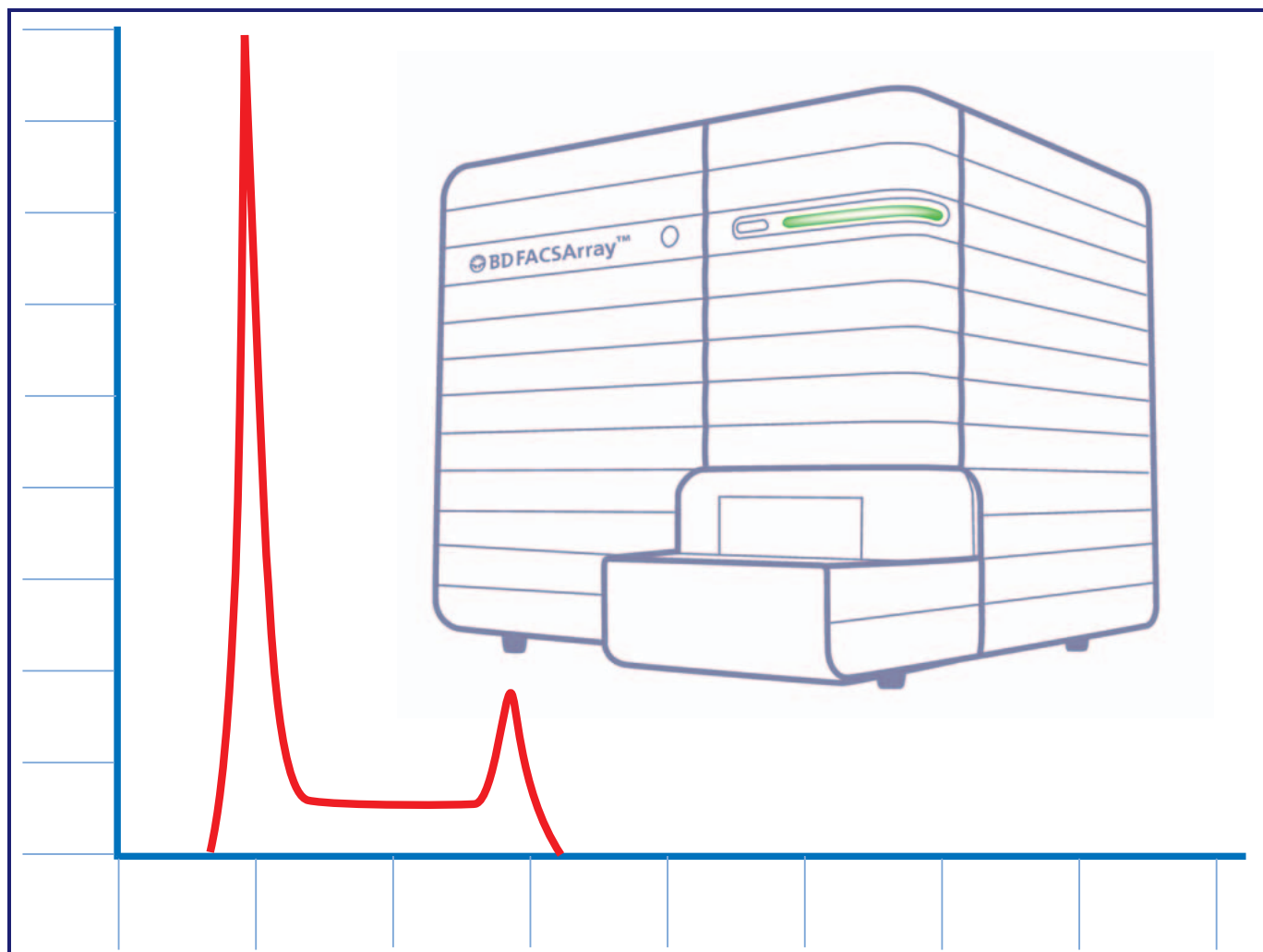BD FACSArray™ Bioanalyzer

Cell-Cycle Analysis Using the BD FACSArray™ Bioanalyzer

This Application Note describes a method for using the BD FACSArray™ bioanalyzer to analyze cells stained for cell-cycle analysis using propidium iodide and an additional intranuclear stain for multiparameter analysis.

Introduction

Somatic cells proliferate to support tissue and organismal growth and to replace damaged cells. In the case of adaptive immunity, T and B lymphocytes divide and differentiate in response to foreign antigenic stimulation, known as clonal expansion and differentiation. This hallmark response ensures that sufficient numbers of antigen-specific effector and memory lymphocytes arise to successfully deal with the offending antigen (eg, pathogenic microbes, viruses). For this reason, flow cytometric analysis of lymphocyte activation and proliferation (cell-cycle entry and progression) and differentiation is featured in many immune function studies. The BD FACSArray™ bioanalyzer is designed to perform analysis of cellular samples that are stained with combinations of up to four different fluorescent dyes or antibodies specific for various cellular markers. This application note gives a concise method of staining cellular DNA with propidium iodide and phosphorylated histones for a detailed cell-cycle analysis using the BD FACSArray bioanalyzer.

BD Biosciences
Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen

The cell-cycle (aka, cell division cycle) consists of a series of processes involved in the growth, replication, and division of cells. The cell-cycle can be divided into two major stages, interphase (a phase between mitotic events) and mitosis. There are three distinct, successive stages within interphase, called G1, S, and G2 phases. During G1 (first gap), cells monitor their environment and grow (synthesize RNA and proteins). If conditions are right, cells commit to DNA synthesis (S phase) and replicate their chromosomal DNA. A G2 phase (second gap) follows in which cells continue to grow and prepare for mitosis (division). The G2 gap allows time for the cell to complete its DNA replication before initiating mitosis. In mitosis, there are four successive phases:

- prophase–marked by chromosomal condensation, dissolution of the nuclear membrane and spindle formation

- metaphase–chromosomal alignment along the metaphase plate

- anaphase–separation of chromosome pairs into opposite poles of the cell

- telophase–creation of two nuclei, accompanied by cytoplasmic division (cytokinesis) giving rise to two daughter cells.

Upon completion of the process, each daughter cell contains the same genetic material as the original cell and roughly half of its G2 level of cytoplasm.

In addition to these specific stages, the G0 phase has been described for cells that exit from the cell-cycle and enter a quiescent, nondividing state. In response to external stimuli, some cells may undergo reactivation and express early response genes. These cells can leave G0 and enter the G1 phase of the cell-cycle (G0–G1 transition) that is marked by cell growth (ie, blast transformation due to measurable increases in RNA and protein synthesis). Another consequence of cellular activation may be the induction of programmed cell death (apoptosis). Together, through the counterbalancing processes of cell proliferation and apoptosis, and the establishment of quiescent or nondividing states, appropriate numbers and various types of somatic cells (including cells of the immune system) are dynamically maintained (homeostasis) throughout the body's life cycle.
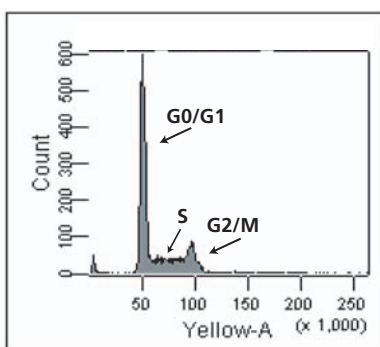

Figure 1. Flow Cytometric Analysis of Cellular DNA Content

Using fluorescent nucleic acid dyes, such as propidium iodode, it is possible to identify the proportions of cells that are in one of the three interphase stages of the cell-cycle by using flow cytometry to measure their relative DNA content (*Figure 1*). Cells that are in the G0/G1 phase (before DNA synthesis) have a defined amount (1×) of DNA (ie, a diploid chromosomal DNA content). During S phase (DNA synthesis), cells contain between 1× and 2× DNA levels. Within the G2 or M phases (G2/M), cells have a 2× amount of DNA (ie, a tetraploid chromosomal DNA content).

Immunofluorescent staining of phosphorylated histones may be used with propidium iodide staining and flow cytometric analysis to further resolve cells within the G2 or M phases. Cells that express phosphorylated Histone 3 at serine-28 are in the M phase. Phosphorylated Histone 3 can thus be used as a cell-cycle marker for screening for affects to the M phase of the cell-cycle pathway. This experiment will utilize Anti Histone H3 (pSer28)-Alexa Fluor® 647 as the Phospho Histone 3 antibody to investigate the affects of a specific compound upon the M phase. The ability to simultaneously and rapidly analyze samples for their cell-cycle status and intracellular signaling markers makes this assay ideal for high content screening.

This application note describes a method for staining and analyzing cells for cell-cycle analysis using the BD FACSArray bioanalyzer. This procedure includes optional steps for how to perform further research of intracellular, intranuclear, or cell-surface proteins along with cell-cycle analysis. Before you begin this procedure, review the entire *Methods and Protocols* section to familiarize yourself with the steps involved. Review the *BD FACSArray™ Bioanalyzer User's Guide*, especially Chapter 7, for a detailed explanation of how to set up the instrument for cellular applications.

Fluorochromes to Use with the BD FACSArray™ Bioanalyzer

The BD FACSArray™ 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.

Fluorochromes that can be used with ethanol-fixed cells stained with propidium iodide (PI)

FLUORESCENT PARAMETER	FLUOROCHROME OR DYE	LASER
Yellow	Propidium Iodide	Green
Far Red	Propidium Iodide	Green
Red	APC or Alexa Fluor® 647	Red
NIR	-	Red

Propidium Iodide

Propidium iodide (PI) is the most widely used fluorescent dye for staining DNA in whole cells (or isolated nuclei). PI intercalates into the DNA helix of fixed and permeabilized cells. Because PI can stain both double-stranded RNA and DNA, cells must be treated with RNase to ensure that PI staining is DNA specific. PI does not cross the plasma membrane of viable cells. However, it can readily enter dead cells (or cells in late stages of apoptosis) that have damaged plasma membranes and can stain their cellular DNA.

Materials

REAGENT	CAT. NO
BD Pharmingen Stain Buffer (FBS)	554656
96-well U bottom plate	BD Falcon 353910
RNase	Sigma R-5500
Propidium Iodide	Sigma P-4170
ModFit *LT*™ Software for PC	*349329
Anti Histone H3 (pSer[28])-Alexa Fluor® 647	inquire
Nocodazole	Sigma M-1404

*BD Biosciences Immunocytometry Systems

Methods and Protocols

Protocol for Staining DNA with Propidium Iodide for Cell-Cycle Analysis Using the BD FACSArray™ bioanalyzer.

The protocol in this application note includes optional steps on how to perform intracellular, intranuclear, or cell-surface staining for further cell-cycle and cellular analysis. Please review the outline for the appropriate steps for your experiment.

Outline of Sample Preparation Steps

Cell-Cycle Experiment

1. Perform cell surface staining (optional)
2. Fix cells
3. Perform intracellular or intranuclear surface staining (optional)
4. RNase treatment
5. PI stain
6. Acquire and analyze cells on the BD FACSArray bioanalyzer

Depending on the experiment that will be run on the BD FACSArray bioanalyzer, the cells will have to be prepared in a specific pattern in the 96-well plate. The following example will demonstrate a method for performing a two-color experiment, using PI and Alexa Fluor® 647. With this two-color experiment, specific setup wells need to be prepared to set up and optimize the instrument. If cell-cycle alone is being run, there is only one color being detected and thus no set-up wells are necessary.

The cell-cycle analysis experiment described here used DNA stained with PI for a general cell-cycle analysis and Anti Histone H3 (pSer28)-Alexa Fluor® 647 to distinguish M phase cells in the cell-cycle. For demonstration purposes cells were arrested with 1 µg/mL of Nocodazole for 4 hours prior to harvesting to allow for cells to be arrested in the M phase.

Sample Preparation

Prepare the 96-well sample plate as follows:
- Well A1: unstained control sample
- Well A2: PI stained control sample
- Well A3: Alexa Fluor® 647 only control sample
- Well A4: PI and APC or Alexa Fluor® 647 stained cells together for the complete cell-cycle analysis

TIP: Additional samples used in the setup wells may be needed for the first time this assay is performed. Prepare additional volumes according to your needs.

Cell-Surface Staining (Optional)

Cells may be stained for surface antigens prior to fixation with ethanol (EtOH). Because PI emits fluorescence in both the Yellow and Far Red channels upon excitation, antibodies conjugated with the fluorochromes phycoerythrin (PE) or PE-cyanine dye Cy7 (PE-Cy7) should not be used. Fluorescent antibodies conjugated to allophycocyanin (APC) or Alexa Fluor® 647 are the recommended conjugates for two-color staining with PI when using EtOH-fixed cells.

> *Note:* APC-Cy-7 conjugates are not recommended for use with EtOH-fixed cells. Cells may be stained prior to EtOH fixation by adding an appropriate concentration of APC- or Alexa Fluor® 647 conjugated antibody to the cells. Each researcher should determine if the experimental antibody will work with EtOH-fixed cells. EtOH can destroy certain fluorochromes and antigenic epitopes on cells.

> *Note:* If the antibody has been shown to stain EtOH -fixed cells, then staining may be done post EtOH fixation.

1. Incubate the cells for 15 – 30 minutes on ice at 0 – 4°C then wash using BD Pharmingen™ staining buffer (BD Pharmingen™ Stain Buffer (FBS) or 1× Dulbecco's PBS, 3% FCS, 0.09% NaN3, pH 7.4).
2. For cells that must be stained prior to EtOH fixation, stain the cells according to the manufacturer's protocol.
3. Wash cells in staining buffer before proceeding to the fixation step.

Fixation of Cells

Cells are fixed using ice-cold 70% EtOH. Cells may be fixed in bulk then transferred to a 96-well U bottom plate or the cells may be fixed in the plate directly. Both procedures are described below.

A. Bulk Cell Fixation

1. Wash cells twice with 1× PBS to remove any residual media or buffers that contain FBS or BSA. It is important so as to avoid aggregates, which can clog the instrument during analysis. Add 100 µL of 1× PBS per 1×10^7 cells and resuspend the cells by vigorous vortexing.
2. Add 5 mL of ice-cold 70% EtOH by dropper while slowly mixing or vortexing. Additional EtOH may be added to adjust cell concentration to 2×10^6 cells/mL. This volume may be added without vortexing.
3. Incubate the cells with EtOH for at least 1 hour at or below 4°C. Cells may be incubated overnight and are stable at –20°C for weeks.
4. Prior to staining, resuspend cell pellets and check for aggregates prior to transferring to the 96-well U bottom plate.
   - If any aggregates are present, filter the cell suspension through a 60 µm pore nylon mesh.
5. Transfer the sample to a 96-well U-bottom plate at $\leq 5 \times 10^5$ cells per well.

B. Cell Fixation in Plates with U Bottom Wells

1. Add $\leq 5 \times 10^5$ cells per well.

2. Wash the cells twice with 100 µL 1× PBS to remove any residual FCS or BSA.

3. Fix cells by adding 10 µL of 1× PBS to each well followed by 100 µL of 70% ice-cold EtOH.

4. Incubate cells for at least 1 hour at or below 4°C.

5. Wash the cells twice with 100 µL 1× PBS. Pellet the cells by centrifugation at $300 \times g$ for 5 minutes.

Intracellular, Intranuclear, or Surface Marker Staining (Optional)

1. If they are to be stained for intranuclear markers such as Phospho Histone, treat EtOH-fixed cells with 0.1% Tween-20 in 1× PBS for 20 minutes at room temperature.

2. Wash cells in staining buffer, then stain with the appropriate concentration of antibody for 20 minutes at room temperature.

3. Wash cells in staining buffer.

4. Pellet the cells by centrifugation at $300 \times g$ for 5 minutes, then aspirate supernatants from the wells.

5. If a secondary staining step is needed, stain the cells with the appropriate amount of secondary step antibody according to the manufacturer's protocol.

6. Wash the cells in staining buffer, and aspirate the supernatant from the wells.

7. **Do not resuspend the cells.** Proceed directly to the RNase treatment.

RNase Treatment

Propidium iodide (PI) is not a DNA-specific dye. PI stains all nucleic acids including RNA. Therefore, treatment with RNase is necessary to ensure specific staining of DNA by PI.

1. Add 50 – 100 µg of a working concentration of RNase in 1× PBS to each well. A working concentration of RNase can be made by diluting the stock of 1 µg/µL in 1× PBS and stored at –80°C.

2. Incubate at 37°C for 30 minutes.

3. Wash the cells in staining buffer and aspirate the supernatant from the wells.

4. Resuspend wells A1 and A3 in 200 µL staining buffer. Do not resuspend the cells in wells A2 or A4, but proceed directly to the DNA staining step.

Staining for DNA

The protocol below specifies volumes needed for staining two wells with PI. Adjust the volumes according to the number of wells in your experiment.

PI stock is diluted to 1 mg/mL in H_2O and stored at 4°C in the dark.

1. Dilute 15 µL of PI stock (1 mg/mL) in 585 µL of staining buffer.

2. Add 200 µL of diluted PI solution at 5 µg/sample to each of the following wells:

   • A2: PI setup well

   • A4: Investigation well

Cell-Cycle Analysis with the BD FACSArray™ Bioanalyzer

The BD FACSArray™ bioanalyzer aspirates cell samples from a 96-well plate during flow cytometric analysis; the maximum sample volume that can be aspirated at one time is limited to approximately 100 µL per well. Additional volume should be added to the wells to allow for instrument setup.

It is important to prepare the cell samples according to the above procedure so that the wells are correctly laid out in the plate for easy acquisition by the BD FACSArray bioanalyzer.

Before setting up the controls and instrument optimization for spillover (compensation), create a new experiment through the Experiment Wizard.

Experiment Wizard

Figure 1. Experiment Wizard


Figure 2

Figure 3

Creating an Experiment using the Wizard

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 approximately 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 provided for cellular applications. The templates contain default loader and instrument settings that serve as a starting point for sample setup and optimization for an immunophenotyping 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*).
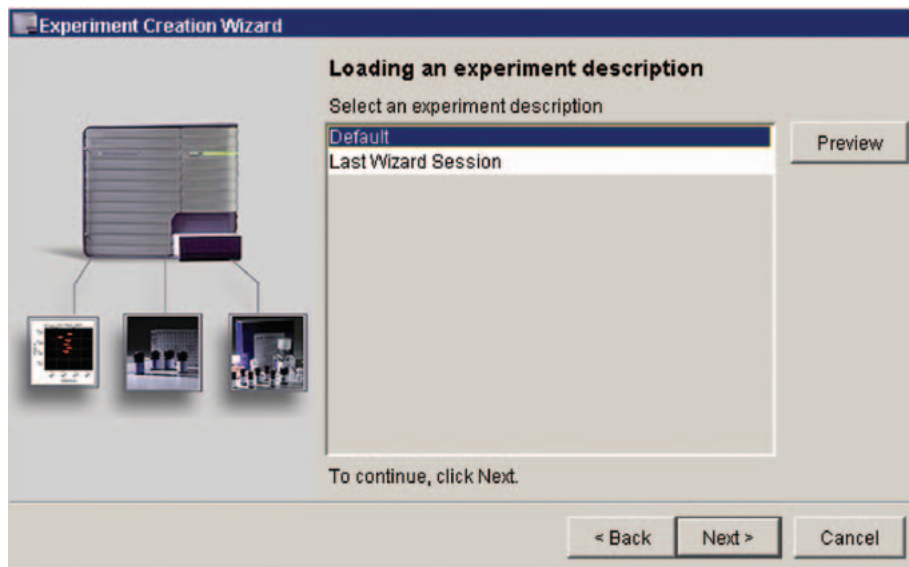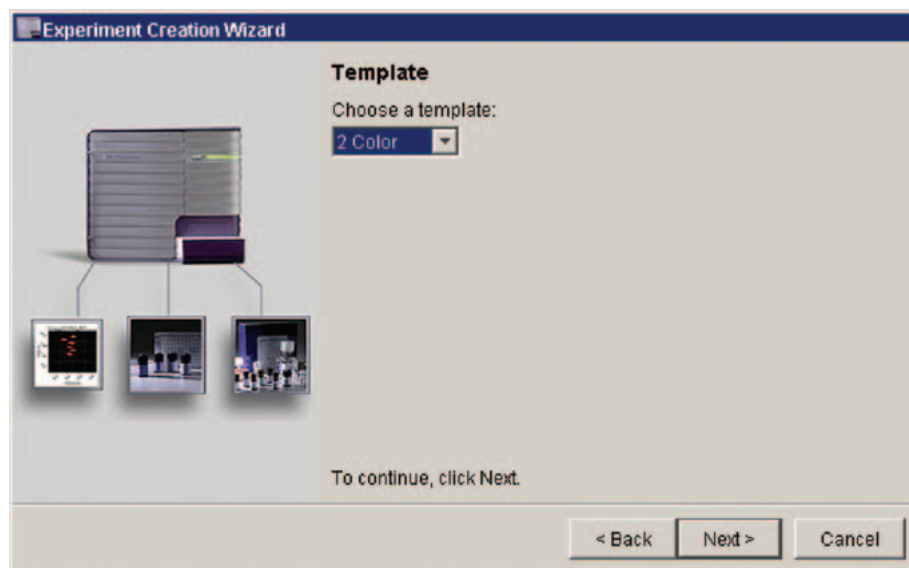
Entering Sample Information

For cellular experiments, you must add setup wells if you want the software to automatically calculate optical spillover. 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 checkboxes.

2. Click in each checkbox for the colors to be used.

Note: Click Yellow for PI and Red for Phospho Histone-Alexa Fluor® 647

Note: When you choose to add setup wells to your experiment, an unstained well is also added. The unstained well is run before the other setup wells. For this 2-color experiment, a total of three setup wells will be added.

3. Click Next.

   The Number of Samples view appears.

4. Enter the number 1 for the sample in A4 that will be analyzed for this experiment; click Next.

   • The Sample Identification view appears.

5. Leave the default the sample name, Sample_001, for each sample in the Sample Identification view.

Entering Well Information

1. Click Next.

   • The Number of Wells per Sample view appears.

2. Leave the default 1 well per sample; click Next.

   • The Well Names View appears (*Figure 5*).

3. To identify the investigation well, enter the name of the antibodies that are in this well.

   • In this example, enter Cell-Cycle as the name for Well 1.

   • To enter text in the text field:

   • Double-click in the white area of the text field to highlight the existing entry.

   • Type 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 labels will appear as axes labels in the plots displayed in the Template view.
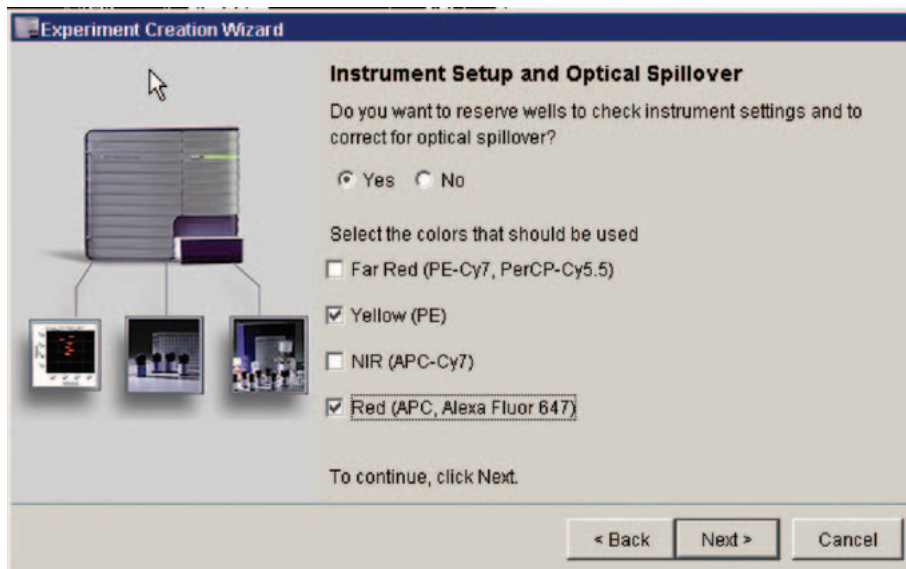
Figure 4
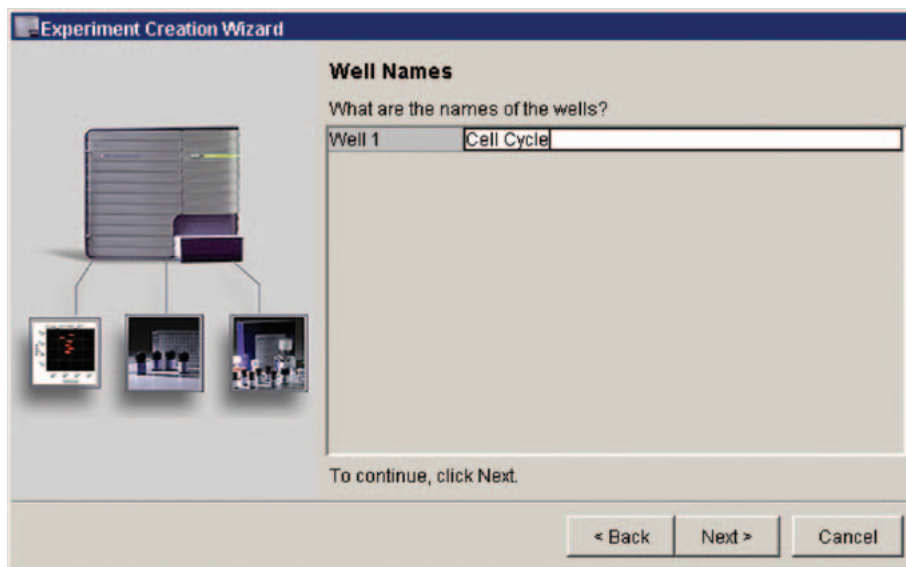
Figure 5
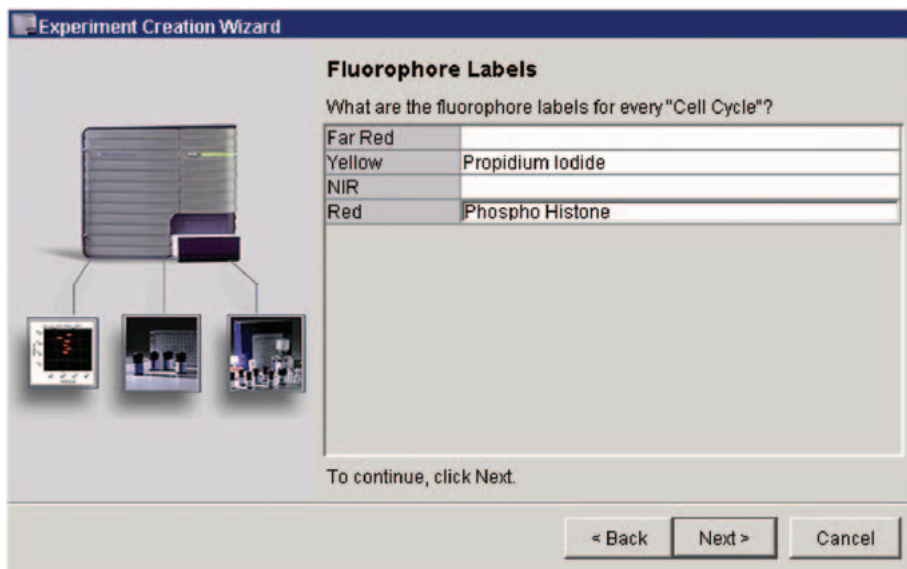
Figure 6

Figure 7

Figure 8

5. Enter the following names for the Yellow and Red parameters:
   - Propidium Iodide (for Yellow)
   - Phospho Histone (for Red)

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.
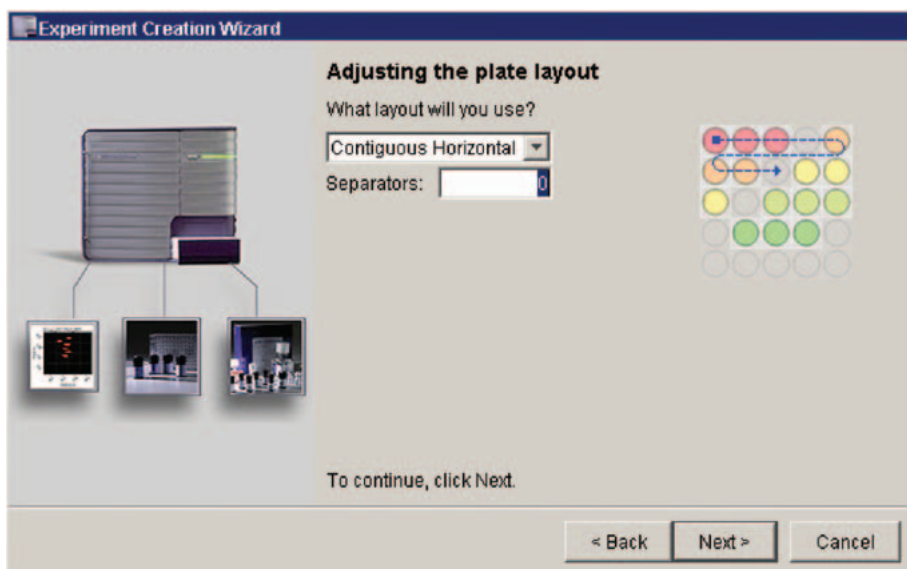
Specifying Loader and Acquisition Settings

1. Click Next.
   - The Loader Settings view appears (*Figure 8*).

2. Change the Sample Flow Rate from 2.0 to 0.5 µL/sec and leave the remaining settings as default; click Next.
   - It is important to use the lowest flow rate when acquiring cell-cycle samples to achieve the most accurate measurement.
   - The Acquisition Settings view appears, (*Figure 9*) where you can choose how many events per well to acquire and whether to use a stopping gate.
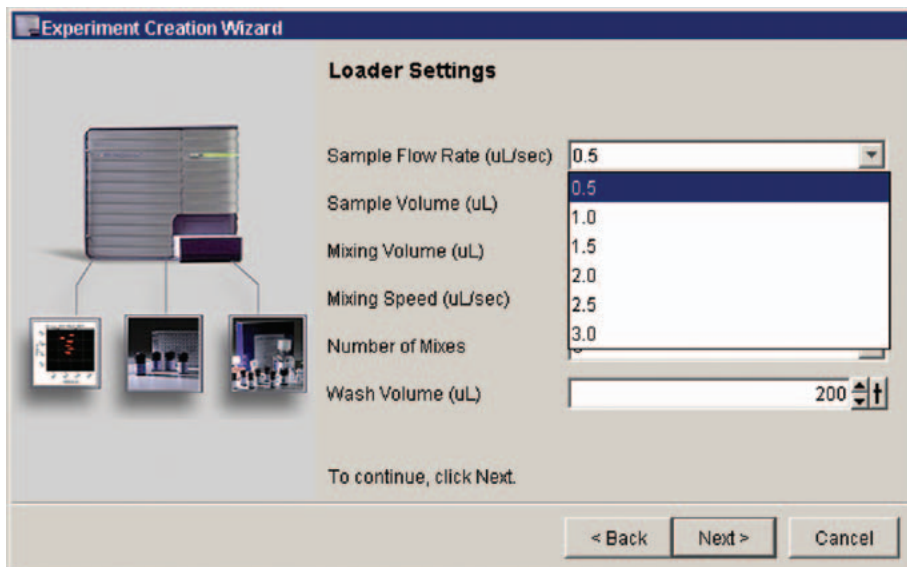
3. Choose the acquisition settings.

- Click the arrow next to the Events To Acquire field and choose 20,000 events from the drop-down menu.

Note: When analyzing cell-cycling data files in a cell-cycle software, like Verity's ModFit *LT™* software, it is preferable to save a data file with at least 10,000 events excluding debris and aggregates. Customize the events to acquire for your experiment

- Choose All Events as the stopping rule.

4. Click Next.

- The Experiment Name view appears (*Figure 10*).

Saving Experiment and Wizard Session

1. Type in Cell-Cycle Phospho Histone 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. You may modify these settings as needed at a later date.

3. Click the Yes radio button and enter the name Cell-Cycle Phospho Histone Wizard for this wizard session; click Next.

- The Completing the Experiment Wizard summary view appears (*Figure 11*).

4. Review the choices that were made.

5. Click Finish.

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

TIP: Go to the application toolbar and select "Reset Positions" under the View menu to see the workspace.
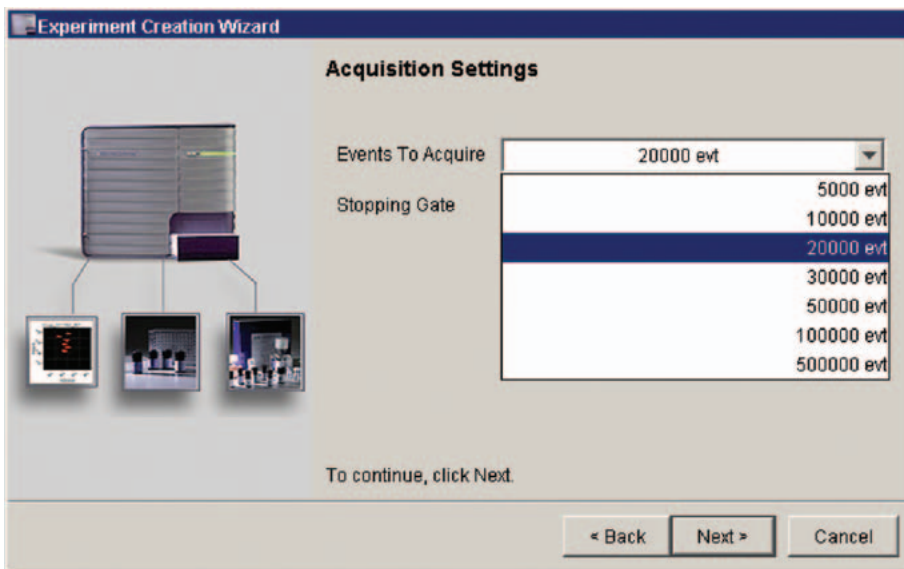
Figure 9
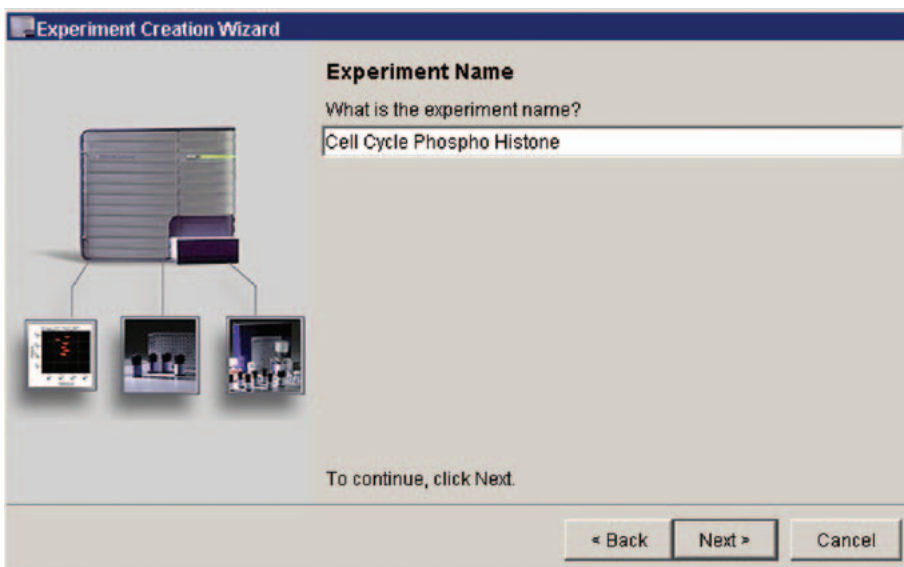
Figure 10
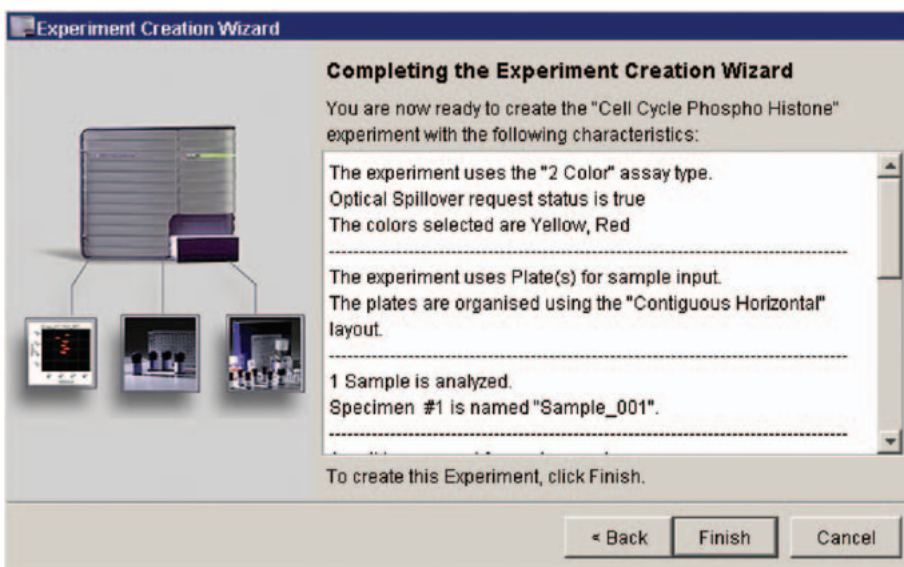
Figure 11
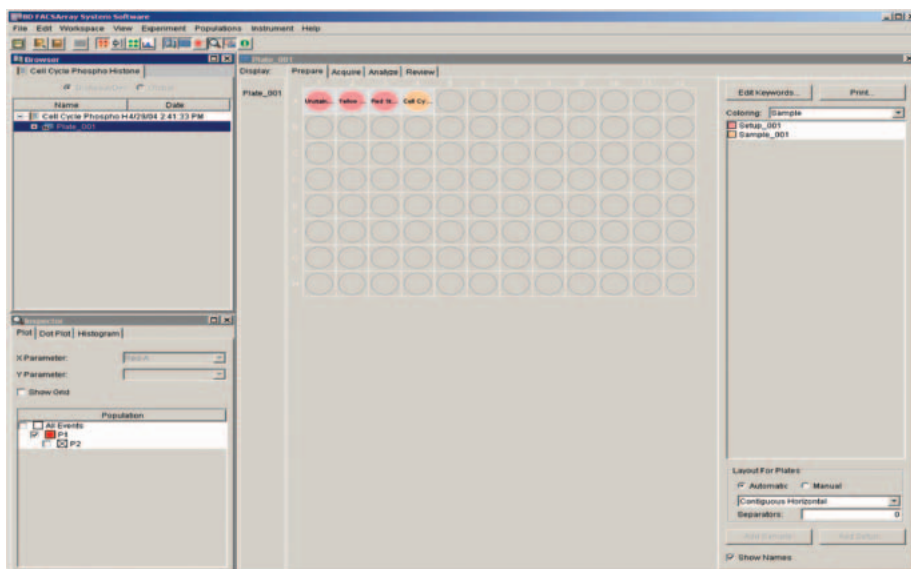
Figure 12

Review the Experiment in the Prepare Workspace

1. In the Prepare workspace (*Figure 12*), review your plate layout. Click the Prepare tool ( ) in the tool bar to choose the Prepare workspace.

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 to calculate automatic optical spillover correction by the software.

2. Click well A1.

3. In the Inspector, choose the Acq. Tab, and then increase the sample volume from 20 µL to 100 µL (the maximum value) by typing 100; press Enter.

   • 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 steps 2 – 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

TIP: Use a printout of the plate view as a guide for transferring prepared samples to the 96-well plate.

1. Print the plate view of the experiment by clicking the Print button in the upper right corner of the Prepare workspace.

2. Ensure that there is approximately 200 µL of the prepared samples in each of the following well.

   • Unstained sample in well A1

   • PI stained sample in well A2

   • Phospho Histone-Alexa Fluor® 647 stained sample in well A3

   • Investigation sample in well A4

Loading the Plate

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

Setup Tool
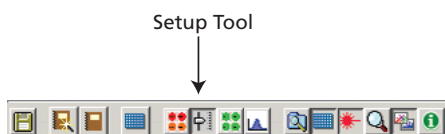
   • The wells you selected for acquisition appear in the plate view, and the setup wells are numbered in the acquisition order.

2. Place the plate containing the stained samples on the plate holder.

   • Position the plate so that well A1 is over the A1 mark on the plate holder.

3. Click the Load button in the Plate Control group.
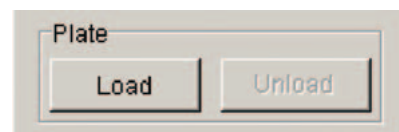
   • If no lid is detected on the plate, the plate is completely retracted into the bioanalyzer.

   • 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 your 2-color cellular sample.

   • Adjust Forward Scatter (FCS), Side Scatter (SSC), and threshold.

   • Gate the population of interest.

   • Adjust parameter voltages.

   • Save data for all the setup wells.

   • Calculate optical spillover.

Optimizing FCS, 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 (*Figure 13*).

2. Click the Setup button in the Acquisition Control group.

   • Events appear in the plots, but data is not being saved.

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.
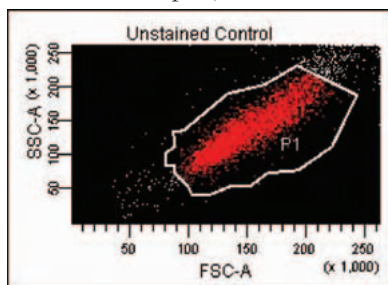
   • Adjust the signal for events displayed in plots by changing voltage settings.

1. Click the Parameters tab in the Instrument frame to display the Parameters pane. (*Figure 14*)

2. 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.

3. Press Enter on your computer keyboard to save the changes once the appropriate scatter profile is achieved.

4. 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 15*).

   • 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 Set Up button to stop acquisition of sample from the A1 well.

TIP: If it is difficult to find the population of interest in the PI histogram, the threshold parameter can be changed to the Yellow parameter. This will allow for the PI stained sample to be more easily visible.
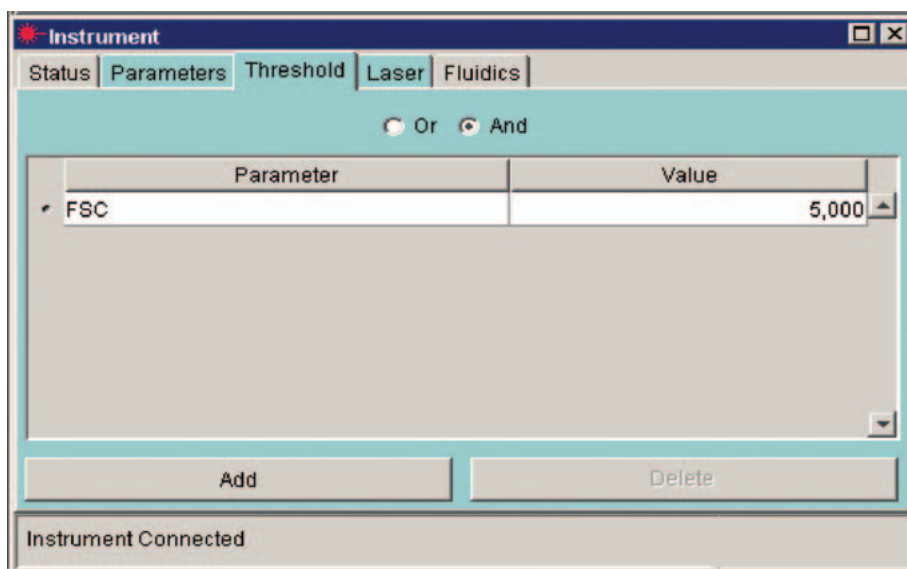

Figure 13
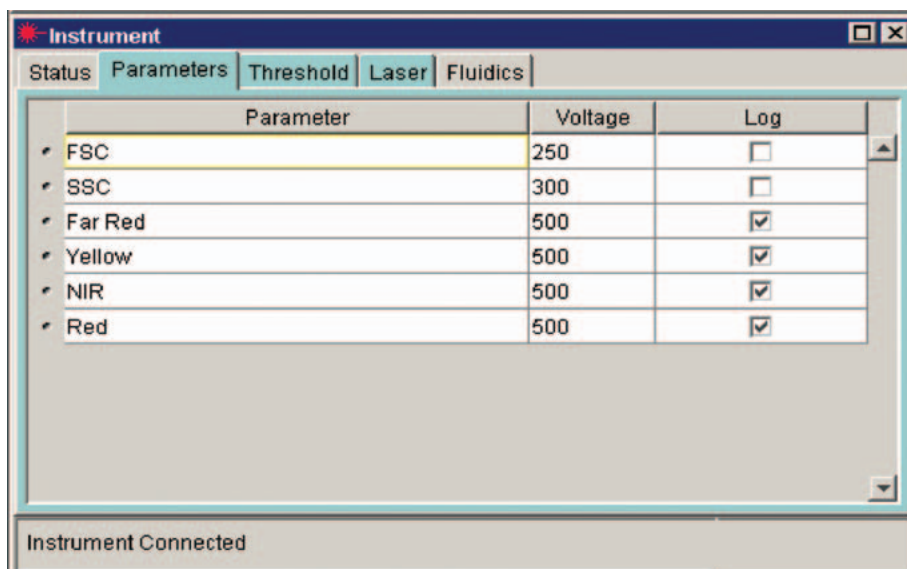
Figure 14

Figure 15

Gating the Population of Interest

The next step is to adjust the gate to surround the cells of interest, while excluding debris. This ensures that only the cellular population is viewed while optimizing fluorescent settings. You may 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.
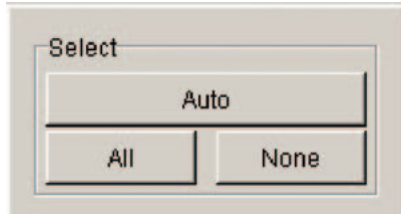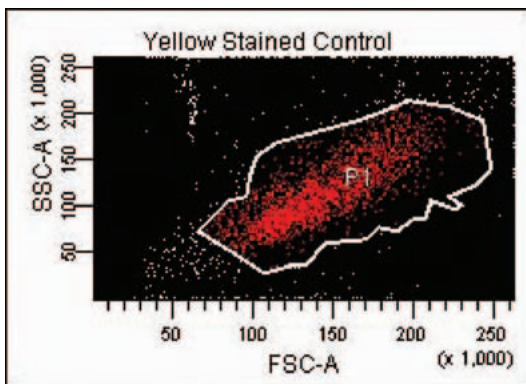

2. Click the Setup button in the Acquisition Control group.
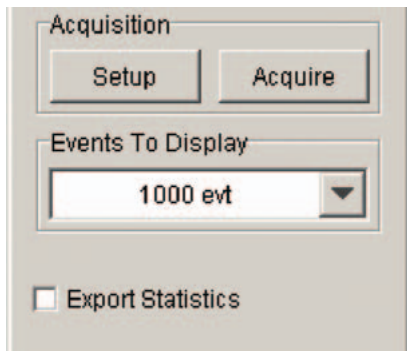
- Events from well A2 appear in the plots, but data is not being saved.

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

    Note: The histogram plot displays only gated events.

Optimizing Fluorescence Settings

For cell-cycle experiments the goal for optimizing fluorescence settings for the DNA is different from the goal for optimizing fluorescence settings for intracellular, intranuclear, or cell-surface staining. For cell-cycle optimization, the DNA profile must be optimized to adjust the G0/G1 peak to appear around channel 50. Adjusting the G0/G1 population to channel 50 will allow for the ability to detect varying amounts of DNA that may be present in some samples.

For intracellular, intranuclear, or cell-surface staining, the goal is to ensure that the brightest stained cells do not appear off-scale while the dimly fluorescent cells remain on-scale. For optimum sensitivity, the brightest and dimmest populations should be as well separated as possible while keeping both on scale. For optimum resolution of dim populations, keep the mean channel of dimly stained (not unstained) populations above channel 300.

1. In the Parameters tab of the Instrument frame, deselect the Log checkbox for Yellow.

    - The Yellow parameter will change to a Linear display.

    - DNA measurements must be displayed on linear scale in order to be able to clearly distinguish between the cell-cycle phases. This will allow for the PI stained cells, measured in the Yellow parameter, to be displayed on linear scale.

2. While observing the DNA histogram, adjust the Yellow voltage if necessary to place the G0/G1 population to around channel 50.
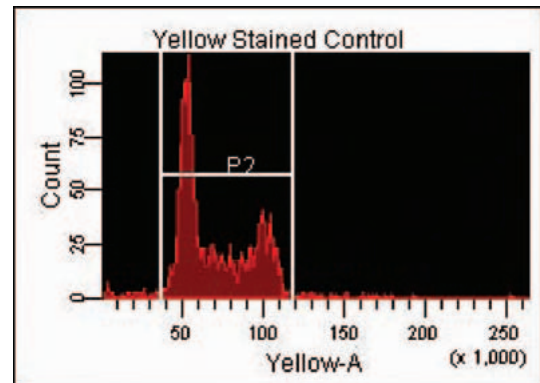
    Note: You do not need to adjust the interval gate at this time.

3. Click Setup to stop acquisition.

4. In the Select Control group, click None to deselect the wells, and then click A3 to view the Phospho Histone setup well.

5. Click the Setup button in the Acquisition Control group.

    - Events from well A3 appear in the plots, but data is not being saved.

6. While observing the Red histogram, perform the following:

    - Adjust the Red voltage if necessary to place the brightest population on scale.

    - Verify the dim fluorescent cells remain on scale.

7. Click Setup to stop acquisition (*Figure 16*).

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

Customizing the Loader Settings for Setup Wells

You have adjusted the instrument setting for the cellular experiment. Before acquiring the setup wells, you will 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.
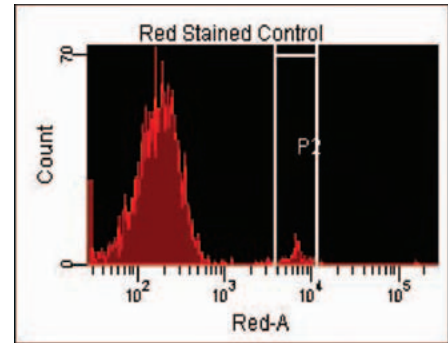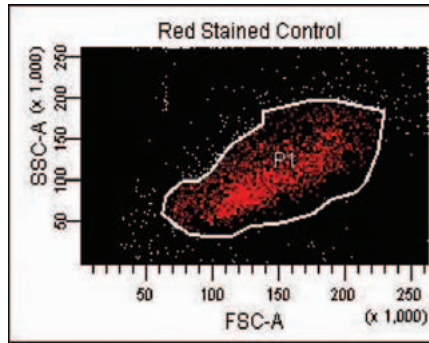
Figure 16

1. Click the Prepare tool () in the application toolbar to open the Prepare workspace.

2. Click well A1; change the sample volume in the inspector 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.

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.

   • Data will be saved for all three wells. After the last setup well is saved, the software automatically calculates and applies optical spillover correction.

   • If optical spillover correction was successfully calculated, the following message appears.
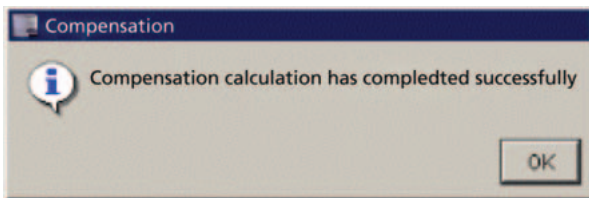
Ensuring Gates Were Properly Set

1. Click the Analyze tab in the Plate Editor.
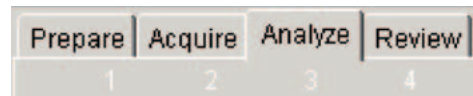
2. Click the first well in the plate view and then examine the plots in the Template view.

3. If needed, perform the following:

   • Click on the gate (P1) in the FCS vs SSC plot and drag it to the cell population while gating out debris.

   • Adjust interval markers in each histogram to enclose the negative population.
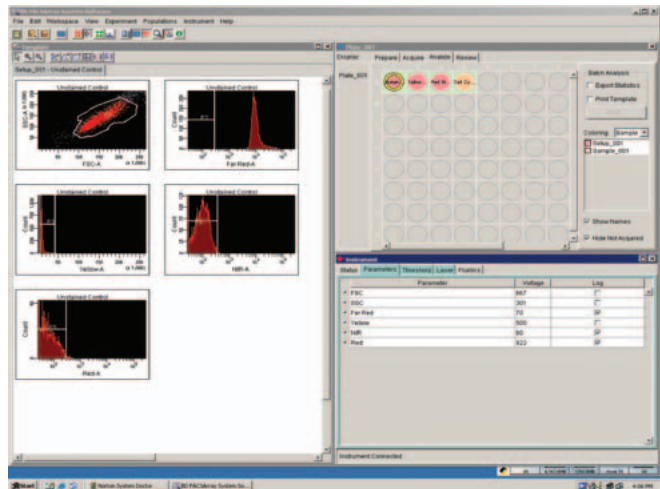
4. Click in each of the remaining setup wells and verify:

   • The gate in the FCS vs SSC plot surrounds the cell population.

   • Interval gates enclose the positive population in each fluorescence parameter
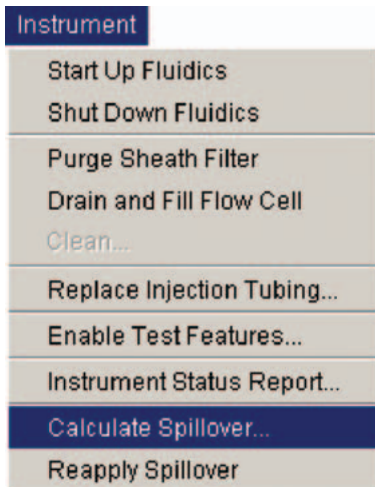
- For the PI positive well, adjust the Interval gate to encompass the entire cell-cycle population.
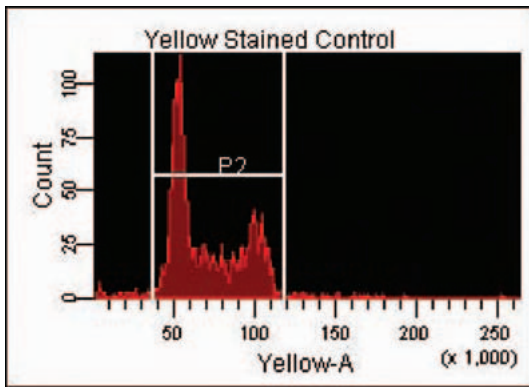
- For the Red positive sample, adjust the Interval to be around the positive cells.
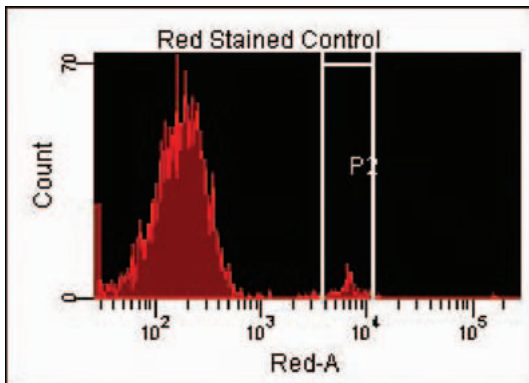
5. If you adjusted any of the gates in this section, choose Instrument > Calculate Spillover to recalculate the optical spillover.


6. Click the Acquire tab in the Plate Editor.

7. Click the Save button ( ) to save your work.
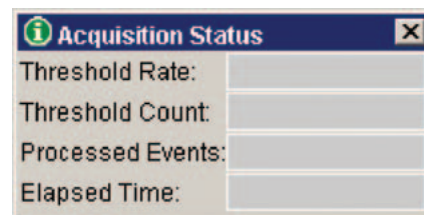
Data Acquisition

1. In the Select Control group, click the Auto button.

   - The remaining sample well (A4) will become selected and will be labeled as number one. The number in each well indicates the run order.

2. Click the Acquire tool in the application toolbar to open the Acquire workspace.

3. Click the Acquire button in the Acquisition Control group.
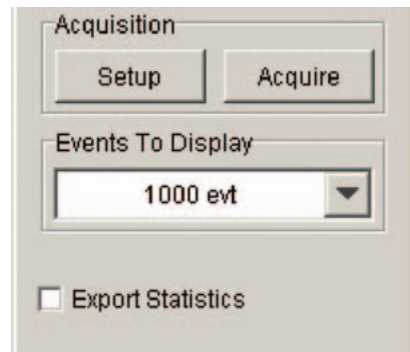
- After a short pause, the following occurs:

   - An orange ring appears in the well selected for acquisition, indicating that sample is being acquired.

   - Events appear in the plots.

   - Data is saved.

4. Click the Acquisition Status tool ( ) in the application workspace to open the Acquisition Status frame.

5. In the Acquisition Status frame, monitor the event rate to ensure that events are being detected and acquired.

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

7. Remove the plate from the Plate Holder. This completes sample acquisition.

Data Analysis Using BD FACSArray™ Software

BD FACSArray™ software can perform cell-cycle analysis estimating the percentage of cells in each cell-cycle compartment. A more accurate cell-cycle analysis should be performed with a cell-cycle modeling software, such as ModFit *LT*™ software. Cell-cycle software uses algorithms that correctly model the three cell-cycle compartments and is able to analyze the overlap that occurs between the G0/G1, S, and G2/M phases. In addition, cell-cycle modeling software provides a more accurate analysis by subtracting out underlying debris and aggregate cells that may interfere with the measurement of the cell-cycle compartments, thus determining an accurate percentages of cells in each cell-cycle phase.

Use the analysis features in BD FACSArray software to create an estimate of the percentages in each cell-cycle compartment as a starting point for your analysis. Perform further analysis in a third-party cell-cycle modeling software for a more complete analysis.

Create a Gate

1. Click on the Analyze tool ( ) in the application toolbar to open the Analysis workspace (*Figure 17*).

2. Display data from the sample well by clicking on well A4.

   • In the template, the plots display the stored data from the selected sample well.

3. Click on the Snap-To Auto polygon gate in the template toolbar.

   Snap-To

4. Click on the cell population in the FCS vs. SSC plot.

   • A Snap-To gate will be automatically created around the population. This gate will be used to gate out the debris.
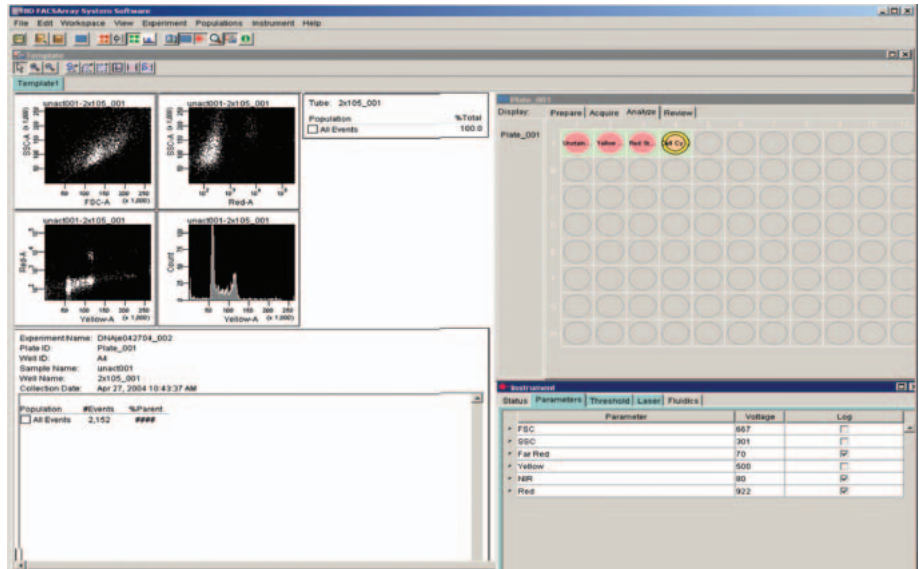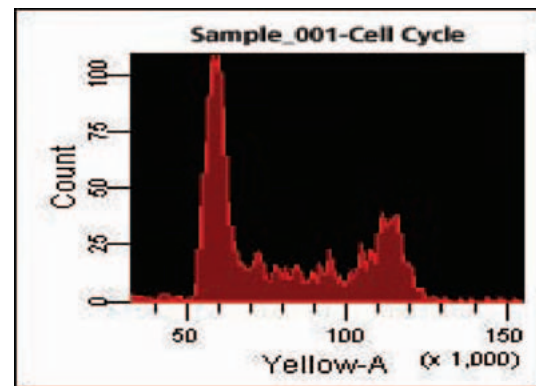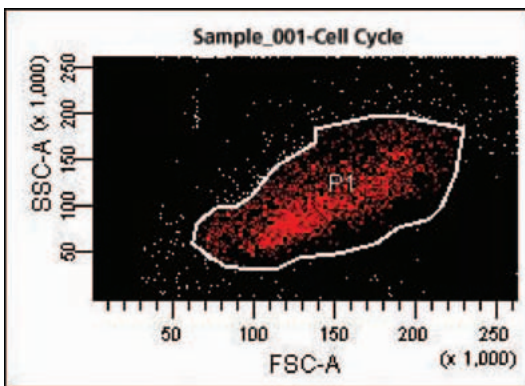
Figure 17

TIP: The P1 gate can be adjusted or moved if necessary. To adjust the position of the gate, click and hold on the gate and drag it to the new position. To adjust the shape of the gate click on the gate first, then click, hold then drag the vertex point you want to move.

5. To display only gated data in the fluorescent plots perform the following:

   • To select multiple plots, click on the border of the plots while holding down the Shift key.

   • Right-click on the border of one of the selected plots.

   • Choose Show Populations, and then select P1 from the menu that appears.

   • Only the cells that are in gate P1 will now appear in the fluorescence plots.

6. Zoom in on the cell-cycle histogram to more easily identify the cell-cycle compartment.

   • Click on the magnifier glass button ( ) to zoom in on the cell-cycle population displayed in the Yellow parameter.

   • Click and drag across the population in the Yellow parameter to enlarge the view.

7. Create three interval gates on the three cell-cycle compartments: G0/G1 peak, S-phase, and G2/M peak.

   • In the template toolbar, click on the interval gate tool ( ) to select an interval gate.

   • Click and drag the gate across the G0/G1 population in the Yellow parameter.

   • Repeat the steps for creating the interval gate (P3) to surround the S-phase cells.

   • Repeat the steps for creating the interval gate (P4) to surround the G2/M population.
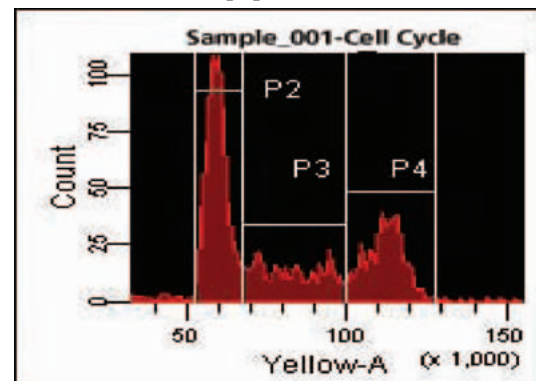
• The statistics for each population will be displayed in the statistics view.

8. Analyze the Phospho Histone positive cells to determine the percentage of cells in the M-phase of the cell-cycle.

   • Click on the rectangle button( ) to select it.

   • Click and drag the rectangle around the Phospho Histone positive population.

Sample_001-Cell Cycle
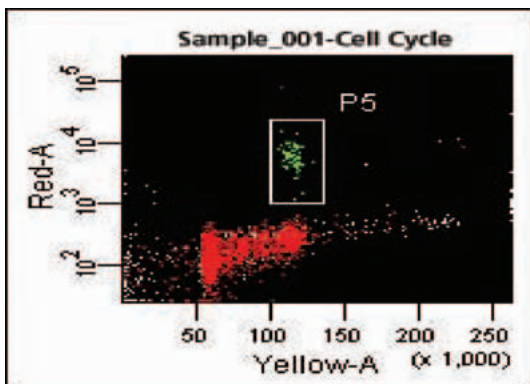
   • The statistics for P5 will be displayed in the statistics view.

9. Review the statistics to get an estimate of the percentage of cells in each cell-cycle compartment.

   • The PI histogram determines the percentage of cells in the G0/G1, S, and G2/M phases of the cell-cycle.

   • The Phospho Histone positive cells determine the percentage of cells in the M-phase only.
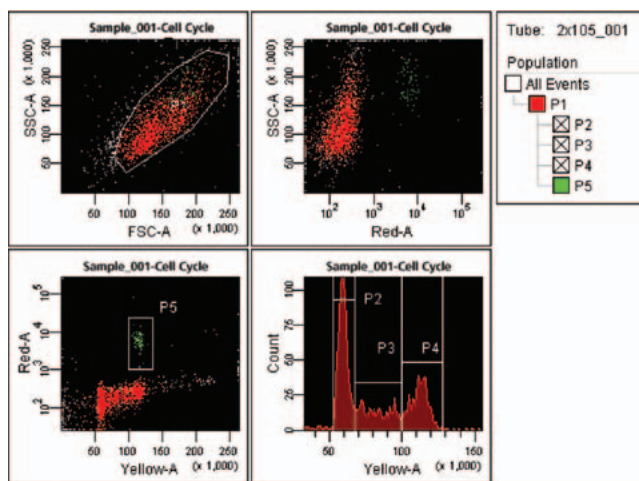
Population	#Events	%Parent
All Events	2,152	####
P1	1,840	85.5
P2	836	45.4
P3	409	22.2
P4	505	27.4
P5	59	3.2

Estimated percentages of the cell-cycle compartments are determined by BD FACSArray™ software as follows:

   • P2 estimates the G0/G1 percentage

   • P3 estimates the S-Phase percentage

   • P4 estimates the G2/M percentage

   • P5 estimates the phospho histone M-phase percentage

These percentages are only estimate the cell-cycle phases; they are based on the interval gates that were set in the histogram. Since the cell-cycle phases overlap, using interval gates does not accurately determine the percentage of cells in each cycle.

Cell-Cycle Analysis Using ModFit *LT* Software

Cell-cycle analysis is more accurately performed by a cell-cycle modeling software by applying specific models to the cell-cycle phases to account for the overlap in the phases. Data files must be exported from BD FACSArray software to be analyzed in a third-party cell-cycle analysis software. For more detailed information on ModFit *LT* software, refer to the ModFit *LT* user guide.

Exporting Data from BD FACSArray™ Software

1. In the BD FACSArray™ software, click once on your cell-cycle experiment icon in the browser to select the experiment that contains the data files to be exported. (Do not double-click to open the experiment).

   • If you performed the experiment described in this Application Note, click on the Cell Cycle Phospho Histone Experiment icon in the Browser.

2. Go to the File menu and choose Export -> FCS.
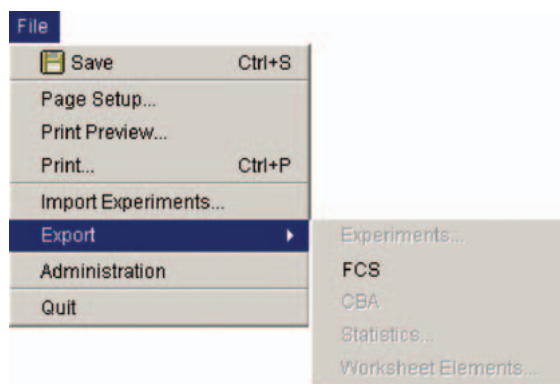
3. Choose the FCS files to be exported as FCS 2.0 files.
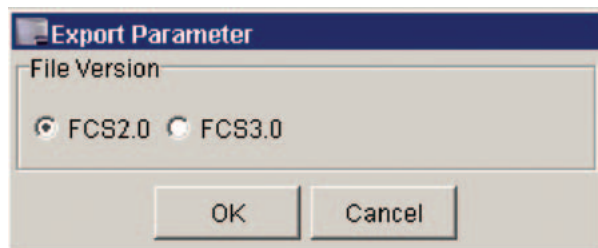
   • The files will be exported to the D:\BDExport\FCS folder

ModFit *LT*™ Software

1. Open the ModFit *LT*™ software application.

2. Log in as a standard user.

3. Open a file by clicking the File button or by using the pull-down menu under File.

4. Navigate to the D:\BDExport\ FCS folder to locate the cell-cycle data file that was exported from the BD FACSArray™ software.

5. Click on the FCS data file and click Open.

   • The Choose Parameter For Analysis dialog appears.

6. Choose Yellow-Area as the parameter for analysis.

   • The Define Gates dialog appears.

7. Leave the gates unselected and click OK.

   • Verity recommends allowing ModFit *LT* software to automatically detect the debris and aggregates, thus creating a gate is not necessary.

8. Click on the Choose Model (**Mod**) button from the tool bar or using the pull down menu under analysis

9. Set the properties for Manual Analysis.

   • Go to Edit-> Properties for Manual Analysis

   • Change the number of cycles to 1 to create a cell-cycle analysis for the diploid sample that was run.

   • Leave the AutoDebris and AutoAggregates features enabled to allow for the software to automatically model and subtract out the debris and aggregates.

   • Click OK.

   • The software will automatically place the model ranges over the detected peaks.

   • Adjust the ranges for the G1 peak, S-phase, or the G2 peak only if needed.

10. Click the Fit Data button (**Fit**) from the toolbar to perform cell-cycle analysis.

    • The software will automatically model the peaks under the model ranges for the G1 peak, S-phase, and the G2 peak.

    • ModFit *LT* displays the results of the cell-cycle analysis in a report (*Figure 18*).

Tip

Extra setup wells:

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

1. Click to select the Prepare Workspace in the application toolbar.

2. In the Layout for Plates field of the plate editor, click the Manual button.

3. Click to select the wells in the plate. Use the Shift key for multiple selections.

4. Click Add Sample or Add Setup.

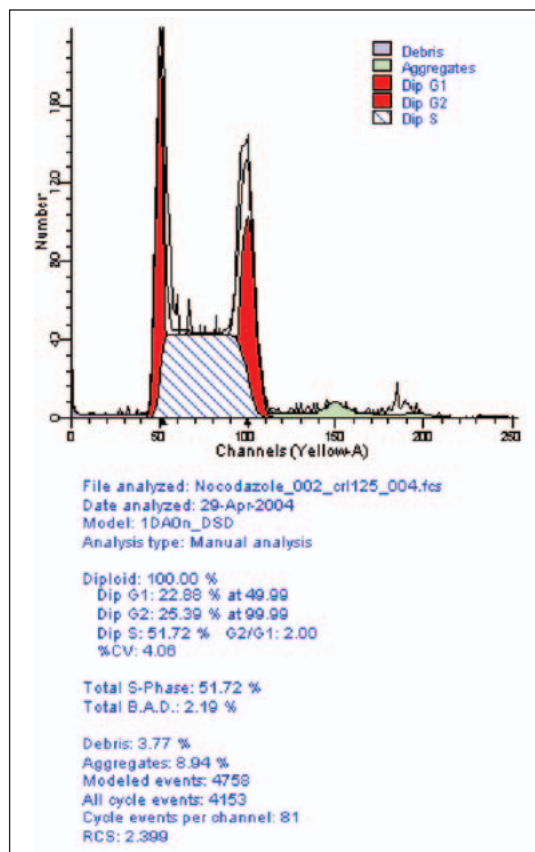   • Repeat steps 3 – 4 for all the samples that you are adding.

Figure 18

References

1. Cell organization, subcellular structure and cell division. 1995. In *Molecular Cell Biology. Third Edition*. H.Lodish, D. Baltimore, A. Berk, S. L. Zipursky, P. Matsudaira, and J. Darnell, eds. W.H. Freeman and Company, New York, pp. 141-188.

2. Noguchi, P. 1991. Use of flow cytometry for DNA analysis. In *Current Protocols in Immunology*. J. Coligan, A. Kruisbeek, D. Margulies, E. Shevach, and W. Strober, eds. Green Publishing Associates and Wilesy-Interscience, New York. Section 5.7.1-5.7.4.

3. Darzynkiewicz, Z., F. Traganos, T. Sharpless, and M.R. Melamed. 1976. Lymphocyte stimulation: A rapid multiparameter analysis. *Proc. Natl. Acad. Sci. USA* 73:2881.

4. Juan,G., F. Traganos,. W. James, J. Ray, M. Roberge, D. Sauve, H. Anderson, and Z. Darzynkiewicz, 1997. Histone H3 Phosphorylation and Expression of Cyclins A and B1 measured in individual cells during their progression through G2 and Mitosis. *Cytometry* 32:71.

5. Goto, H., Y. Yasui, E. Nigg, and M. Inagaki. 2002. Aurora-B phosphorylates Histone H3 at Serine28 with regard to the mitotic chromosome condensation. *Genes to Cells* 7:11.

6. Goto,H., Y. Tomono, K. Ajiro, H. Kosako, M. Fujita, M. Sakurai, K. Okawa, A. Iwamatsu, T. Okigaki, T. Takahashi, and M. Inagaki. 1999. Identification of a Novel Phosphorylation Site on Histone H3 Coupled with Mitotic Chromosome Condensation. *J. Biol. Chem.* 274: 25543.

Contributors
Jeanne Elia, David Ernst, Dennis Sasaki

Notes

Notes

Notes

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

Europe
Belgium
Tel 32.53.720.211
Fax 32.53.720.450
contact_bdb@europe.bd.com

Japan
Nippon Becton Dickinson
Toll free 0120.8555.90
Tel 81.24.593.5405
Fax 81.24.593.5761
Clontech Products
Tel 81.3.5324.9609
Fax 81.3.5324.9637

United States
BD Biosciences
Customer/Technical Service
Toll free 877.232.8995
Clontech
Fax 650.354.0775
Discovery Labware
Fax 978.901.7493
Immunocytometry Systems
Fax 408.954.2347
Pharmingen
Fax 858.812.8888
www.bdbiosciences.com

Local Offices and Distributors

Argentina/Paraguay/Uruguay
Tel 54.11.4551.7100 x106
Fax 54.11.4551.7400

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 57.1.244.1363

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
Tel 852.2575.8668
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

Turkey
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

BD Biosciences

Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen