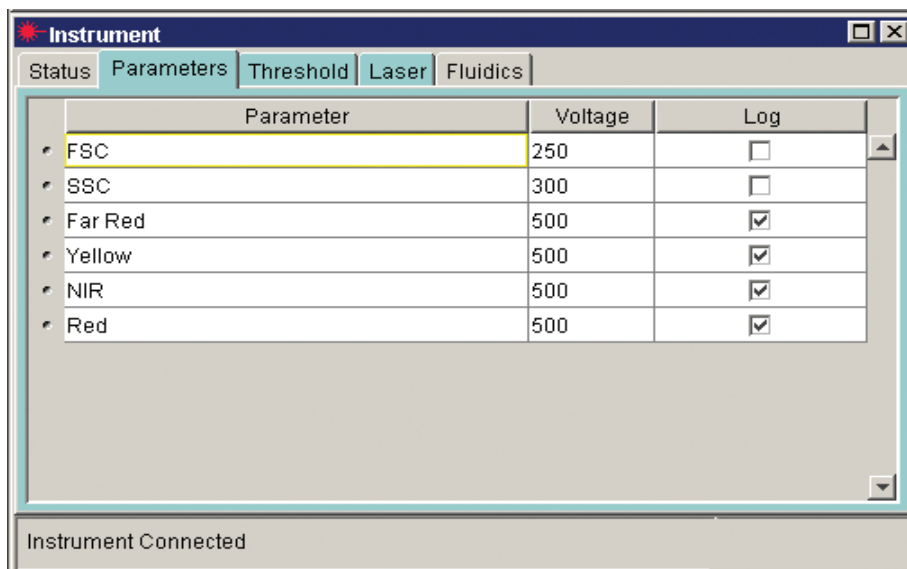


Figure 12

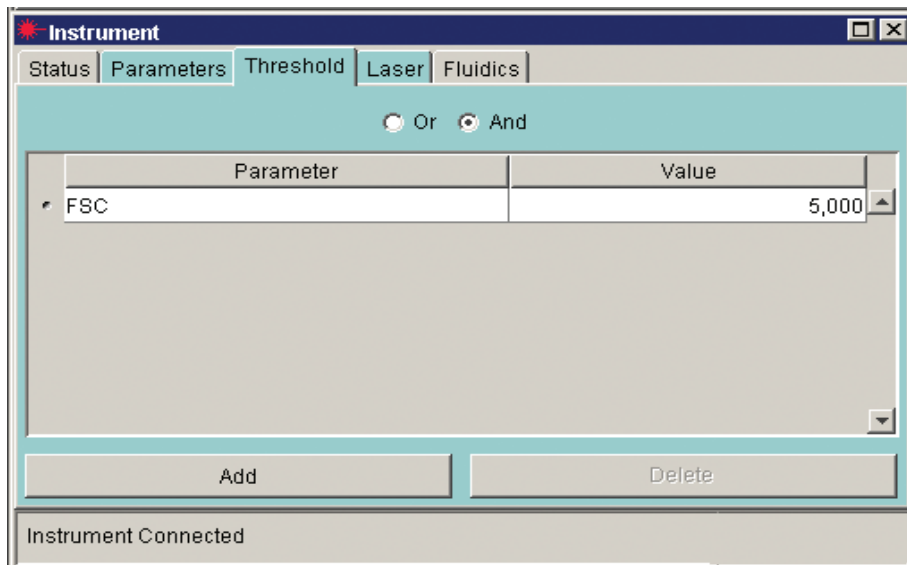


Figure 13

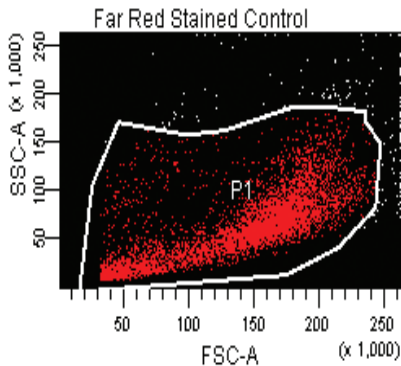
Gating the Population of Interest

The next step is to adjust the gate to surround the cells of interest. This ensures that only the cell population is viewed while optimizing the fluorescence settings. You will need to readjust the gate for each of the single-color setup wells.

1. In the Select Control group, click None to deselect the wells, and then click A2.
2. Click the Setup button in the Acquisition Control group.

After a short pause, events from well A2 appear in the plots, but data is not being saved.

3. If the gate does not surround the Jurkat population, click on the gate in the FSC vs SSC plot and drag it to the cell population.

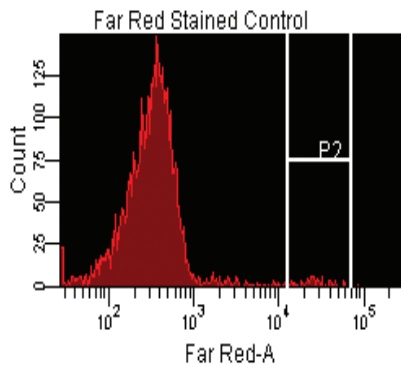


Note: The histogram plot displays only gated events. Include high scatter events as well. Dead cells tend to exhibit higher side scatter and reduced forward scatter measurements. Non-viable cells need to be evaluated in this experiment.

Optimizing Fluorescence Settings

In this experiment, it is important to verify that the brightest stained cells do not appear off scale while the dimly fluorescent cells remain on scale. For best sensitivity, the brightest and dimmest populations should be as well separated as possible while keeping both on scale. For best resolution of dim populations, keep the mean channel of dimly stained (not unstained) populations above channel 300.

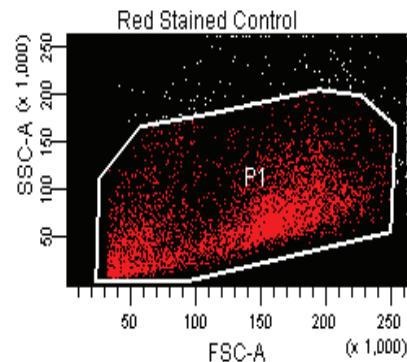
1. While observing the Far Red histogram (well A2 should still be acquiring), optimize the fluorescence settings for the positive peak as follows:



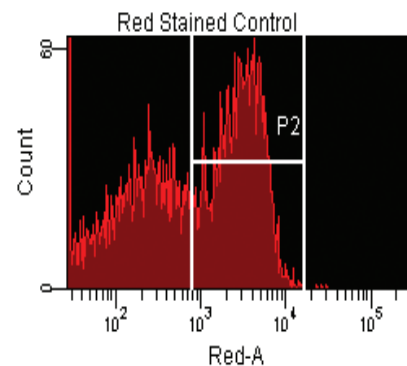
- Adjust the detector voltages if necessary to place the brightest population on scale.
- Verify the dim fluorescent cells remain on scale.


Note: You do not need to adjust the interval (histogram) gate at this time.

2. Click Setup to stop acquisition.
3. In the Select Control group, click None to deselect the wells, and then click A3.
4. Click Setup in the Acquisition Control group. Events from well A3 appear in the plots, but data is not being saved.




5. Verify that the FSC vs SSC gate is positioned correctly. If it is not, reposition the gate as before, making sure to include the high SSC events.
6. While observing the Red histogram, optimize the fluorescence settings for the Red Parameter. Refer to step 1 if necessary.



7. Click Setup to stop acquisition.
8. Click the Save button () to save your work.

Customizing the Loader Settings for Setup Wells


The instrument settings for the cellular experiment have been adjusted. Before acquiring the setup wells, adjust the loader settings for the sample volume to ensure optimal sample acquisition. Decreasing the sample volume for the setup wells allows you to speed up acquisition and ensure that the aspirated sample volume does not exceed the available volume.

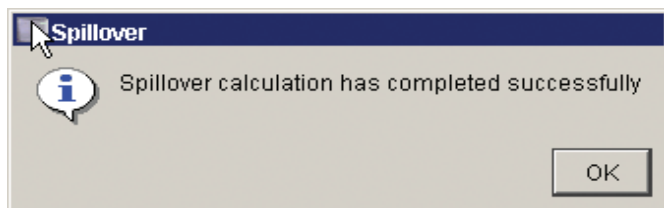
1. Click the Prepare tool () in the Application Toolbar to open the Prepare workspace.
2. Click well A1; change the sample rate and the sample volume in the Inspector:
 - In the Sample Rate field, change the rate from 0.5 $\mu\text{L}/\text{sec}$. to 2.0 $\mu\text{L}/\text{sec}$.; press Enter.
 - In the Sample Volume field, change the volume from 100 μL to 20 μL ; press Enter.
3. Repeat step 2 for the remaining setup wells (A2 and A3).

Tip: Always verify that you have enough volume in the wells prior to acquisition by unloading the plate and visually inspecting the volume. A minimum volume of 50 μL is recommended for this experiment.

Acquiring Data to Calculate Optical Spillover

The last step is to save the setup well data so that the software can use the data to automatically calculate and apply optical spillover correction to the experimental samples.

1. Click the Setup tool () in the application toolbar to open the Setup workspace.
2. In the plate view, verify setup wells A1 – A3 are selected.
3. Click Acquire.
4. Data will be saved for all three wells. After the last setup well is saved, the software automatically calculates and applies optical spillover correction.
5. If optical spillover correction is successfully calculated, the following message appears.

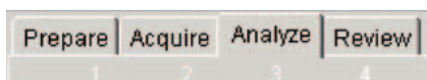


Ensuring Gates Were Properly Set.

For most applications, the Snap-To gate will surround the population of interest. However, in this experiment, the Snap-To gate will most likely exclude the high side-scatter events that need to be included for both setup and analysis. To ensure that the calculation is generated from appropriate data, you will need to expand the FSC vs SSC gate to include the high SSC events. After expanding the gate, you will need to verify that the histogram markers for each of the single channel set-up wells are positioned correctly.

Complete the following steps to ensure that all gates are properly set.

1. Click the Analyze tab in the Plate Editor.



2. Click on the unstained setup well (A1) in the Plate View and examine the plots in the Template view.
3. Expand the FSC vs SSC gate (P1) to include high SSC events.
4. Verify that the histogram interval gates in the Far Red and Red parameters are appropriately set on the negative populations (*Figure 14*).

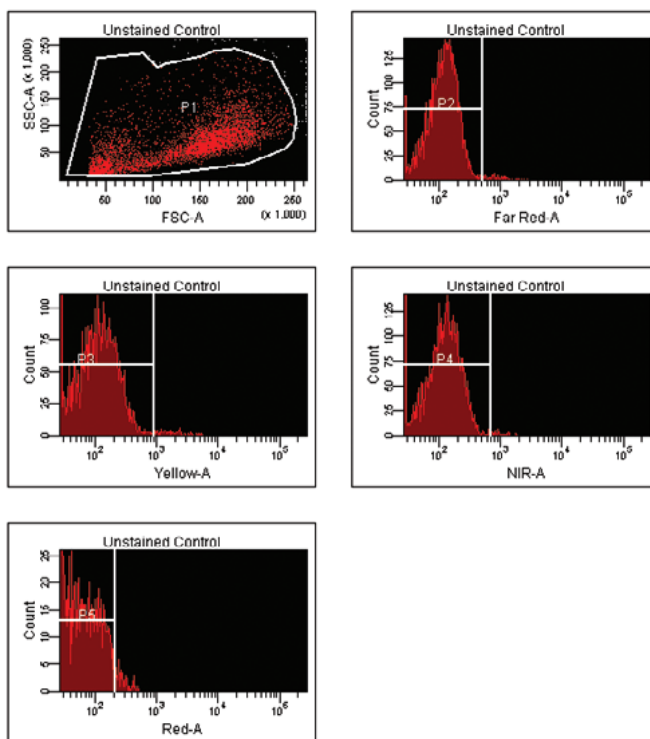
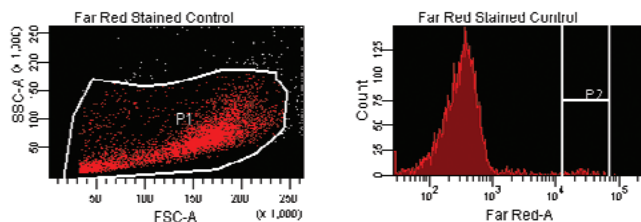


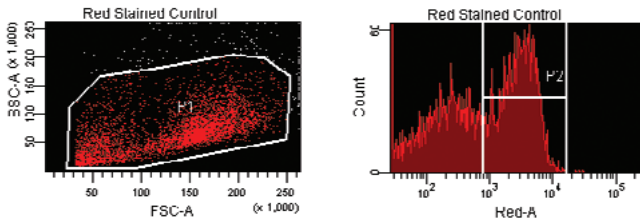
Figure 14

5. Click in Far Red (7-AAD) setup well (A2).
 - Expand the FSC vs SSC gate as before.
 - Verify that the P2 histogram interval gate is correctly centered on the 7-AAD positive population.

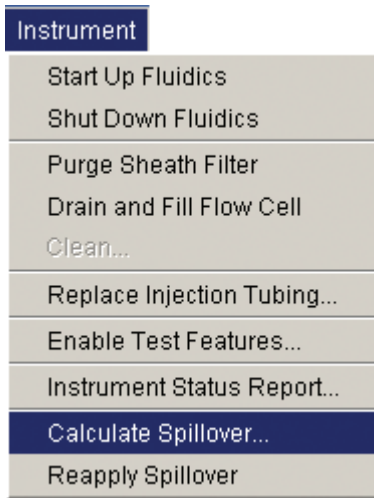


Note: It is especially important to include high side-scatter events for this well. This will ensure a sufficient number of 7-AAD positive cells to successfully calculate the spillover correction. 7-AAD positive cells may show a broad distribution of events. Select the brightest events and center the interval gate on them.

- Click on the Red (Annexin) setup well (A3).
 - Expand the FSC vs SSC gate as before.
 - Verify that the histogram interval gate (P2) is centered on the Annexin V positive population.




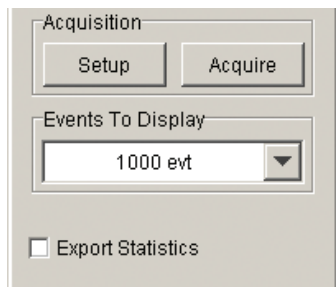
- After redefining gates in this section, choose Instrument > Calculate Spillover to recalculate optical spillover.




- Click the Save button () to save your work.

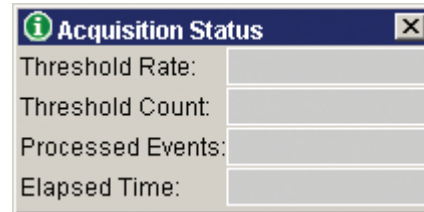
Data Acquisition

- Click the Acquire tab in the Plate Editor.
- In the Select Control group, click the Auto button.
The remaining sample wells will become selected and well A4 is designated as number one. The number in each well indicates the run order.
- Click the Acquire tool () in the application toolbar to open the Acquire workspace.
- Click the Acquire button in the Acquisition Control group.

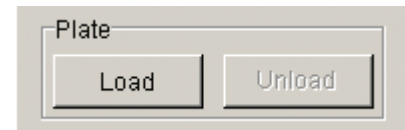


After a short pause, the following occurs:

- An orange ring appears around the first well, indicating that sample is being acquired.
 - Events appear in the plots.
 - Data is saved.
- Click the Acquisition Status tool () in the application workspace to open the Acquisition Status frame.
 - In the Acquisition Status frame, monitor the event rate to ensure events are being detected and acquired.



- Once the plate has been acquired, click the Unload button in the Plate Control group to eject the plate from the instrument.




- Remove the plate from the Plate Loader.

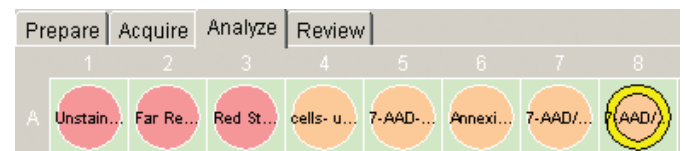
Data Analysis Using the BD FACSArray™ Software

Comparative analysis between the untreated and camptothecin treated investigation wells can be performed using the BD FACSArray™ software. Most cells will exhibit background levels of apoptotic and dead cells. The levels present in your treated investigation well (A8) include both background levels and camptothecin induced levels of apoptosis and cell death. Use the untreated investigation well (A7) to determine the background levels of dead and apoptotic cells in your Jurkat population. Subtract these background levels from the total levels in your treated well (A8) in order to determine only those cells that have undergone induced apoptosis.


Set Gates and Quadrants

Use the Untreated and Treated Investigation Wells to Create a Light Scatter Gate

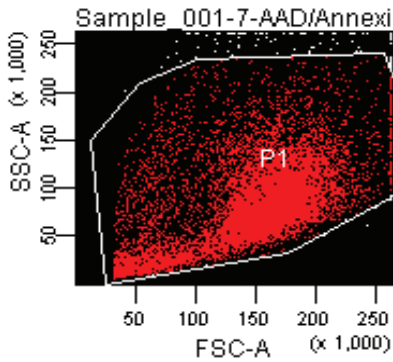
- Click on the Analyze tool () in the application toolbar to open the Analysis workspace.
- Click on well A8 to display data from the treated investigation well. A yellow circle will appear around the outer edge of the well. The yellow circle identifies the well under analysis.



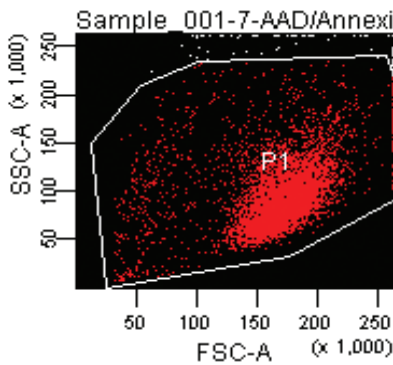
3. Create a P1 Gate on the FSC-A and SSC-A plot. Create a

new gate by selecting the Polygon Gate icon () and clicking around the perimeter of the Jurkat Cell population. As before, be sure to include the high-side scatter events in your FSC vs. SSC gate.

Note: The polygon gate will remain the same for all of the experimental wells. If a Snap-To gate is used, the gate will snap to a different position for each well analyzed, according to the data obtained for each well. The Snap-To gate will return to its default position each time you select that well for analysis and will most likely exclude the high side scatter events that need to be included in this analysis.



4. Once you have created the P1 gate using the treated investigation well, click on well A7 in the plate view to view the untreated investigation well. Verify that the P1 gate includes all of the relevant events for the untreated cells. Expand or reposition the gate if necessary.




5. Analyze acquired data through the P1 Gate.

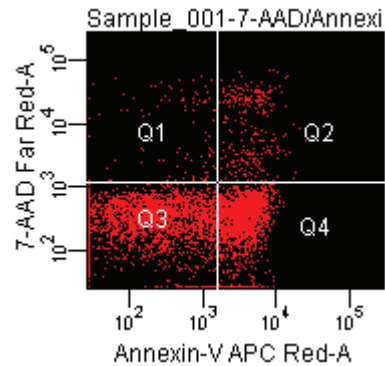
- Right Click on the border of the 7-AAD vs Annexin V APC dot plot.
- Choose Show Populations, and then select P1 from the menu that appears.

Only the cells that are in the gate will now appear in the dot plot.

Note: Parameters for dot plots can be changed by clicking on the axis and selecting appropriate parameter from the drop down menu

Use the Treated Investigation Well to Set a Quadrant Marker

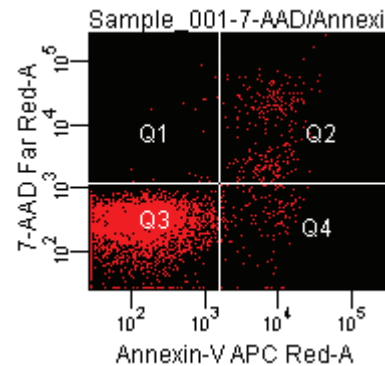
1. Set a quadrant marker using the treated investigation well (A8).
 - View well A8.
 - Click the quadrant marker tool () to select a quadrant marker, then click and drag across the Annexin V vs 7-AAD dot plot to create the quadrant marker. Position the crosshair (+) between the three distinct populations (double negative cells, Annexin V positive cells and double positive cells).
 - Verify that the double positive cells are in Q2, the double negative cells are in Q3 and the Annexin V positive cells are in Q4. If necessary the gate can be repositioned by clicking in the center crosshair and dragging the center to a new position.



Note: The untreated, single stained control wells (A4, A5 and A6) can also be used to setup the quadrant marker. While we do not use these wells in this analysis, they are important experimental controls that can be referred to if necessary.

Compare Treated vs. Untreated Investigation Wells.

1. On the plate view, select well A7. Display data from the untreated 7-AAD/Annexin V well.



Note: The BD FACSAarray™ software uses templates to analyze the data acquired. Once the gates are established on a template, they gates will remain the same for all wells unless changed or modified by the user. For this experimental example, once the gates are established on well A8, they will not change, thus the analysis gates will be identical for both well A8 and well A7.

- Observe the statistics at the bottom of the analysis window. These numbers represent the background levels of apoptosis and cell death present in the cell Jurkat population. Print or record these numbers for easy reference later in the analysis.

Population	#Events	%Parent
<input type="checkbox"/> All Events	5,000	###
<input checked="" type="checkbox"/> P1	4,912	98.2
<input checked="" type="checkbox"/> Q1	20	0.4
<input checked="" type="checkbox"/> Q2	264	5.4
<input checked="" type="checkbox"/> Q3	4,480	91.2
<input checked="" type="checkbox"/> Q4	148	3.0

- Select well A8. Observe the statistics at the bottom of the analysis window. These numbers represent both background and induced levels of apoptosis and cell death.

Population	#Events	%Parent
<input type="checkbox"/> All Events	5,000	###
<input checked="" type="checkbox"/> P1	4,943	98.9
<input checked="" type="checkbox"/> Q1	101	2.0
<input checked="" type="checkbox"/> Q2	283	5.7
<input checked="" type="checkbox"/> Q3	2,632	53.2
<input checked="" type="checkbox"/> Q4	1,927	39.0

- Determine the levels of early and late apoptosis/cell death induced by the treatment with camptothecin. Subtract the background levels (obtained from untreated investigation well A7) from the levels obtained after treatment with camptothecin (well A8). This calculation can be performed to determine the level of induction for early apoptosis (Q4), late apoptosis/cell death (Q2) and total apoptotic and dead cells (Q2 + Q4).

- In the experiment shown the levels of apoptosis are as follows:

- Early Apoptosis:**

Well 8, Q4 – Well 7, Q4 = Induction of Early Apoptosis
 $39.0\% - 3.0\% = 36.0\%$

- Late Apoptosis/Cell Death:**

Well 8, Q2 – Well 7, Q2 = Induction of Late Apoptosis/Cell Death
 $5.7\% - 5.4\% = 0.3\%$

- Total apoptotic and dead cells:**

Well 8 (Q2+Q4) – Well 7 (Q2+Q4) = Induction of Total Apoptosis/Cell Death
 $(5.7\% + 39.0\%) - (5.4\% + 3.0\%) = 36.3\%$

Other Fluorochrome Formats That Can Be Used With This Experiment.

Annexin V-APC and Propidium Iodide (PI) can also be used as alternative reagents to perform an apoptosis and viability analysis. PI is another commonly used vital dye. An experiment was conducted using PI in the place of 7-AAD and similar results were obtained. If PI is used, you may receive an error message when calculating spillover. This message will state that the compensation for the Yellow channel is unable to be set. Click OK and proceed through the experiment. As long as the other formats used in the experiment do not have their emissions read by the Yellow detector (PE is an example of one that does), the results will appear similar to those obtained with 7-AAD. Because of this spillover, the use of Annexin V-PE and PI for this experiment is not recommended.

Tips

Extra Setup Wells:

After an experiment is created with the Experiment Wizard, extra setup or sample wells can be added to the plate layout by performing the following steps:

- Click to select the Prepare Workspace in the application toolbar.
- In the Layout for Plates field of the plate editor, click the Manual button.
- Click to select the wells in the plate. Use the Shift key for multiple selections.
- Click Add Sample or Add Setup.
 - Repeat steps 3 – 4 for all the samples you are adding.

Export/Import Blank Experiments

If you will be performing the same experiment many times, performing the following steps can shorten software input:

- Create an experiment through the wizard or manually. Fill in all the appropriate labels for sample, well, parameter names.
- Create the appropriate gating strategy and name the gates.
- Edit the statistics box.
- Export the blank experiment.
- Acquire data into your experiment.
- The next time you run a similar experiment, import the blank experiment. Rename the experiment. Acquire data.

References

- Raynal P and Pollard HB. Annexins: The problem of assessing the biological role for a gene family of multifunctional calcium and phospholipid-binding proteins. *Biochimica et Biophysica Acta*. 1994;1197:63-93.
- BD Biosciences Technical Data Sheet 7-AAD.
- BD Biosciences Technical Data Sheet Annexin V-APC.
- BD Biosciences Technical Data Sheet Annexin V-PE Apoptosis Detection Kit.
- BD Biosciences Annexin V Staining online protocol <http://www.bdbiosciences.com/pharming/en/protocols/>

Contributors

Jacob Rabenstein, John Lee, Jeanne Elia, and Natalie Golts.

Regional Offices

Asia Pacific

BD Singapore
Tel 65.6861.0633
Fax 65.6860.1590

Australia/New Zealand

Australia
Tel 61.2.8875.7000
Fax 61.2.8875.7200
bd_anz@bd.com
New Zealand
Tel 64.9.574.2468
Fax 64.9.574.2469
bd_anz@bd.com

Canada

BD Biosciences
Toll free 888.259.0187
Tel 905.542.8028
Fax 888.229.9918
canada@bd.com

Japan

Nippon Becton Dickinson
Toll free 0120.8555.90
Tel 81.24.593.5405
Fax 81.24.593.5761
Clontech Products
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United States

BD Biosciences
Customer/Technical Service
Toll free 877.232.8995
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Austria

SCIENTIFIC SUPPORT
Tel 43.1.706.36.60.44
Fax 43.1.706.36.60.45
BDBsupport_GSA@europe.bd.com
CUSTOMER SERVICE
Tel 43.1.706.36.60
Fax 43.1.706.36.60.11
bd_austria@europe.bd.com

Belgium

CUSTOMER SERVICE
Tel 32.53.720.550
Fax 32.53.720.549
customer_service_bdbelgium@europe.bd.com

Brazil

Tel 55.11.5185.9995
Fax 55.11.5185.9895
biosciences@bd.com.br

Central America/Caribbean

Tel 506.290.7318
Fax 506.290.7331

Chile

Tel 56.2.460.0380 x16
Fax 56.2.460.0306

China

Tel 8610.6418.1608
Fax 8610.6418.1610

Colombia

Tel 57.1.572.4060 x244
Fax 57.1.572.3650

Denmark

Tel 45.43.43.45.66
Fax 45.43.43.41.66
bdbnordic@europe.bd.com

East Africa

Tel 254.2.341157
Fax 254.2.341161
bd@africaonline.co.ke

Eastern Europe

Tel 49.6221.305.161
Fax 49.6221.305.418
bdb.ema@europe.bd.com

Egypt

Tel 202.268.0181
Fax 202.266.7562

Finland

Tel 358.9.88.70.7832
Fax 358.9.88.70.7817
bdbnordic@europe.bd.com

France

Tel 33.4.76.68.36.40
Fax 33.4.76.68.35.06
SCIENTIFIC SUPPORT
Tel 33.4.76.68.34.25
Fax 33.4.76.68.55.71
bdb_france_scientific_support@europe.bd.com
CUSTOMER SERVICE
Tel 33.4.76.68.37.32
Fax 33.4.76.68.35.06
customerservice.bdb.france@europe.bd.com

Germany

SCIENTIFIC SUPPORT
Tel 49.6221.305.525
Fax 49.6221.305.530
BDBsupport_GSA@europe.bd.com
CUSTOMER SERVICE
Tel 49.6221.305.551
Fax 49.6221.303.609
customerservice.bdb.de@europe.bd.com

Greece

Tel 30.210.940.77.41
Fax 30.210.940.77.40

Hong Kong

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Fax 852.2803.5320

Hungary

Szerena
Tel 36.1.345.7090
Fax 36.1.345.7093

India

Tel 91.124.238.3566.77
Fax 91.124.238.3225

Indonesia

Tel 62.21.577.1920
Fax 62.21.577.1925

Italy

Tel 39.02.48.240.1
Fax 39.02.48.20.33.36

Japan

Fujisawa Pharmaceutical Co., Ltd.
(Reagents from Immunocytometry Systems & Pharmingen)
Tel 81.6.6206.7890
Fax 81.6.6206.7934

Korea

Tel 822.3404.3700
Fax 822.557.4048

Malaysia

Tel 603.7725.5517
Fax 603.7725.4772

Mexico

Tel 52.55.5999.8296
Fax 52.55.5999.8288

Middle East

Tel 971.4.337.95.25
Fax 971.4.337.95.51
bdb.ema@europe.bd.com

The Netherlands

CUSTOMER SERVICE
Tel 31.20.582.94.20
Fax 31.20.582.94.21
customer_service_bdholland@europe.bd.com

North Africa

Tel 33.4.76.68.35.03
Fax 33.4.76.68.35.44
bdbiosciences_maghreb@europe.bd.com

Norway

Laborel S/A
Tel 47.23.05.19.30
Fax 47.22.63.07.31

Peru/Bolivia/Ecuador

Tel 51.1.430.0323
Fax 51.1.430.1077

Philippines

Tel 632.807.6073
Fax 632.850.1998

Poland

Tel 48.22.651.75.88
Fax 48.22.651.75.89
bdb.ema@europe.bd.com

Portugal

Enzifarma
Tel 351.21.421.93.30
Fax 351.21.421.93.39

Saudi Arabia

Tel 966.1.26.00.805/806
Fax 966.1.26.00.804

South Africa

Tel 27.11.807.15.31
Fax 27.11.807.19.53
bdb.ema@europe.bd.com

Sweden

Tel 46.8.775.51.10
Fax 46.8.775.51.11
bdbnordic@europe.bd.com

Switzerland

SCIENTIFIC SUPPORT
Tel 41.61.485.22.91
Fax 41.61.485.22.92
BDBsupport_GSA@europe.bd.com
CUSTOMER SERVICE
Tel 41.61.485.22.22
Fax 41.61.485.22.00
customerservice.bdb.ch@europe.bd.com

Taiwan

Tel 8862.2722.5660
Fax 8862.2725.1768

Thailand

Tel 662.643.1374
Fax 662.643.1381

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Tel 90.212.222.87.77
Fax 90.212.222.87.76

United Kingdom & Ireland

Tel 44.1865.78.16.88
Fax 44.1865.78.16.27
bduk_customerservice@europe.bd.com

Venezuela

Tel 58.212.241.3412 x248
Fax 58.212.241.7389

West Africa

Sobidis
Tel 225.20.33.40.32
Fax 225.20.33.40.28



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