

BD FACSCalibur Operator Course Workbook



For In Vitro Diagnostic Use
Class I (1) Laser Product

bdbiosciences.com
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BD Biosciences
2350 Qume Drive
San Jose, CA 95131-1807
USA
Tel (877) 232-8995
Fax (800) 325-9637
facsservice@bd.com

Asia Pacific
Tel (65) 6861-0633
Fax (65) 6860-1593

Europe
Tel (32) 2 400 98 95
Fax (32) 2 401 70 94
help.biosciences@europe.bd.com

Brazil
Tel (55) 11-5185-9995/9941
Fax (55) 11-5185-9895
bdbiosciences@bd.com.br

Japan
Nippon Becton Dickinson Company, Ltd.
Toll Free 0120-8555-90
Tel 81-24-593-5405
Fax 81-24-593-5761

Canada
Toll Free (888) 259-0187
Tel (905) 542-8028
Fax (888) 229-9918
canada@bd.com

Mexico
Toll Free 01-800-236-2543
Tel (52) 55 5999 8296
Fax (52) 55 5999 8288

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FCC Information

WARNING Changes or modifications to this unit not expressly approved by the party responsible for compliance could void the user's authority to operate the equipment.

NOTICE This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to Part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, can cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his or her own expense.

Shielded cables must be used with this unit to ensure compliance with the Class A FCC limits.

This Class A digital apparatus meets all requirements of the Canadian Interference-Causing Equipment Regulations.

Cet appareil numérique de la classe A respecte toutes les exigences du Règlement sur le matériel brouilleur du Canada.

Limitations

Unless otherwise specified, applications described in this guide are for research use only, and are not for use in therapeutic or diagnostic procedures.

History

Revision	Date	Change Made
23-10509-00 Rev. A	11-14-08	Initial release

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About This Workbook

This workbook contains presentations, exercises, and worksheets used during the operator course for the BD FACSCalibur™ cytometer, and is for training purposes only.

Additional BD FACSCalibur Information

For more information about the cytometer and its software, as well as instructions for use, refer to:

- *BD FACSCalibur Flow Cytometer Reference Manual*
- *BD CellQuest Pro Software Reference Manual*
- *BD FACSComp Software Reference Manual*
- *BD MultiSet Software Reference Manual*

Conventions

Hazard Alerts

The following symbols and formats are used to alert you to a potential hazard.



General hazard or unsafe practice that could result in material damage, data loss, minor or severe injury, or death.



Laser radiation hazard or unsafe practice that could result in minor or severe injury, or death.



Biological hazard or unsafe practice that could result in minor or severe injury, or death.



Electrical danger or unsafe practice that could result in minor or severe injury, or death.

Text and Keyboard Conventions

Convention	Use
TIP	Highlights features or hints that can save time and prevent difficulties.
<i>Italics</i>	Italics are used to highlight book titles, to indicate text to be typed in a dialog, and to clarify some user interface labels.
>	The arrow indicates a menu choice. For example, “select File > Print” means to select Print from the File menu.
Ctrl+X	When used with key names, a plus symbol means to press two keys simultaneously. For example, Ctrl+p means to hold down the Control key while pressing the letter <i>p</i> .

Technical Assistance

For technical questions or assistance in solving a problem:

- Read the section of the workbook or user's guide specific to the operation you are performing.
- Refer to the troubleshooting section in the appropriate user's guide.

If additional assistance is required, contact your local BD Biosciences technical support representative or supplier.

When contacting BD Biosciences, have the following information available:

- Product name, part number, and serial number
- Any error messages
- Details of recent system performance

For instrument support from within the US, call (877) 232-8995.

For support from within Canada, call (888) 259-0187.

Customers outside the US and Canada, contact your local BD representative or distributor.

Limitations

For in vitro diagnostic (IVD) use when used with IVD reagents. Refer to the appropriate reagent package insert for application-specific limitations.

Course Objectives

After completing this course, you will be able to:

- Describe basic concepts in flow cytometry.
- Operate and maintain the cytometer, and troubleshoot basic instrument problems.
- Use BD FACSComp™ software to perform quality control on the cytometer.
- Acquire and analyze multicolor data using BD CellQuest™ Pro software.
- Organize data files with Mac OS X.

If applicable, you will be able to:

- Sort cells and analyze the sorted sample to determine sort purity.
- Use the BD FACS™ Loader option (with BD™ WorklistManager software and BD™ LoaderManager software) to acquire and analyze data.
- Use the BD™ High Throughput Sampler (HTS) option (with BD™ PlateManager software) to acquire and analyze data.
- Use BD Multiset™ software to acquire and analyze data.

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BD FACSCalibur Cytometer Overview and Operation

This chapter reviews general flow cytometry concepts and introduces concepts specific to your BD FACSCalibur™ cytometer.

After completing this chapter, you will be able to:

- Describe cytometer components.
- Describe the main cytometer systems and how each works.
- Perform cytometer startup and shutdown procedures.
- Log in to your BD FACStation™ computer system.

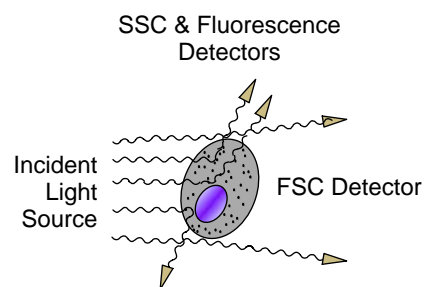


Introduction to the BD FACSCalibur Flow Cytometer



What Can a Flow Cytometer Tell Us About a Cell?

- Relative size:
Forward Scatter (FSC)
- Relative granularity or
internal complexity:
Side Scatter (SSC)
- Relative fluorescence
intensity



2

Cytometer Subsystems

Fluidics

To introduce and focus the cells for interrogation

Optics

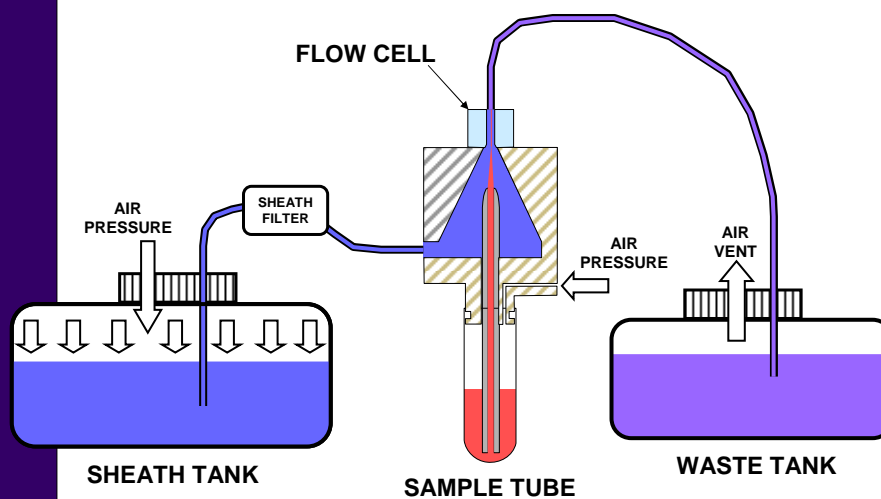
To generate and collect the light signals

Electronics

To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer

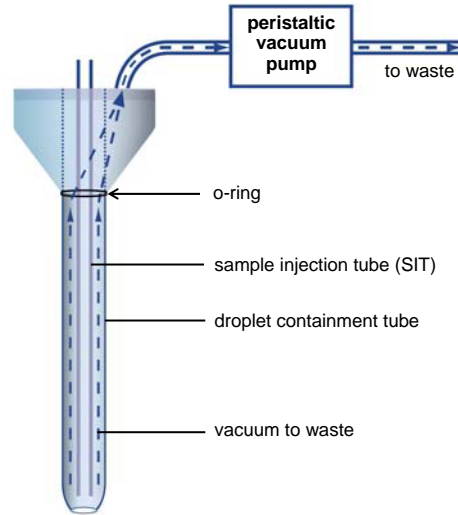
3

Basic Fluidics Principles



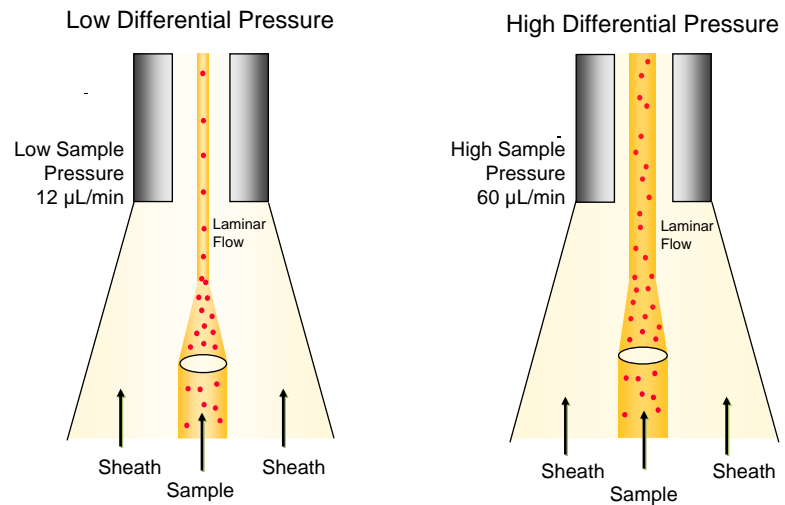
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Droplet Containment System



5

Sample Flow



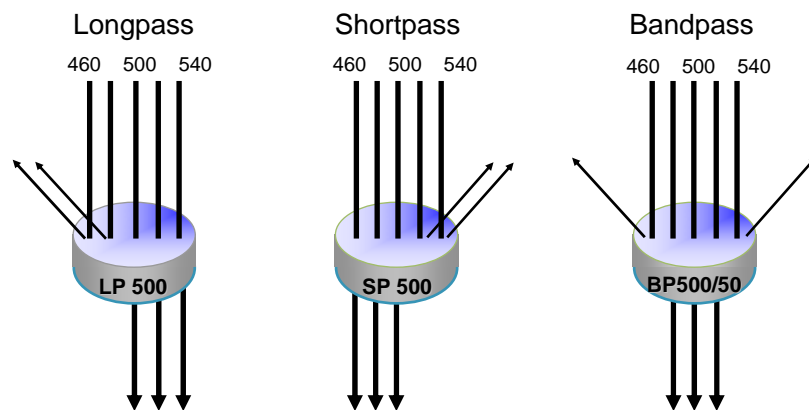
6

Optics

- **Excitation optics** consist of:
 - A laser
 - Lenses to shape and focus the laser beam
- **Collection optics** consist of:
 - A collection lens
 - A system of optical mirrors and filters
 - Optical detectors

7

Collection Optics: Filters



8



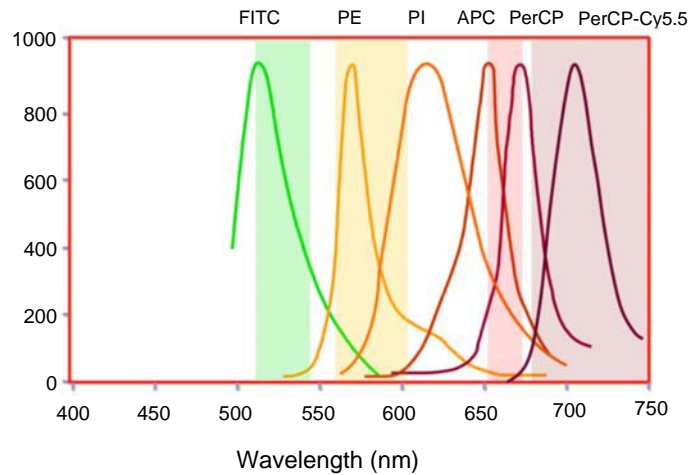
Collection Optics: Detectors

	<u>Detector</u>	<u>Filter</u>	<u>Color</u>	<u>Fluorochrome</u>
Blue Laser 488 nm	FL1	530/30 nm	Green	FITC
	FL2	585/42 nm	Yellow/Orange	PE
	FL3	670 nm LP	Dark Red	PerCP, PerCP-Cy5.5
Red Laser 635 nm	FL4	661/16 nm	Red	APC

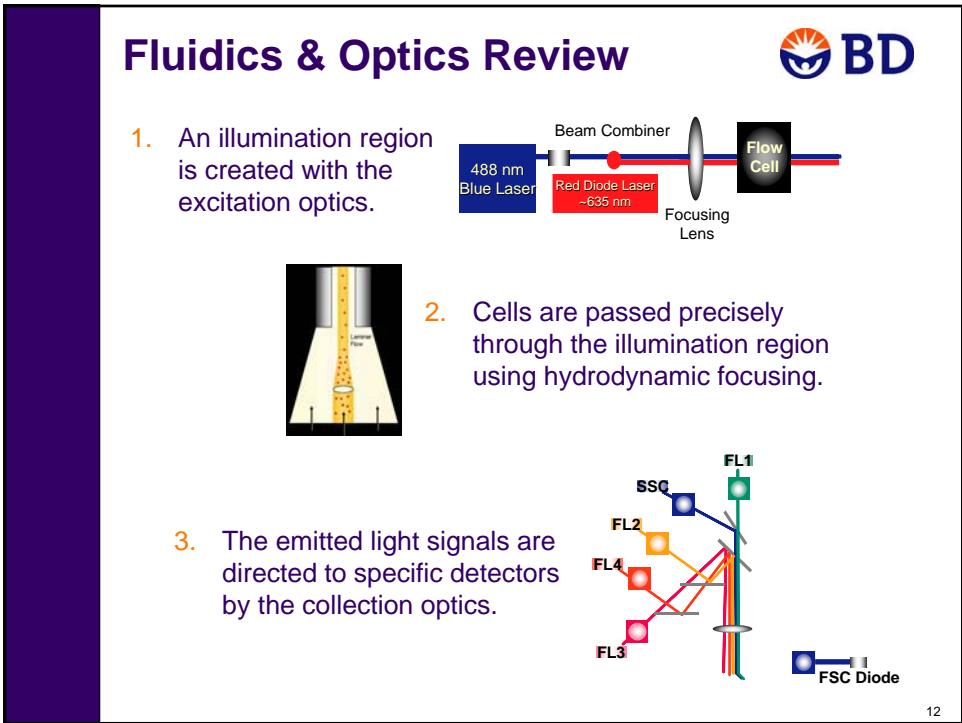
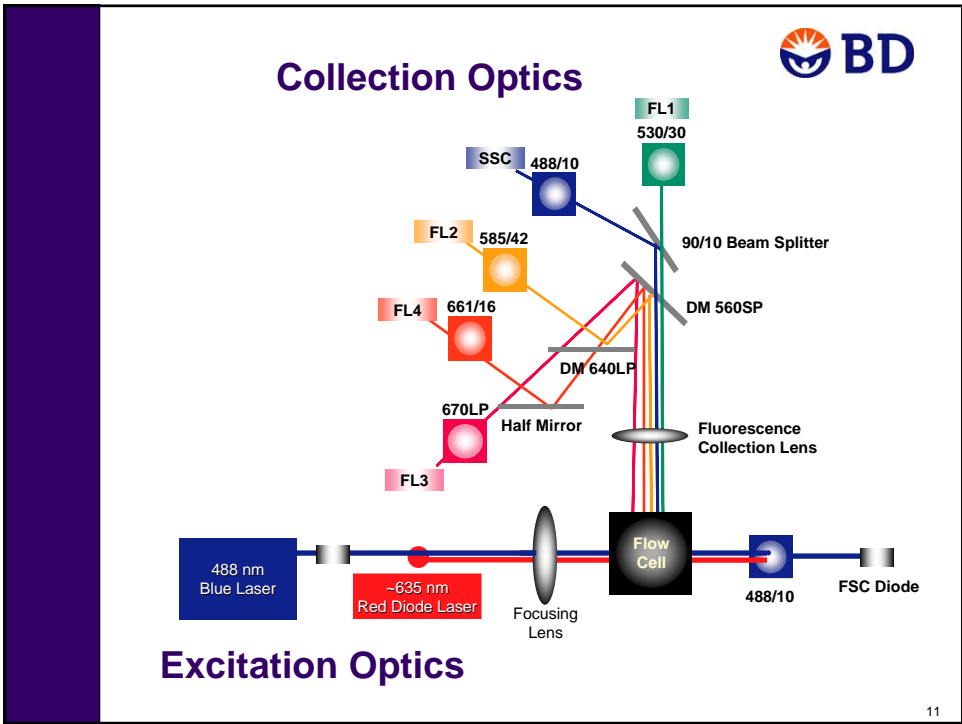
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Emission Spectra



10

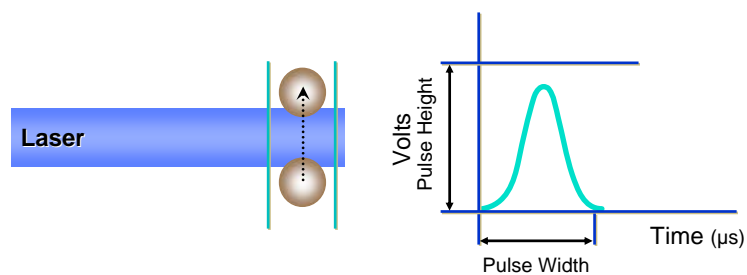


Electronics

- Optical signals are converted to electronic signals.
- Voltage pulse height, area, and width are analyzed.
- Analog signals are converted to digital signals.
- Data is transferred to the computer.

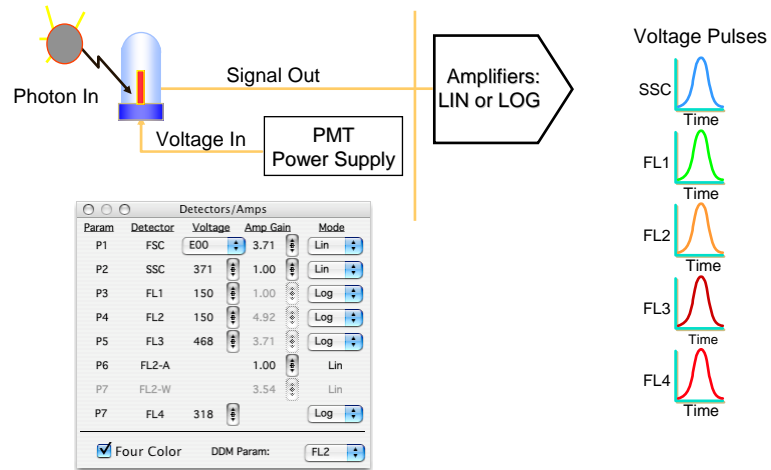
13

Creation of a Voltage Pulse



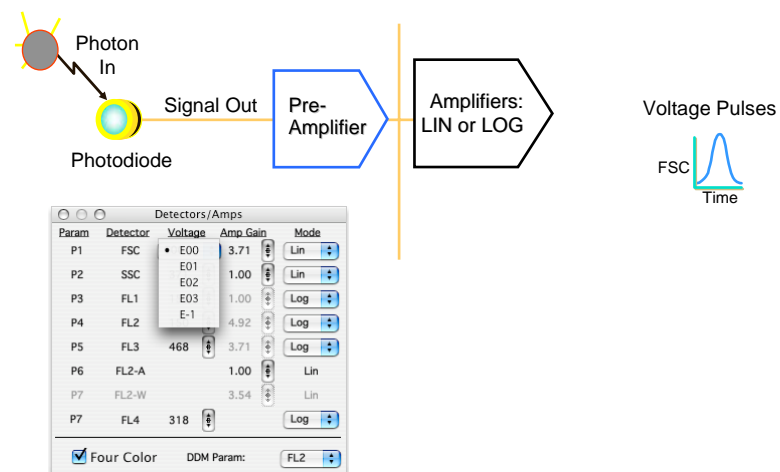
14

Conversion of Optical Signals to Proportional Electronic Signals



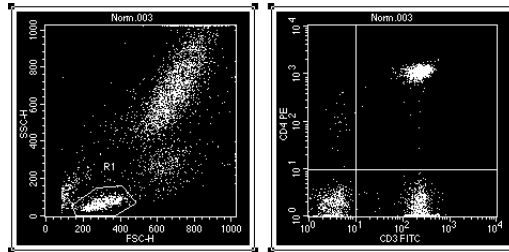
15

Conversion of Optical Signals to Proportional Electronic Signals



16

Linear v. Log Amplification

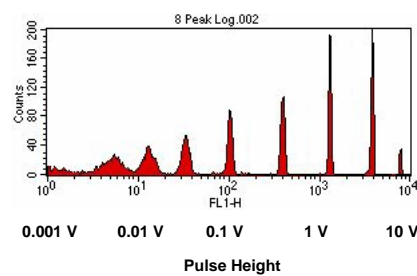
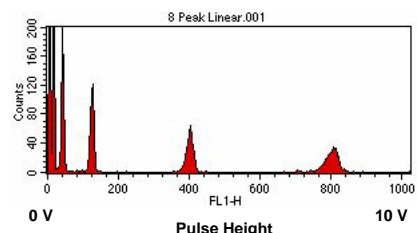
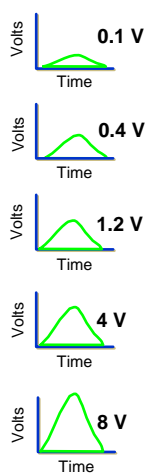


- **Linear** amplification is used for light scatter parameters. Linear mode amplifies signals 1.00 to 9.99 times.
- **Log** amplification is used for fluorescence signals with a large dynamic range. The log scale has a range of four decades.

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Pulse Height Analysis

FL1 Voltage Pulses

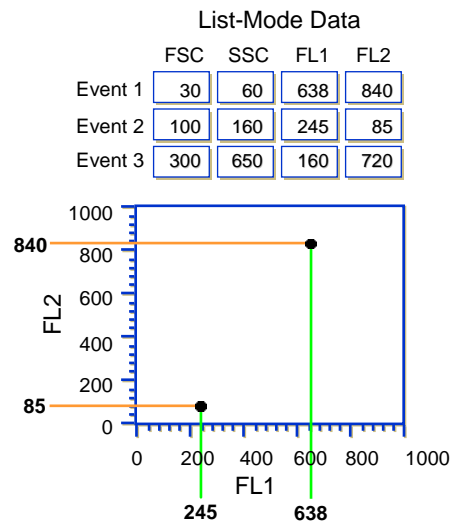


18



FCS Files

- Keywords
- Instrument Settings
- List-Mode Data



Review

- The flow cytometer can provide information about a particle's relative size, relative complexity, and relative fluorescence intensity.
- Particles are carried in a fluid stream to the flow cell where they scatter laser light and emit fluorescent light.
- Detectors produce electronic signals proportional to the optical signals striking them.
- Data is collected and processed on every event and then stored in the computer.

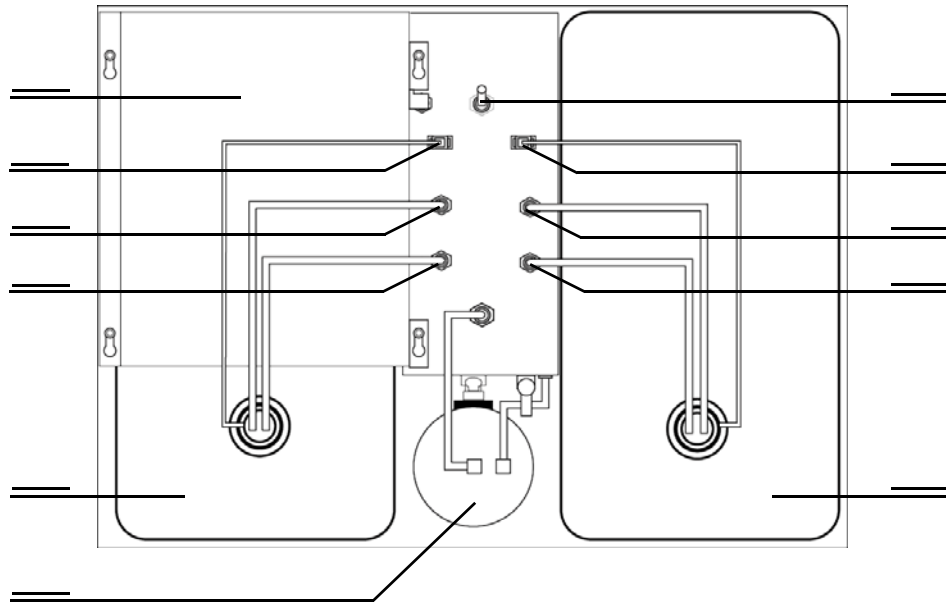
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Cytometer Components

Fluidics Drawer

Fill in the missing labels on the fluidics drawer illustration using the following options:

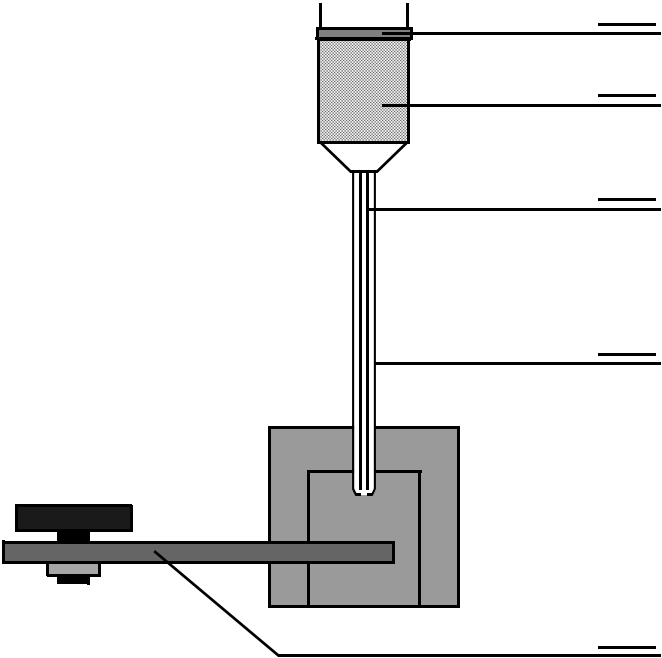
- | | |
|--|----------------------------------|
| A. Sheath filter | G. Sheath tank |
| B. Metal bracket | H. Waste probe sensor connector |
| C. Air connector (pressurize sheath) | I. Sheath connector |
| D. Toggle switch (vent valve) | J. Waste tank |
| E. Air connector (vent waste pressure) | K. Sheath probe sensor connector |
| F. Waste connector | |



Sample Injection Port (SIP)

Fill in the missing labels on the SIP illustration using the following options:

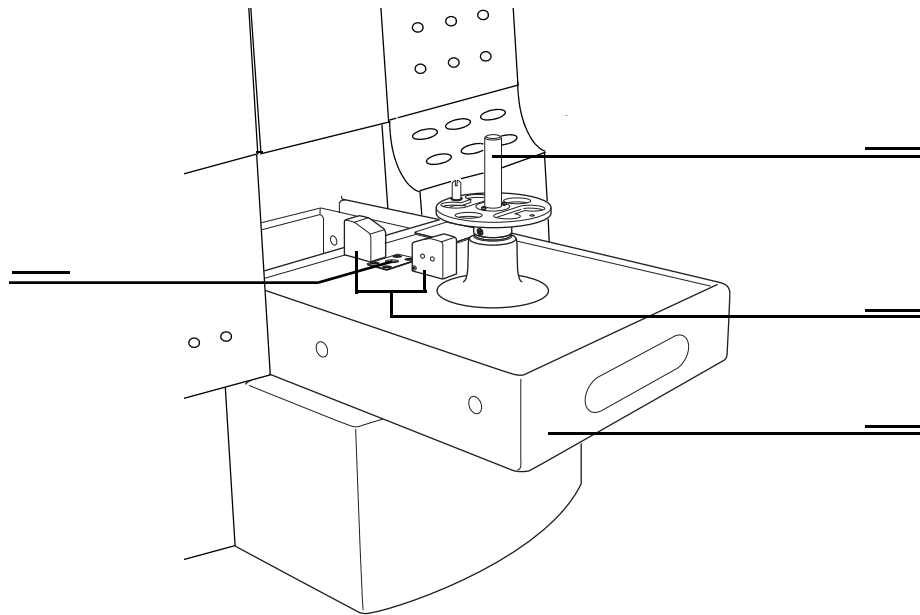
- A. Droplet containment sleeve
- B. Support arm
- C. Tube retainer
- D. Sample injection tube
- E. Bal seal



Loader

Fill in the missing labels on the Loader illustration using the following options:

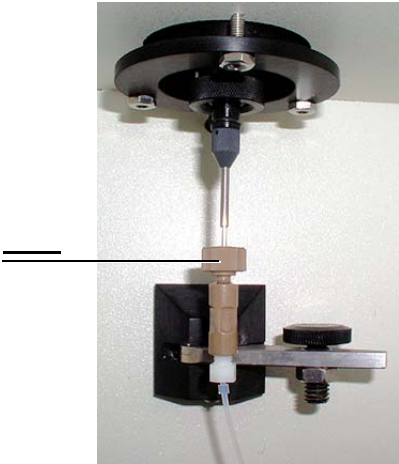
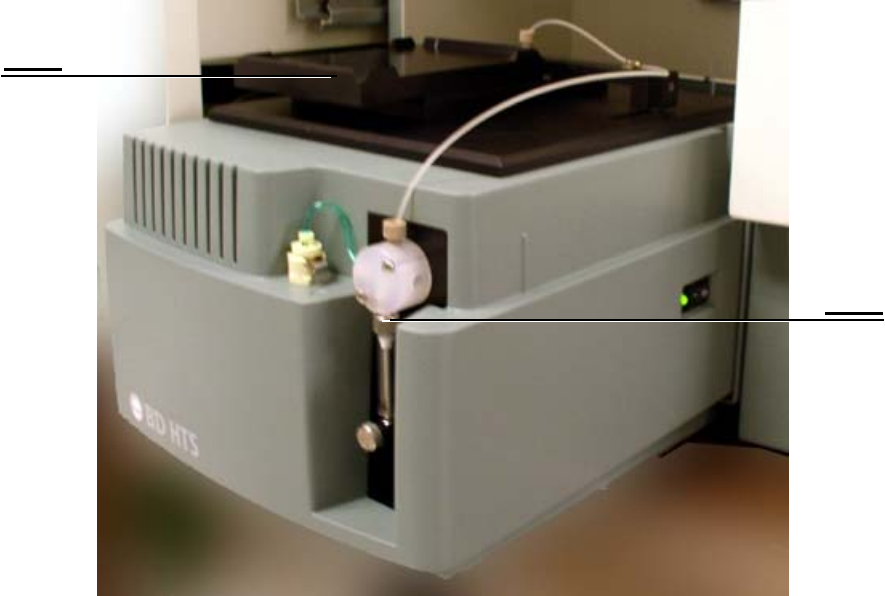
- A. Drawer
- B. Center spindle
- C. Optical sensors
- D. Tube lifter



BD High Throughput Sampler

Fill in the missing labels using the following options.

- A. Sampler coupler
- B. Primary syringe and valve
- C. Plate holder

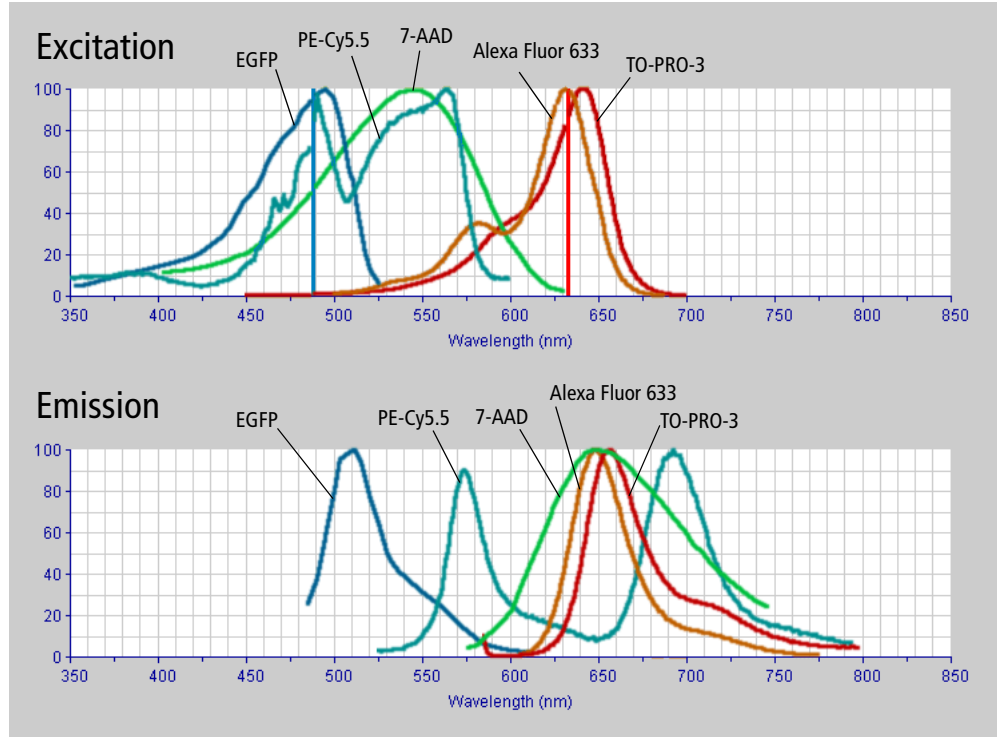


Optics Exercise

Use the BD FACSCalibur optics information and the excitation and emission graphs to determine the laser and PMT to use for each of the following fluorochromes.

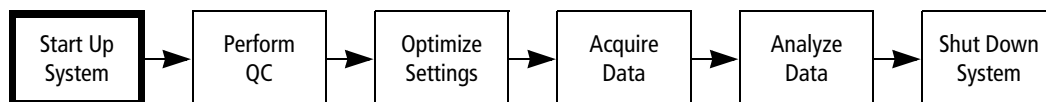
Fluorochrome	Laser (Red or Blue)	Detector
7-AAD	_____	_____
Alexa Fluor® 633	_____	_____
EGFP	_____	_____
PE-Cy™5.5	_____	_____
TO-PRO-3	_____	_____

Laser	Detector	Bandpass Filter	Wavelength Range
488 nm (blue)	FL1	530/30	515 to 545
	FL2	585/42	564 to 606
	FL3	670LP	670 and higher
635 nm (red)	FL4	661/16	653 to 669



System Startup

Acquisition of flow cytometry data is a multistep process. The following figure outlines the main steps involved. This exercise will focus on starting up the cytometer and the computer.



Always turn on the cytometer before turning on the computer when acquiring data. This enables the computer to recognize that the cytometer is connected. When analyzing data, it is not necessary to turn on the cytometer.

- 1 Turn on the cytometer. The cytometer power switch is located in the lower right corner of the instrument.

The LO fluid control button lights green and the STNDBY button lights orange.

The system allows the laser to warm up for 5 minutes after the instrument is powered on, before it will acquire data.

- 2 Turn on the computer.
- 3 Select your user ID and enter the password.

Filling the Sheath Tank

Recommended Sheath Fluid

- BD FACSTFlow™ sheath fluid (BD Biosciences) for nonsorting applications.
- Phosphate-buffered saline (PBS) (Dulbecco's Ca²⁺ and Mg²⁺-free) for sorting applications.

Non-recommended Sheath Fluids

- Fisher Hematology Diluent
- Isoton III
- Isolac D
- Deionized (DI) water

IMPORTANT If you prepare your own sheath fluid, pass it through a 0.22- μ m filter before running it on the instrument.

- 1** Slide the fluidics drawer out. Flip the vent valve toggle switch, located between the sheath and waste tanks, in the direction of the arrow.

Toggling the switch relieves the sheath tank of air pressure.

- 2** Disconnect the sheath tubing (white) and the air tubing (blue) from the instrument by squeezing the metal clip on the quick-disconnect and pulling each connector from the fitting.
- 3** Disconnect the fluid detection probe connector by squeezing the sides while pulling the plastic connector.
- 4** Slide the metal sheath tank bracket away from you, and lift up to remove it.
- 5** Remove the sheath tank, remove the fluid detection probe, and fill the tank to 3/4 its capacity with the recommended sheath fluid.



Avoid filling the sheath tank to its maximum capacity.

When a full tank is pressurized, fluid might be forced into the air supply tubing, preventing proper pressurization.

Additionally, since there is approximately 400 mL of bleach in the 4-L waste tank, filling the sheath tank to maximum capacity will overflow the waste tank.

- 6** Replace the sheath tank.
- 7** Replace the sheath bracket by sliding the metal bracket toward you. Make sure the square lip on the right side of the bracket is covering the ball valve.
- 8** Return the connectors to their original positions.
 - a** Insert the sheath fluid detection probe connector into its housing.
 - b** Insert the blue quick-disconnect into the blue port until you hear a click.
 - c** Insert the white quick-disconnect into the white port until you hear a click.
 - d** Verify that there are no kinks in the sheath and waste tubing lines.
- 9** Flip the vent valve toggle switch to pressurize the tank.
- 10** Check the sheath tank to ensure that it is properly pressurized. It should not be able to move around under the bracket.
- 11** Check the sheath filter to be sure no air bubbles are trapped inside.
 - If bubbles are visible, gently tap the filter body to dislodge them and force them to the top. Push the roller in the roller clamp forward to allow the pressurized sheath fluid to force the air bubbles into the waste tank. Return the roller clamp to closed position. Repeat steps if necessary.

12 Check the sheath line and the sheath filter line for bubbles.

- If bubbles are in either line, disconnect the tubing at the quick-disconnect port and press the tip of the valve against the side of a waste beaker. The pressurized sheath fluid will force bubbles and sheath fluid out of the tubing.

Emptying the Waste Tank

It is good practice to empty the waste tank when you fill the sheath tank. This prevents the waste tank from overflowing.



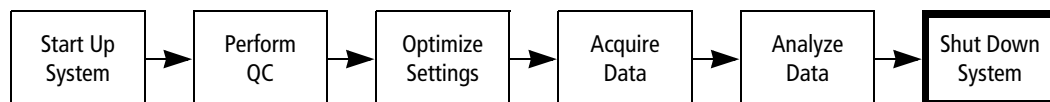
Observe universal precautions when handling waste materials.

- 1** Disconnect the waste tubing (orange) and the air tubing (white) from the instrument by squeezing the metal clip on the quick-disconnect and pulling.
- 2** Disconnect the fluid detection probe connector by squeezing the sides and pulling.
- 3** Remove the waste tank, remove the fluid detection probe, and empty the tank according to local, state, and federal hazardous waste handling regulations.
- 4** Fill the waste container with 400 mL of undiluted household bleach.

This will make a 10% solution of bleach in the waste container once it is full.
- 5** Replace the waste tank.
- 6** Return the connectors to their original positions.
 - a** Insert the waste fluid detection probe connector into its housing.
 - b** Insert the orange quick-disconnect into the orange-colored port until you hear a click.
 - c** Insert the white quick-disconnect into the white-colored port until you hear a click.
 - d** Verify that there are no kinks in the waste and air vent tubing lines.

System Shutdown

Always clean the instrument before you power it off at the end of the day. Proper cleaning will ensure that your instrument will function consistently.



To prevent the sample tube from becoming clogged and to remove adhesive dyes that could remain in the tubing (causing carryover), run a 10% bleach solution through the SIP at the end of each day, followed by a DI water rinse. Also perform this procedure immediately after running viscous samples or dyes such as propidium iodide (PI), acridine orange (AO), or thiazole orange (TO).

- 1 Place the cytometer in RUN mode and set the flow rate to HI.
- 2 Install a tube containing 3 mL of a 10% bleach solution on the SIP with the support arm to the side (DCM pump is on), and let it run for 1 minute.
- 3 Move the support arm under the tube (DCM pump is off). Allow the bleach solution to run for 5 minutes with the flow rate set to HI.
- 4 Remove the tube of bleach solution from the SIP.
- 5 (Optional) Repeat steps 2 through 4 using BD FACSTM rinse solution.
- 6 Repeat steps 2 through 4 using DI water.
- 7 Place the cytometer in STNDBY mode.
- 8 Place a tube containing no more than 1 mL of DI water on the SIP.



Sheath fluid will backflush into the tube and might cause the tube to overflow if more than 1 mL of DI water is in the tube. This could affect instrument performance.

- 9 If you are finished running samples, shut off the power to the cytometer.

The tube of DI water should remain on the SIP to prevent salt deposits from forming in the injection tube.

Periodic Maintenance

The following periodic maintenance procedures are necessary to maintain optimal instrument performance.

- Monthly cleaning
- Sheath filter replacement
- Air filter cleaning
- Bal seal replacement
- Sample O-ring replacement

The monthly cleaning procedure is described in the following section. Refer to the *FACSCalibur System User's Guide* for instructions for other maintenance procedures.

Monthly Cleaning

Perform overall system fluidics cleaning at least once a month or more frequently if you are running high volumes of samples or dyes such as propidium iodide (PI), acridine orange (AO), or thiazole orange (TO).



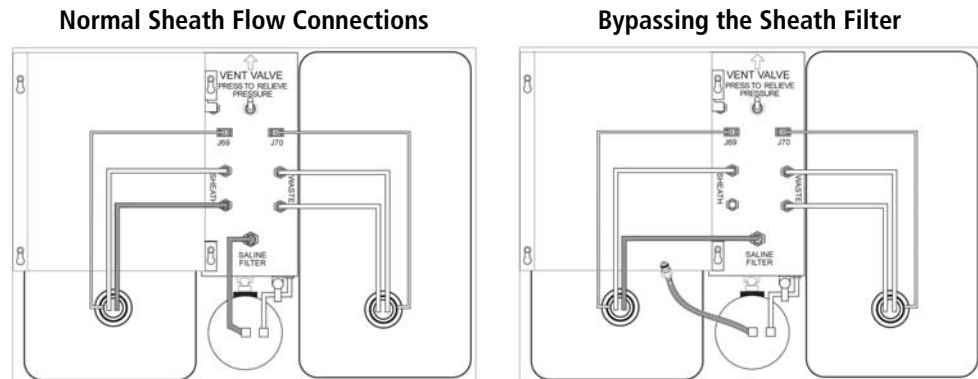
Running bleach or detergent through the sheath filter will break down the filter paper within the filter body. These paper fragments might clog the flow cell and require replacement of the filter.

When performing monthly cleaning, bypass the sheath filter as described in this procedure.

- 1** Turn on the cytometer.
- 2** Remove the sheath tank.

- 3 Disconnect the upper tubing of the sheath filter from the SALINE FILTER port by squeezing the metal clip on the quick-disconnect and pulling the connector from the fitting.
- 4 Connect the sheath tubing (white) from the tank to the port labeled SALINE FILTER.

This bypasses the sheath filter and allows fluid to travel from the sheath tank through the tubing directly to the flow cell and to the waste tank.



- 5 Install a spare tank with 1 to 2 L of a 10% bleach solution.
- 6 Set the flow rate to HI, then install a tube containing 3 mL of 10% bleach solution on the SIP.
- 7 Place the cytometer in RUN mode and allow the solution to run for 20 to 30 minutes.
- 8 Remove the tube of bleach solution from the SIP.
- 9 (Optional) Repeat steps 5 through 8 using BD FACSTTM rinse solution.
- 10 Repeat steps 5 through 8 using DI water.
- 11 Replace the original sheath tank and reconnect the sheath filter. Place a tube containing no more than 1 mL of DI water on the SIP.
- 12 Place the cytometer in STNDBY mode.
- 13 Turn off the power to the cytometer. The tube of DI water should remain on the SIP to prevent salt deposits from forming in the injection tube.

IMPORTANT After a monthly cleaning, you will need to run sheath fluid for approximately 5 minutes before acquiring samples. If you will not be using your instrument for a week or longer, perform monthly maintenance and keep the DI water in the fluidic system until you use the instrument again.

Review

- 1 Name the three subsystems of the BD FACSCalibur flow cytometer.

- 2 What would cause the cytometer status to remain in Standby (RUN button remains orange) when a tube has been placed on the SIP and the RUN button has been pressed?

- 3 What should you do to avoid running bleach through the sheath filter during monthly cleaning?

BD Supplies Part Numbers

Part Name	Part Number
BD FACSFlow sheath fluid	342003
BD FACSTM sheath solution with surfactant	336524
BD FACS Rinse Solution	340346
BD FACS Clean Solution	340345

Instrument Controls in BD CellQuest Pro Software

After completing this chapter, you will be able to:

- Open an Experiment document
- Adjust instrument settings
- Perform manual compensation
- Load and save instrument settings

Introduction

In this chapter, you will learn how to use the instrument controls in BD CellQuest Pro software. Also, you will investigate the concepts of spectral overlap and fluorescence compensation.

Materials

BD Calibrite™ beads will be used in the instrument control exercises in this chapter. The following bead tubes will be provided by your instructor for these exercises:

- Unlabeled beads
- Mixed beads (Unlabeled beads, FITC beads, and PE beads)

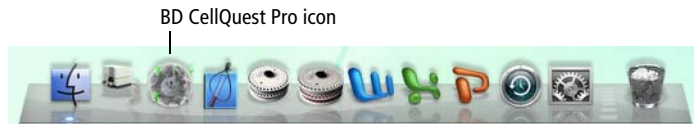
Before You Begin

Before beginning the exercise, perform System Startup on page 33.

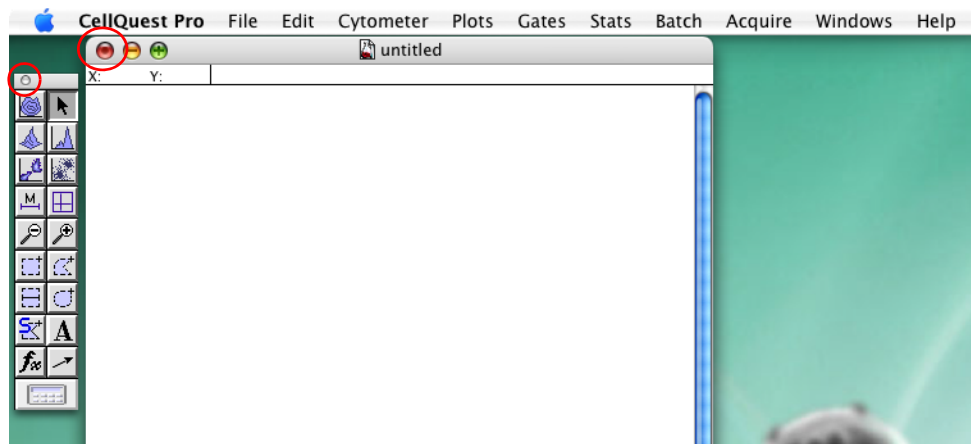
Opening an Experiment Document

In this section, you will start BD CellQuest Pro software, open an Experiment document that has been prepared for you, and connect the software to the cytometer.

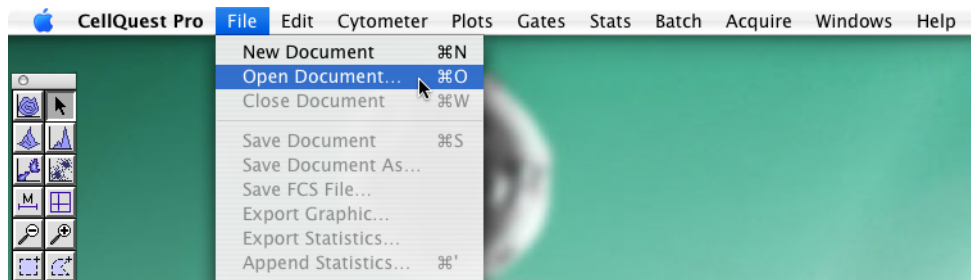
- 1 Start BD CellQuest Pro software by clicking its icon on the dock.



When the software starts, the BD CellQuest Pro menu is displayed, and the Tool Palette and default Experiment document appear.

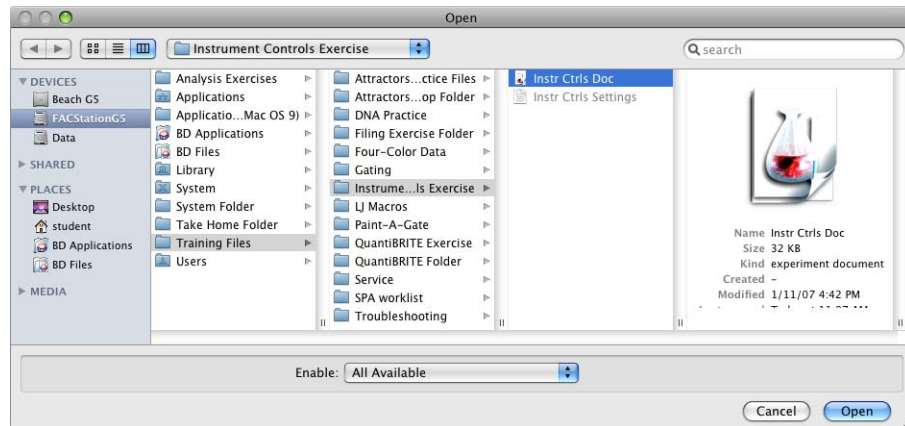


- 2 Before opening another Experiment, close the default document by clicking its close button (red). Click Don't Save when asked if you want to save changes to the document.
- 3 Select File > Open Document.




A standard file open dialog appears.

- Navigate to FACStationG5/Training Files/Instrument Controls Exercise and open the Instr Ctrl Doc file.



An Experiment document named *untitled 2* appears.

TIP Instr Ctrl Doc is a stationary pad, so a copy of the protected document is opened in place of the original.

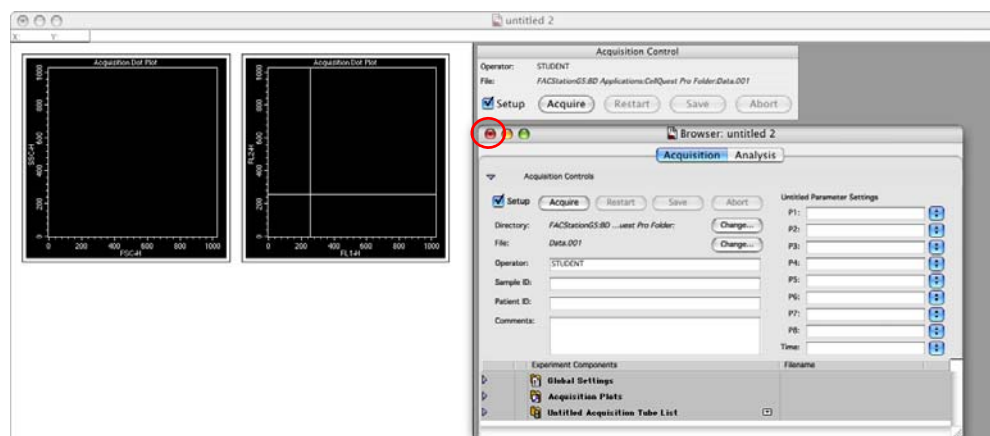
- Expand the Experiment window to full size by clicking its (green) zoom button (). Drag the Tool Palette to a clear space below the plots.

- Select Acquire > Connect to Cytometer (**⌘B**).

The Acquisition Control window and the Acquisition view of the Browser appear.

TIP If you click the Experiment document, the Browser might be obscured from view. Select Windows > Browser:*title* to bring the Browser window back to the front.

- Click and drag the Acquisition Control window to a clear area of the screen.
- Close the Browser by clicking its close button (red).

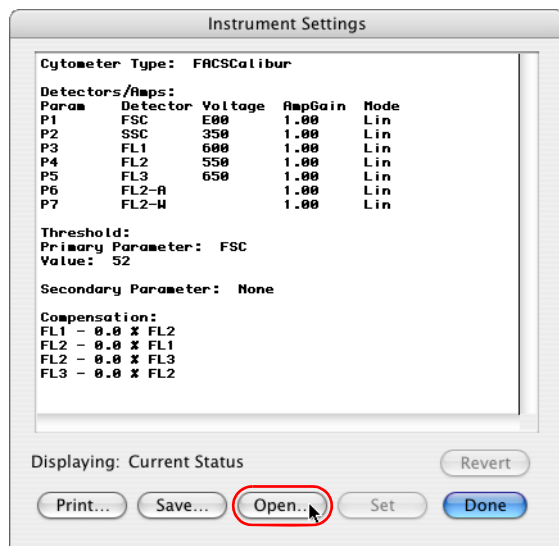


Loading Instrument Settings

To initialize the instrument settings for this exercise, you will open and set an instrument settings file. You will learn more about saving and loading instrument setting files in Chapter 4, BD CellQuest Pro Workflow.

- 1 Select Cytometer > Instrument Settings.

The Instrument Settings dialog is displayed.



- 2 Click Open.
- 3 Navigate back to the folder from which you opened Instr Ctrl's Doc (FACStationG5/ Training Files/Instrument Controls Exercise) and open the Instr Ctrl's Settings file.

The settings from the Instr Ctrl's Settings file are displayed in the Instrument Settings window.

- 4 Click Set in the Instrument Settings window to send these settings to the cytometer.

The Set button changes to blue to indicate that the settings are being sent to the cytometer. When the Set action has completed, the button changes back to white.

IMPORTANT You must click Set to download the instrument settings.

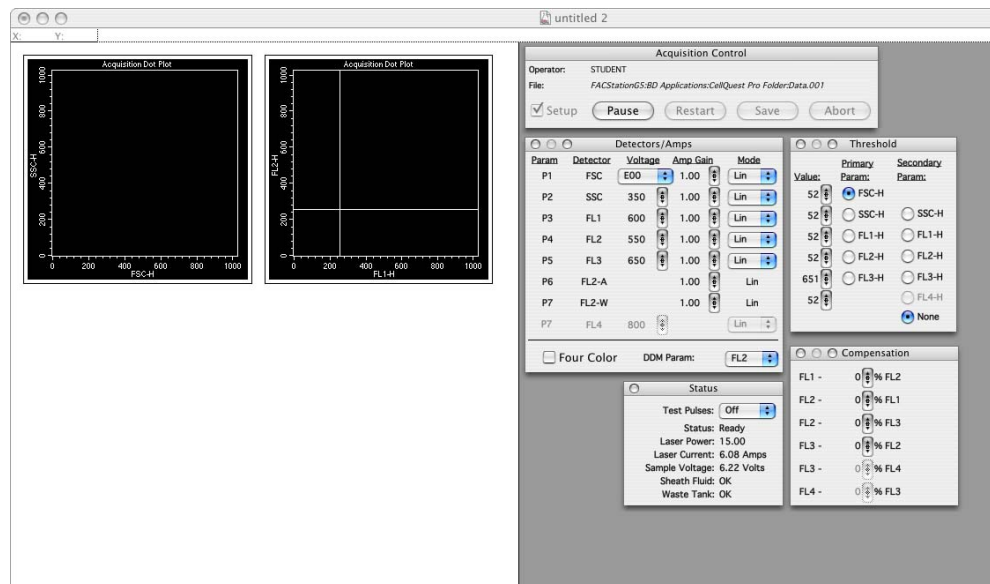
- 5 Click Done to close the Instrument Settings window.

Displaying the Instrument Controls

In this section you will display the BD CellQuest Pro software instrument controls:

- Detector/Amps
 - Threshold
 - Compensation
 - Status
- 1 Select Cytometer > Detectors/Amps to display the detectors and amps controls (⌘1).
 - 2 Select Cytometer > Threshold to display the threshold controls (⌘2).
 - 3 Select Cytometer > Compensation to display the compensation controls (⌘3).
 - 4 Select Cytometer > Status to display the status controls (⌘4).
 - 5 Click and drag each of the instrument control windows to a clear area of the screen.

TIP When you save your BD CellQuest Pro Experiment document, the locations of the instrument settings windows (Detectors/Amps, Threshold, and Compensation) will also be saved. The next time you open these windows, they will appear in the location where you last saved them.



Using Instrument Controls

You will use the BD CellQuest Pro software instrument controls to adjust:

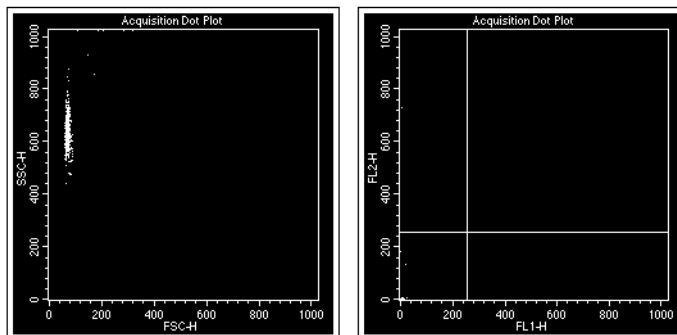
- FSC amp gain and SSC detector voltage
- Threshold setting
- FL1 and FL2 detector voltages
- Fluorescence compensation values

Two tubes are provided: Unlabeled and Mixed.

- The Unlabeled tube contains unlabeled BD Calibrite beads.
- The Mixed tube contains unlabeled, FITC-labeled, and PE-labeled BD Calibrite beads.

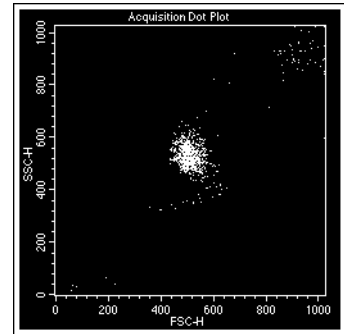
Adjusting FSC Amp Gain and SSC Detector Voltage

- 1 Set the flow rate to MED, and place the cytometer in RUN mode.
- 2 Install the Unlabeled bead tube on the cytometer.
- 3 Confirm that the Setup checkbox is selected in the Acquisition Control dialog, and then click Acquire.



4 Center the unlabeled BD Calibrite beads on the FCS vs SSC plot.

- a** Verify that the FSC and SSC amplifiers are in Linear mode.
- b** Adjust the FSC Amp Gain control to position the beads on the FSC-H scale.
- c** Adjust the SSC Voltage control to position the beads on the SSC-H scale.



5 Change the FSC Voltage control (preamp) to E01. Which direction do the events move?

6 Change the FSC Detector to E-1. Click Pause, then Restart. What happens? Why?

7 Change the FSC Detector back to E00.

8 Change the SSC Amplifier to Log. What happens to the events?

9 Change the SSC Amplifier back to Lin.

10 Verify that the unlabeled bead events are again centered on the FCS vs SSC plot.

Adjusting the Threshold Setting

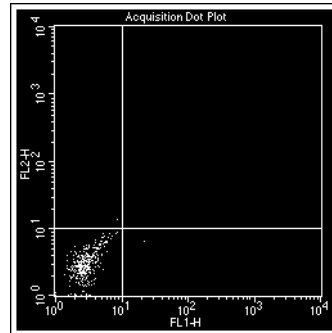
- 1 Change the FSC-H threshold to 800. Click Pause, then Restart. Why do most of the bead events disappear?

- 2 Change the FSC-H threshold back to 52.
- 3 Add a secondary threshold of FL3-H. Click Pause, then Restart. Why do most of the bead events disappear?

- 4 Remove the secondary FL3-H threshold.

Adjusting FL1 and FL2 Detector Voltages

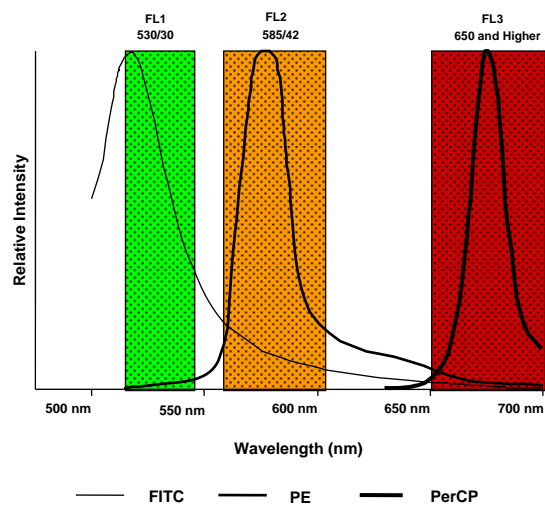
- 1 Position the unlabeled beads in the lower left quadrant of the FL1 vs FL2 plot.
 - a Verify that the FL1 and FL2 amplifiers are in Log Mode. Change, if needed.
 - b Adjust the FL1 Voltage control to position the beads on the FL1-H scale.
 - c Adjust the FL2 Voltage control to position the beads on the FL2-H scale.
- 2 Change the FL1 amplifier to Lin (linear) Mode. Click Pause, then Restart. What happens? Why?



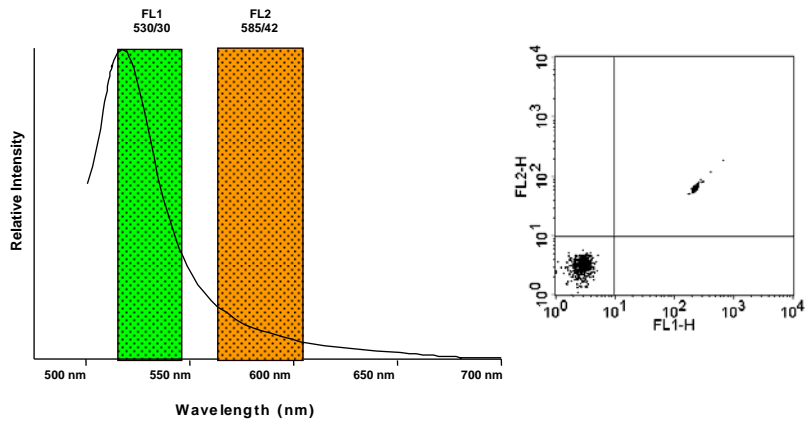
-
-
- 3 Change the FL1 amplifier back to Log Mode.
 - 4 In the Acquisition Control dialog, click Pause, then Abort.
 - 5 Remove the Unlabeled bead tube from the cytometer.
 - 6 Install a tube containing no more than 1 mL of DI water on the SIP and place the cytometer in STNDBY mode.

Compensation

Emission Spectra



FITC Fluorescence Overlap



3

FITC Compensation

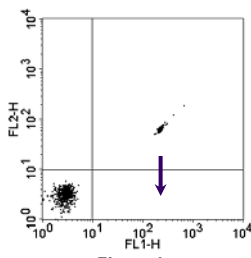


Figure A

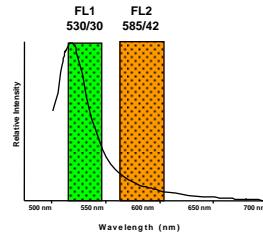


Figure B

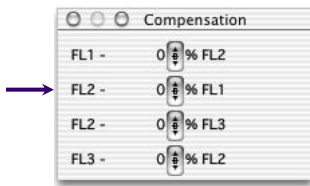
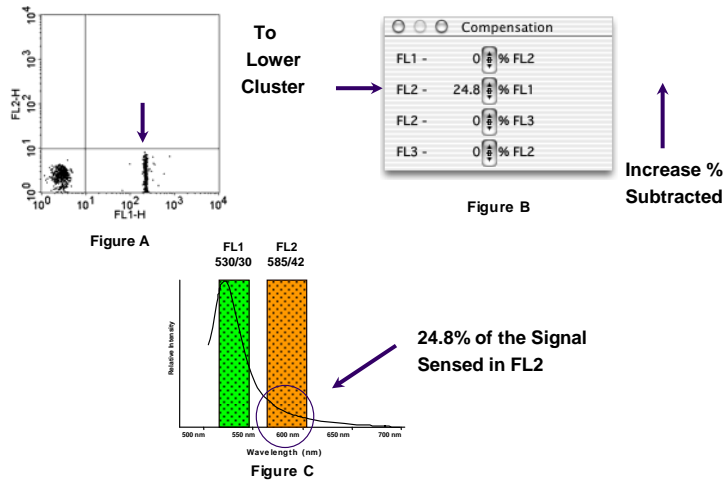


Figure C

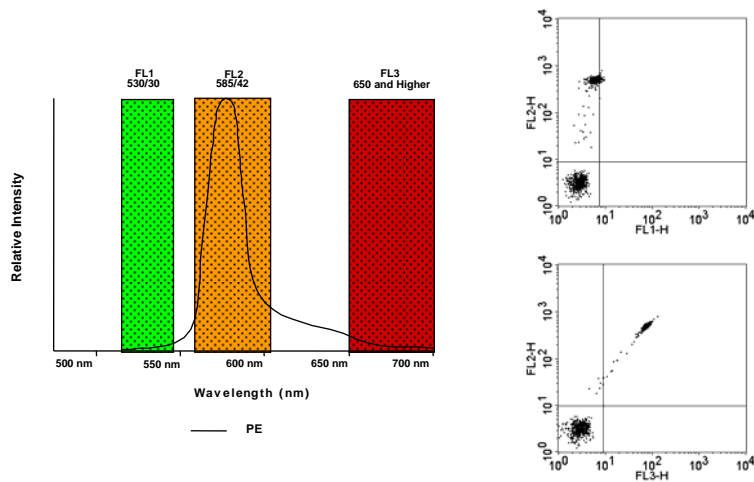
Detector - % Signal

4

FITC Compensation

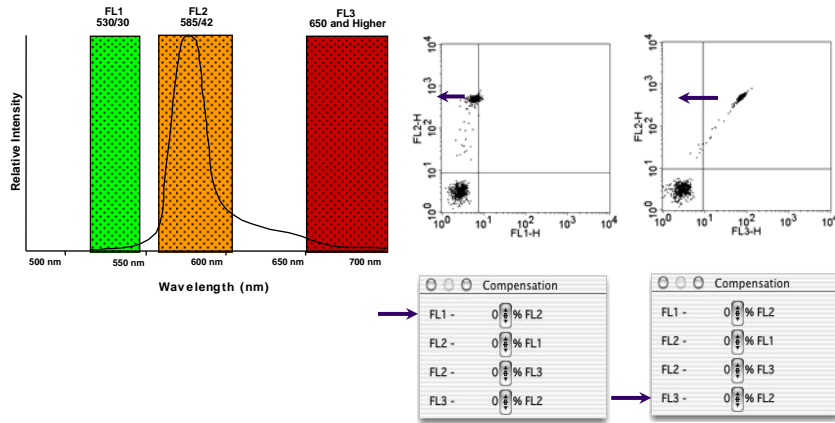


PE Fluorescence Overlap





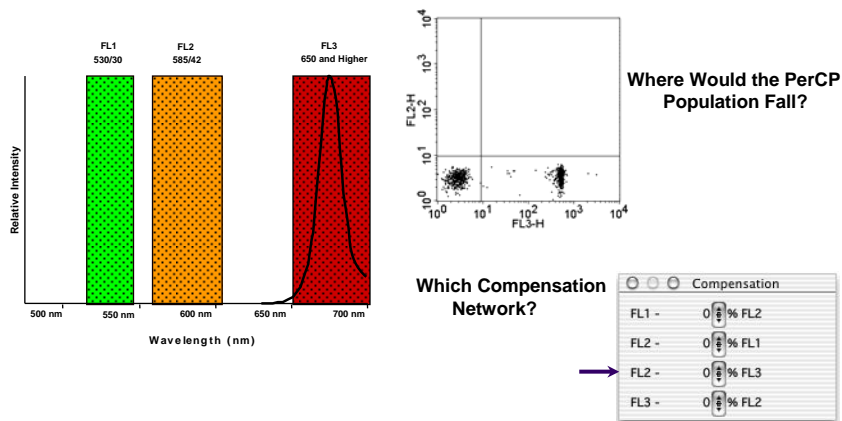
PE Compensation



7



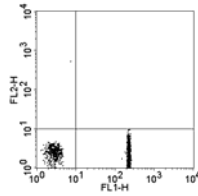
PerCP Fluorescence



8

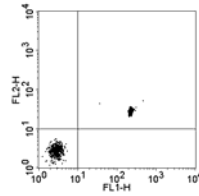
Compensation Examples

Optimal Compensation



Compensation	
FL1 -	0% FL2
FL2 -	25.0% FL1
FL2 -	0% FL3
FL3 -	0% FL2

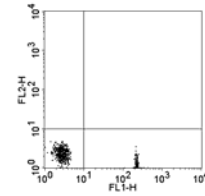
Undercompensation



Compensation	
FL1 -	0% FL2
FL2 -	15.0% FL1
FL2 -	0% FL3
FL3 -	0% FL2

Not enough subtracted

Overcompensation

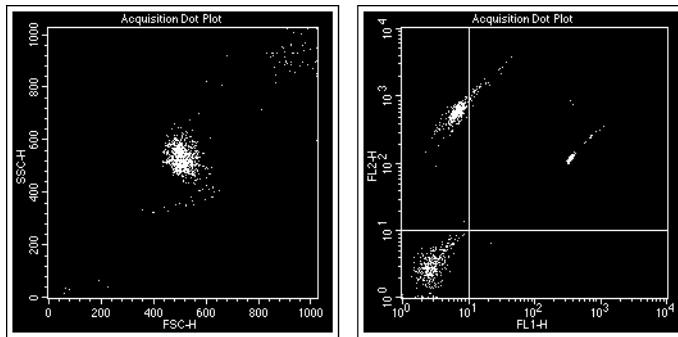


Compensation	
FL1 -	0% FL2
FL2 -	50.0% FL1
FL2 -	0% FL3
FL3 -	0% FL2

Too much subtracted

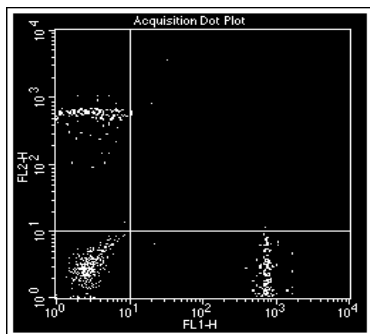
Adjusting Fluorescence Compensation Values

- 1 Remove the DI water tube from the cytometer.
- 2 Set the flow rate to MED, and place the cytometer in RUN mode.
- 3 Install the Mixed bead tube on the SIP.
- 4 Confirm that the Setup checkbox is selected in the Acquisition Control dialog, and then click Acquire.



- 5 Position the FITC⁺ beads in the lower right quadrant of the FL1-H vs FL2-H plot. Which compensation network was adjusted?
-

- 6 Position the PE⁺ beads in the upper left quadrant of the FL1-H vs FL2-H plot. Which compensation network was adjusted?
-



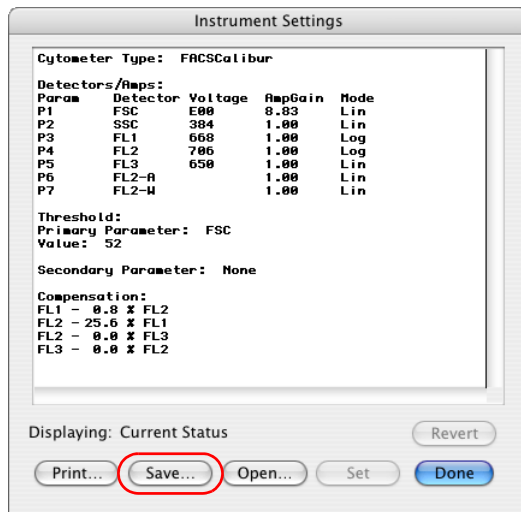
- 7** Increase the FL1-%FL2 Compensation. The PE bead population is now _____ compensated. Compensate correctly.
- 8** Decrease the FL2-%FL1 Compensation. The FITC bead population is now _____ compensated. Compensate correctly.
- 9** Decrease the FL1 PMT voltage by 20 volts. What happens to the compensation?
The FITC bead population is now _____ compensated.
The PE bead population is now _____ compensated.
Restore the FL1 PMT voltage to its adjusted value (increase by 20 volts).
- 10** Click Pause and then Abort in the Acquisition Control dialog.
- 11** Remove the Mixed bead tube from the cytometer.
- 12** Install a tube containing no more than 1 mL of DI water on the SIP and place the cytometer in STNDBY mode.

Saving Instrument Settings

The optimized instrument settings can be saved to a file and later restored.

- 1 Select Cytometer > Instrument Settings.

The Instrument Settings dialog appears.



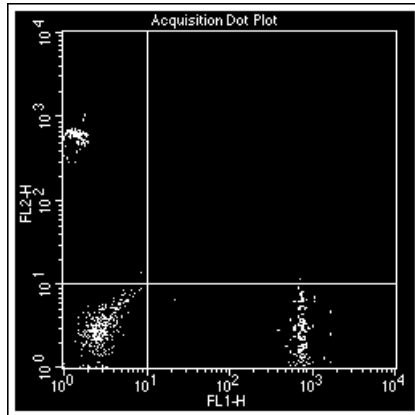
- 2 Click Save.
- 3 The Save Instrument Settings dialog appears.



- 4 Specify your student folder as the destination.
- 5 Type 2-color Beads in the Save As field.
- 6 Click Save to save the settings file and close the Save Instrument Settings dialog.
- 7 Click Done to close the Instrument Settings window.

Compensation Review

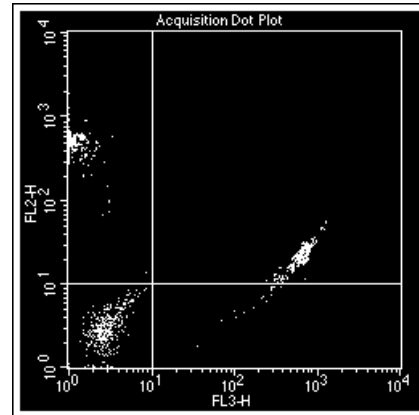
The following plots show populations with compensation settings that may need correction. Draw an up or down arrow in each box to show how the compensation network should be adjusted. If no change is needed, leave the box empty. For each population determine whether it is under-, over-, or optimally compensated, and circle the correct choice



FL1 – %FL2 FL2 – %FL1

FL1⁺: **over-** **under-** **optimally** compensated.

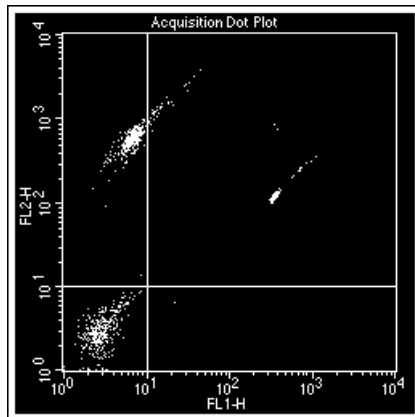
FL2⁺: **over-** **under-** **optimally** compensated.



FL3 – %FL2 FL2 – %FL3

FL2⁺: **over-** **under-** **optimally** compensated.

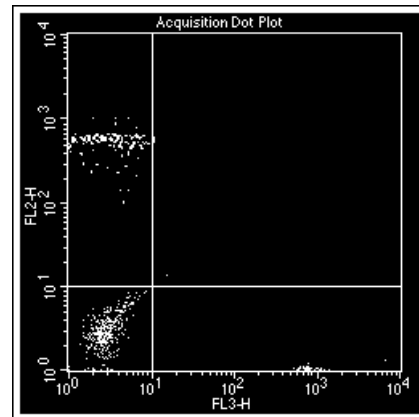
FL3⁺: **over-** **under-** **optimally** compensated.



FL1 – %FL2 FL2 – %FL1

FL1⁺: **over-** **under-** **optimally** compensated.

FL2⁺: **over-** **under-** **optimally** compensated.



FL3 – %FL2 FL2 – %FL3

FL2⁺: **over-** **under-** **optimally** compensated.

FL3⁺: **over-** **under-** **optimally** compensated.

BD FACSComp Software

After completing this chapter, you will be able to:

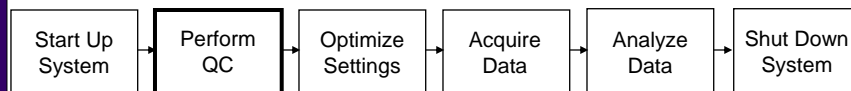
- Use BD FACSComp™ software with or without the BD FACSTM Loader.
- Perform instrument quality control and set up the cytometer for immunophenotyping of human peripheral blood.



BD FACSComp™ Software



The Big Picture





FACSCComp — Uses

- To monitor daily cytometer performance.
- To obtain instrument settings for immunophenotyping of human cells.

3



How BD FACSCComp Software Works

- It adjusts the cytometer settings to position BD Calibrite beads in target channels.
- It checks the sensitivity of the cytometer by evaluating the channel separation of BD Calibrite beads.

4



Functions of FACSCComp

- PMT voltage adjustment
- Time-delay calibration
- Fluorescence compensation adjustment
- Sensitivity testing

5



Preparing CaliBRITE Bead Suspensions

- Add 1.0 mL of sheath fluid to Tube A.
- Add 3.0 mL of sheath fluid to Tube B.
- Add one free-falling, complete drop from each of the BD Calibrite bead vials specified

Beads in Tube A	Beads in Tube B
Unlabeled, APC	Unlabeled, FITC, PE, PerPC, APC

6



FACS Loader Tube Positions

Position	Tube
1	Tube A (unlabeled + APC beads)
2	Tube B (mixed beads)
39	3 mL of 10% bleach solution
40	3 mL of DI water

7



PMT Adjustments

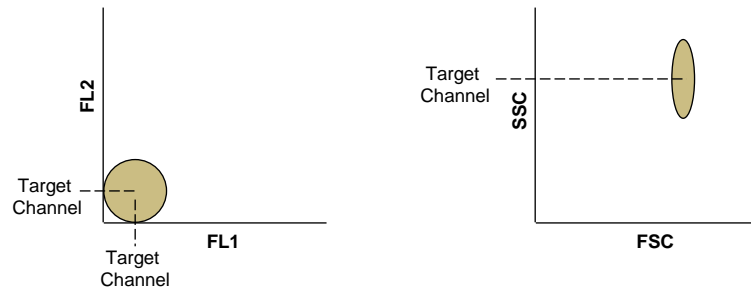
Adjusts voltages to place a bead population at a specified target channel

- Unlabeled beads:
SSC, FL1, FL2, and FL3 PMT adjustment
- APC beads:
FL4 PMT adjustment

8



PMT Adjustments

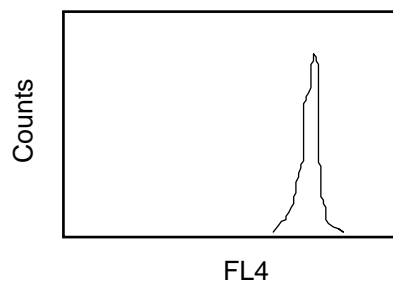


SSC, FL1, FL2, and FL3 PMTs are set with unlabeled CaliBRITE beads.

9



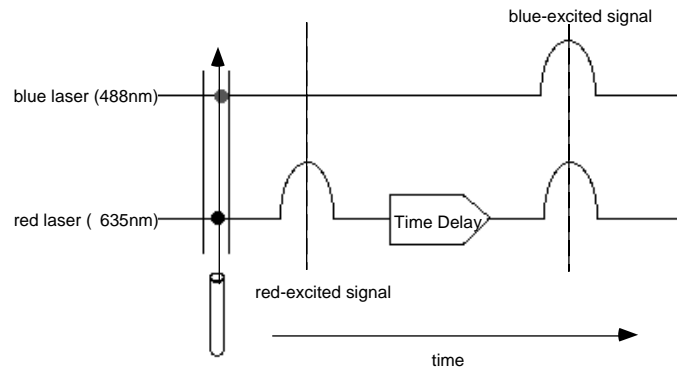
PMT Adjustments



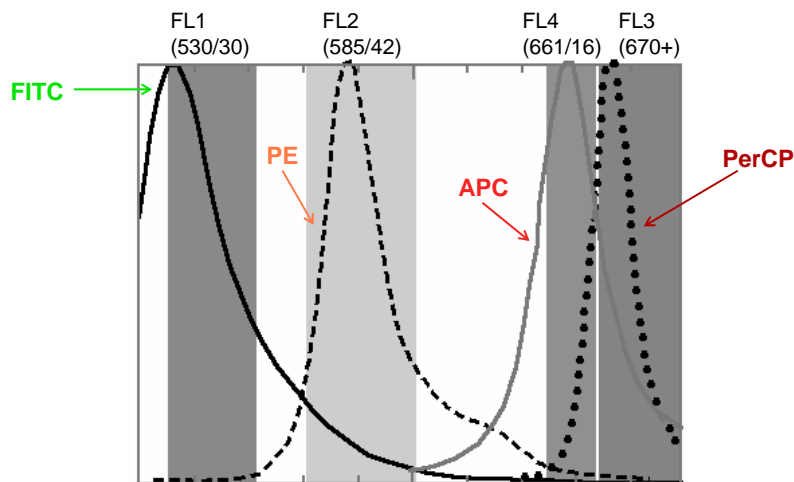
FL4 PMT is set with APC CaliBRITE beads.

10

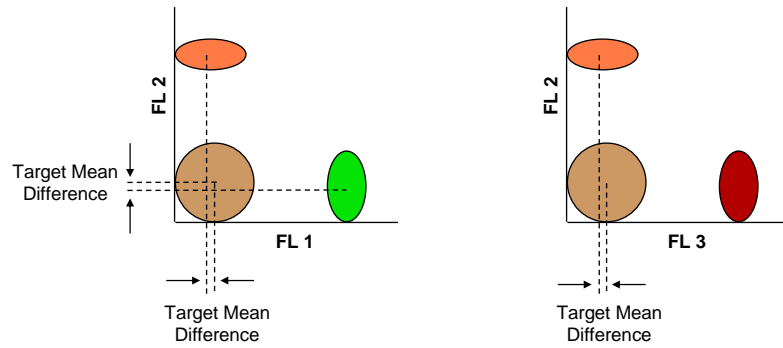
Time-Delay Calibration



Compensation

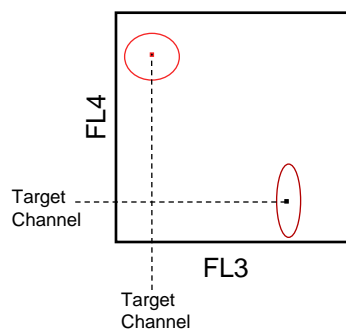


Fluorescence Compensation



13

Fluorescence Compensation



FL3-%FL4

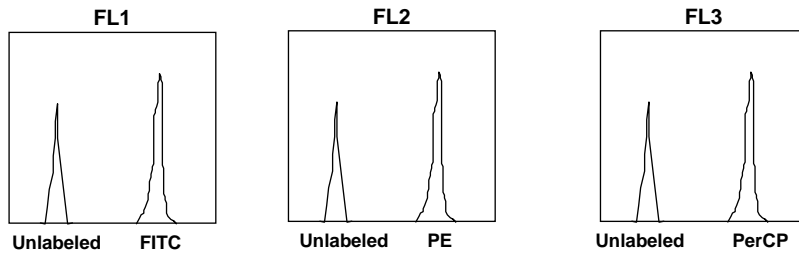
FL4-%FL3

14



Sensitivity

Evaluates labeled-to-unlabeled signal separation for FL1, FL2, and FL3 parameters

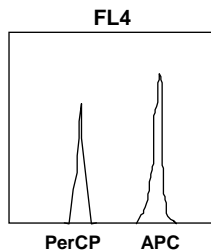


15



Sensitivity

Evaluates PerCP-to-APC or PerCP-Cy5.5-to-APC signal separation for the FL4 parameter

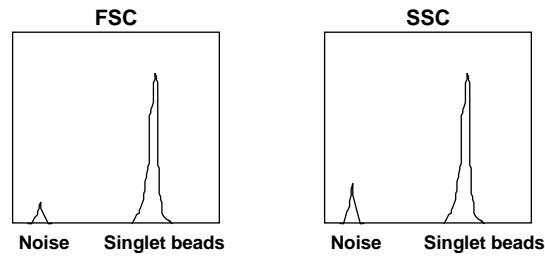


16



Sensitivity

Evaluates signal-to-noise separation for FSC and SSC parameters.



17










Assay Options

- Lyse/Wash (LW)
- LW PerCP-Cy5.5
- Lyse/No-Wash (LNW)
- LNW PerCP-Cy5.5
- HLA-B27 Calibration

18

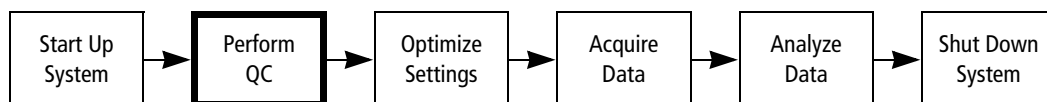
Calibration Files

Assay	Choose In	Calib File Name
<input type="checkbox"/> LyselWash (LW) 	Set Up View	Calib File
<input type="checkbox"/> LW PerCP-Cy5.5 	Set Up View	Calib File.PerCP-Cy5.5
<input type="checkbox"/> Lysel/No-Wash (LNW) 	Set Up View	Calib File.LNW
<input type="checkbox"/> LNW PerCP-Cy5.5 	Set Up View	Calib File.LNW PerCP-CY5.5
<input type="checkbox"/> HLA-B27 Calib 	Set Up View	Calib File.B27
 BD CBA	Preferences	CBA Calib File.LNW
 LeucoCOUNT	Preferences	LeucoCOUNT Calib File.LNW

file path: BD Files/Instrument Settings Files

Introduction

Acquisition of flow cytometry data is a multistep process. The following figure outlines the main steps involved. In this chapter, you will perform quality control (QC) using BD FACSComp software.



Instrument QC is performed by running BD Calibrite™ beads with BD FACSComp software. The software monitors instrument performance and provides automated instrument setup. This software, when used with BD Calibrite beads, provides routine instrument setup for 2-, 3- and 4-color immunophenotyping applications of human cells.

A calibration file is created each time a BD FACSComp assay is successfully completed. Calibration files contain all instrument settings as well as a sensitivity test result (TRUE if all parameters pass, FALSE if any parameter fails). Each time an assay is run, the new calibration file overwrites the existing calibration file of the same type. Refer to the *BD FACSComp Software Reference Manual* for additional details about BD FACSComp assays and calibration files.

NOTE BD FACSComp software versions 5.1 and later include Loader support. Refer to the *BD FACSComp Software Reference Manual* or the *BD FACS Loader Reference Manual* for additional details about using BD FACSComp software with the Loader.

Before You Begin

IMPORTANT The instructions in this chapter are for running the BD FACSComp software Lyse/Wash (LW) 4-color assay. The same procedure can be used to run the Lyse/No-Wash (LNW) 4-color assay, by making the appropriate choices at the Set Up view.

Before beginning the following exercise, start up the cytometer and computer if they are not already on. See System Startup on page 33 for details.

Performing QC and Instrument Setup

The exercises in this section include instructions for both manual tube loading and automatic tube loading with the BD FACS Loader. Unless otherwise noted, instructions apply to both methods. When details differ, they are listed side-by-side, as in the following.

To perform QC and instrument setup, you will:



For Manual Tube Loading

- Prepare BD Calibrite beads.
- Enter information in BD FACSComp software.
- Run a 4-color assay.
- Print and review the Summary Report.



For Automatic Tube Loading

- Prepare BD Calibrite beads.
- Enter information in BD FACSComp software, including Loader preferences.
- Load the tube rack.
- Run a 4-color assay using the Loader in walkaway operation.
- Print and review the Summary Report.

Preparing BD Calibrite Beads

In this section, you will prepare two tubes for a 4-color assay.



When PerCP beads are included in the mixed bead suspension (tube B), the beads are stable for 1 hour at 4°C. At room temperature, the beads are stable for a shorter time.

- 1 Gently vortex the stock vials of BD Calibrite beads to thoroughly suspend them.
- 2 Label one 12 x 75-mm tube *A* and another *B*.
- 3 Add 1 mL of BD FACSFlow sheath fluid to tube *A*.
- 4 Add 3 mL of BD FACSFlow sheath fluid to tube *B*.
- 5 Using the information in the following table, add one free-falling, complete drop from each of the BD Calibrite bead vials specified.

Beads in Tube A	Beads in Tube B
unlabeled, APC	unlabeled, FITC, PE, PerCP, APC

- 6 Mix the bead suspensions in both tubes by gentle vortexing.

Preparing the Software

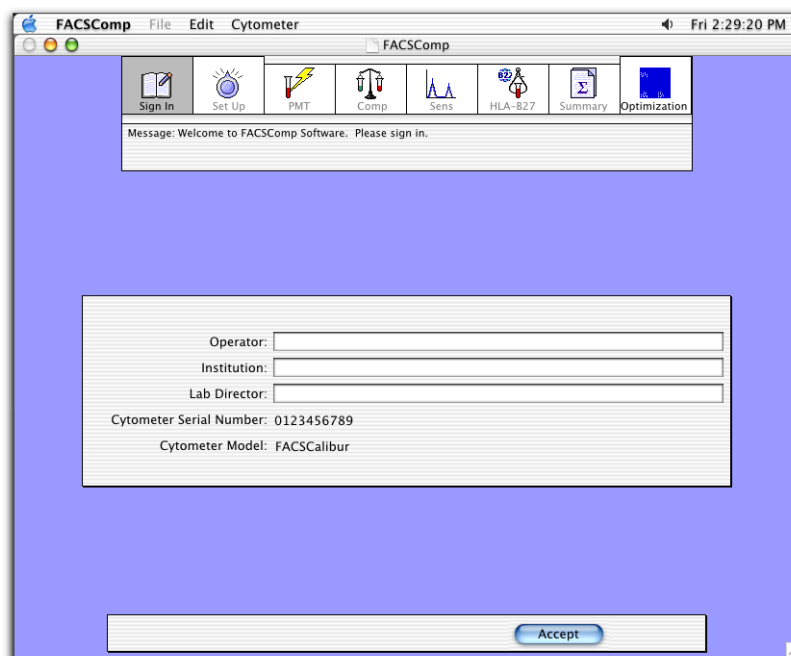
Before running a BD FACSCComp assay, you will make selections and enter information at the Sign In and Set Up views. You will also verify or make additional selections in the BD FACSCComp Preferences dialog. BD FACSCComp software will retain these settings from the previous session, so you will not always need to reenter them.

Sign In and Set Up Views

- 1 Start BD FACSCComp software by clicking its icon on the dock.



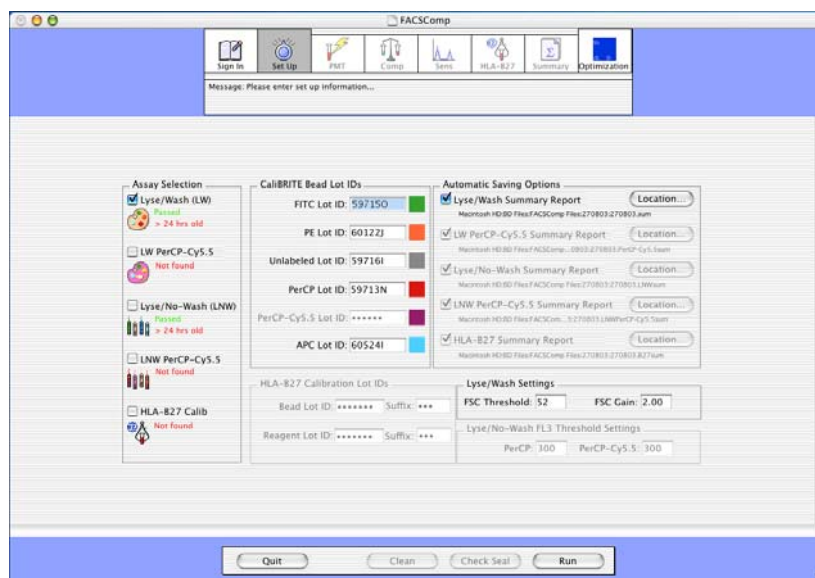
A startup screen appears, and then the application window opens at the Sign In view.



- 2 Type your name in the Operator field.
Only the operator field is required.
- 3 Verify that the Cytometer Model and Serial Number are correct.
- 4 (Optional) Fill in the Institution and Lab Director fields.

- 5 Click Accept or press Return.

The application window advances to the Set Up view. Under Assay Selection, the status for each type of calibration file is displayed.



- 6 Under Assay Selection, select the Lyse/Wash (LW) checkbox. Clear the other assay checkboxes, if needed.

TIP BD FACSComp software performs one or more selected assays in the order they are listed.

- 7 Under CaliBRITE Bead Lot IDs, verify the correct bead lot IDs for the FITC, PE, PerCP, APC, and Unlabeled beads. Modify bead lot IDs as needed.

The bead lot ID, consisting of five numbers and one letter, is found on the sticker included with the BD Calibrite kit. Type the bead lot ID exactly as shown. Do not add a space between the numbers and letter. The letter is case sensitive—type an uppercase letter.

- 8 Under Automatic Savings Options, select the Lyse/Wash Summary Report checkbox to automatically save a copy of the Summary report. Do not modify the Location information.

TIP By clicking Location, you can specify the folder location, name, and format of the report. The default location is BD Files: FACSComp Files:DDMMYY folder. The default name of the Summary Report is *DDMMYY.sum* for a text file and *DDMMYY.sum.PDF* for a PDF file.

TIP The Lyse/Wash Settings and the Lyse/No-Wash FSC Threshold Settings sections allow you to enter instrument settings, if needed. These values are not used by BD FACSComp software, but are added to the appropriate Calib File. Refer to the *BD FACSComp Software Reference Manual* for more details.

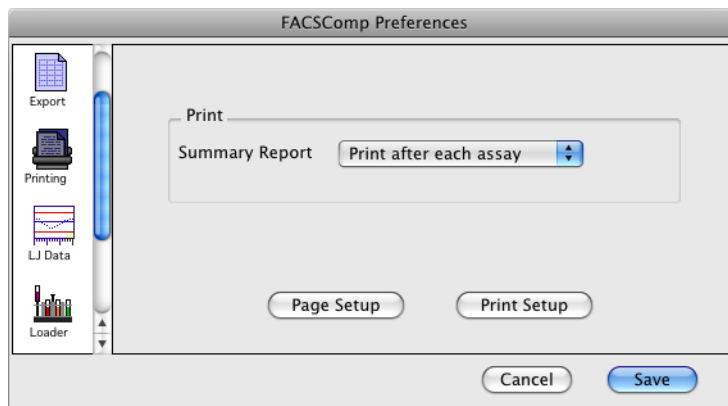
Setting Printing Preferences

REMINDER BD FACSComp software will retain these settings from the previous session, so you will not always need to reenter them.

- 1 Select FACSComp > Preferences.

The Preferences dialog appears.

- 2 Click the Printing icon.



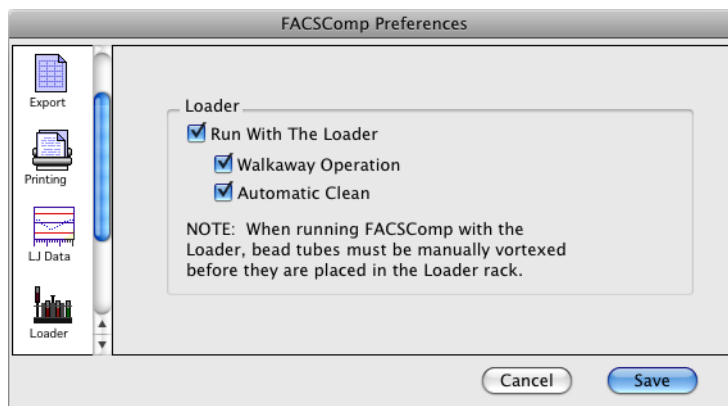
- 3 Verify that Summary Report is set to *Print after each assay*, to automatically print a Summary Report at the conclusion of each assay.
- 4 If you are using manual tube loading, click Save to accept changes and close the Preferences dialog, and skip ahead to Running BD FACSComp Assays on page 76.

Setting Loader Preferences



For Automatic Tube Loading Only

- 1 Click the Loader icon.



- 2 Select the Run With The Loader checkbox.
- 3 Select the Walkaway Operation checkbox.

In walkaway operation, the software will automatically load tubes and advance through PMT, time-delay calibration (4-color assays), compensation, and sensitivity testing without any user intervention.

TIP When more than one assay has been selected in walkaway operation, the software proceeds to the next assay after a 5-second countdown at the Summary report view. You can pause to view the summary report before continuing with the next assay.

- 4 Select the Automatic Clean checkbox.

A 3-minute clean cycle will run automatically when you quit the software. The clean cycle expects a bleach tube in position 39 and DI water in position 40. Each tube runs for 1-1/2 minutes.

- 5 Click Save to accept changes and close the Preferences dialog.

Loading Tubes



For Automatic Tube Loading Only

The following tube positions are used for the Lyse/Wash 4-color assay with a cleaning cycle.

Position	Tube
1	Tube A (unlabeled and APC beads)
2	Tube B (mixed beads)
39	3 mL of 10% bleach solution
40	3 mL of DI water

- 1 Place tube A in rack position 1 and tube B in rack position 2.
- 2 Place a tube containing bleach solution (3 mL) in position 39 and a tube containing DI water (3 mL) in position 40 on the rack.
- 3 If necessary, remove the DI water tube from the SIP and move the support arm to the side for Loader operation.
- 4 Place the rack on the Loader.
- 5 Push the drawer in, and put the Loader cover in place.

The Loader rack cycles and Tube 01 is displayed on the Loader keypad control.

- 6 Verify that the sample flow rate is set to HI and the cytometer is in RUN mode.
- 7 Click Check Seal at the bottom of the Set Up view.

The rack spins and the Loader raises tube 40 to verify that a seal can be formed by the lifter. Once the seal is verified, the tube is lowered and a dialog displays the results of the Check Seal test.

NOTE Refer to the *BD FACSComp Software Reference Manual* for rack positions used when running other BD FACSComp assays.

Running BD FACSComp Assays

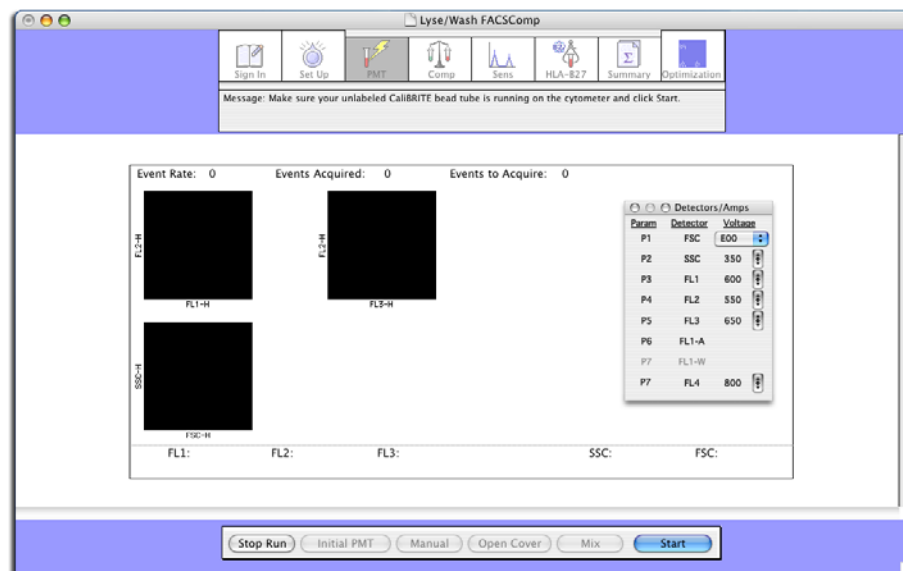
In this section, you will run the selected assay. During the run, the software will:

- Adjust PMT settings until target values are reached.
- Perform time-delay calibration (for 4-color assays).
- Adjust compensation settings until target values are reached.
- Test sensitivity in all parameters.

Starting the Run

- 1 Click Run at the Set Up view.

The application window advances to the PMT view.



- 2 Place the cytometer in RUN mode and set the flow rate to HI.

PMT Adjustments

- 1 To begin PMT adjustments with the unlabeled tube (tube A):



For Manual Tube Loading

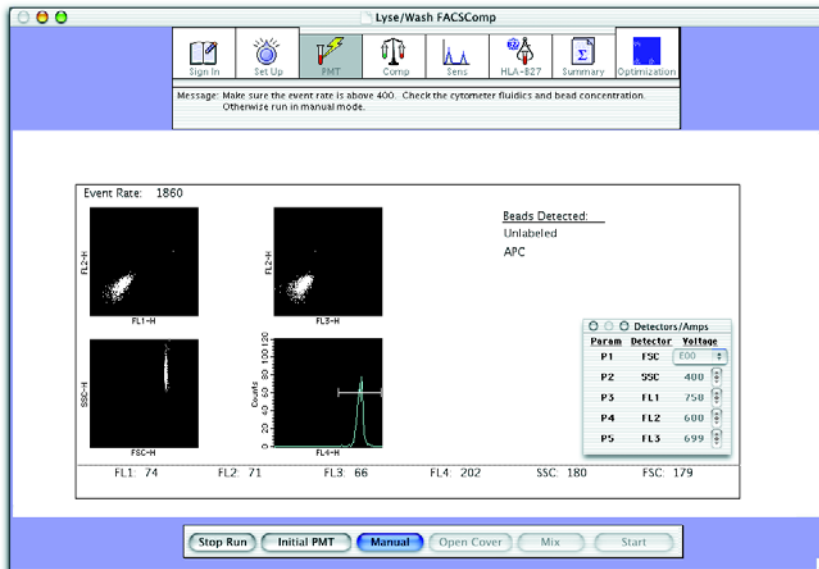
- a Gently mix tube A and install it on the cytometer.
- b Click Start.
Acquisition begins.



For Automatic Tube Loading

- No action is required.
The Loader lifts tube 1 to the SIP.
Acquisition begins.

A status message at the bottom of the PMT view continuously displays the % Complete for the singlet data acquisition.



The software adjusts PMT values so that the singlet bead mean fluorescence values are close to their respective target values.

NOTE If no APC beads are detected or an APC bead lot ID was not entered at the Set Up view, the second laser is turned off and PMTs are adjusted for 3-color setup.

When the message *PMTs Set Successfully* appears, the software counts down for 5 seconds, allowing you to pause the assay before the application window automatically advances to the next step.

Time-Delay Calibration

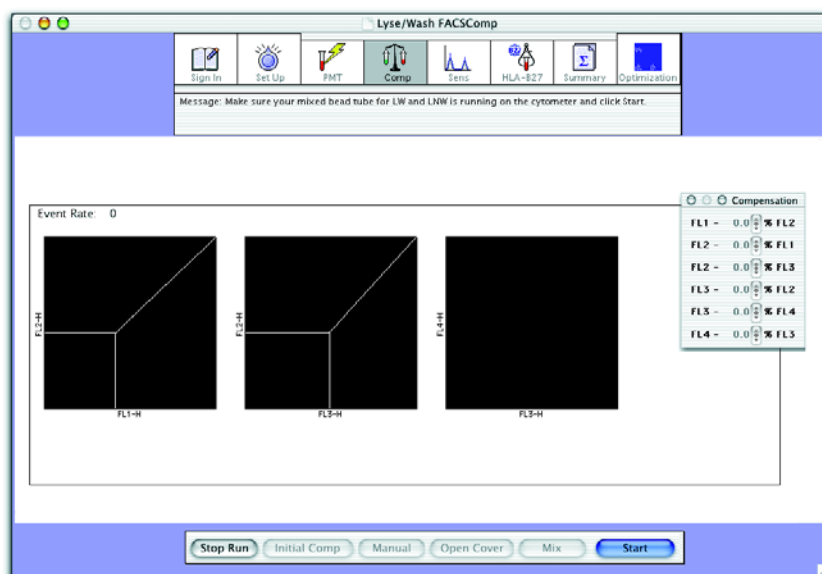
A message appears indicating time-delay calibration is being performed.

If the calibration is successful, a completion message appears. After 3 seconds, the application window automatically advances to the Compensation view.

NOTE If no APC beads are detected or an APC bead lot ID was not entered at the Set Up view, time-delay calibration is not performed, and the assay continues with compensation.

Compensation Adjustment

After the PMTs are successfully set and time-delay calibration is complete (if needed), the application window automatically advances to the Compensation view.



- 1 To begin compensation adjustments with the mixed beads tube (tube B):



For Manual Tube Loading

- a Remove tube A from the cytometer.
- b Gently mix tube B, and install it on the cytometer.
- c Click Start.
Acquisition begins.



For Automatic Tube Loading

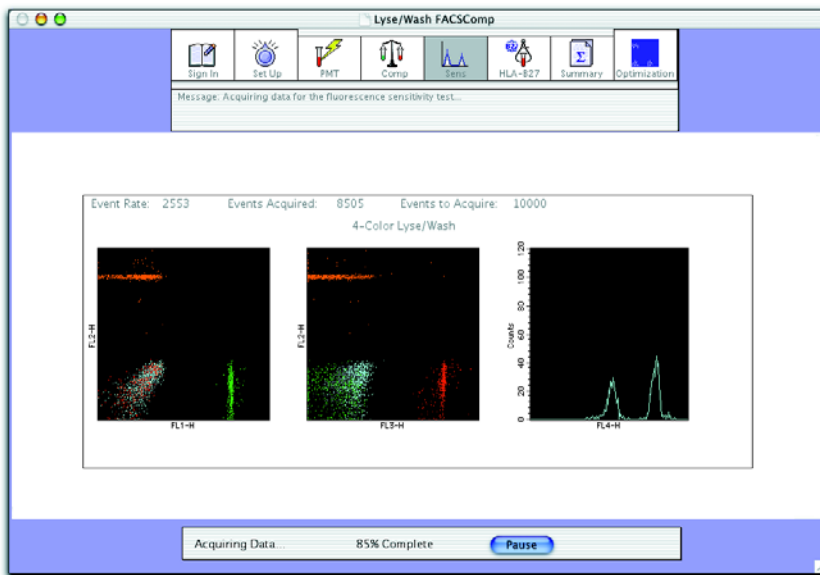
- No action is needed.
- The Loader lowers tube 1 and lifts tube 2 to the SIP.
- Acquisition begins.

The software adjusts compensation values until the mean differences (or mean for FL4) are close to their respective target values.

NOTE If APC beads are not detected, 3-color compensation is performed. If PerCP beads are not detected, 2-color compensation is performed.

When compensation adjustment is complete, the message *Compensation Set Successfully* appears. The software counts down for 5 seconds, allowing you to pause the assay before the application window automatically advances to the Sensitivity view.

Sensitivity Tests



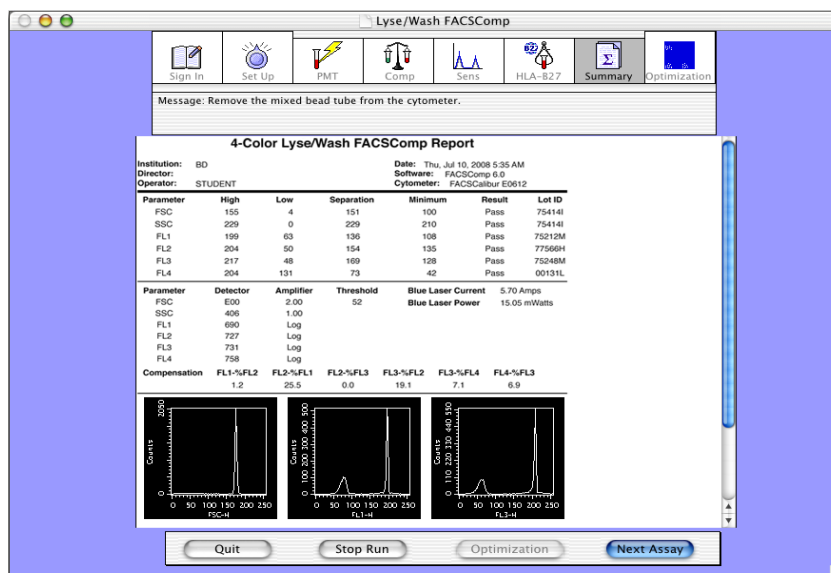
Three sensitivity tests are performed: the Fluorescence Sensitivity Test, the Side Scatter Sensitivity Test, and the Forward Scatter Sensitivity Test. The Summary Report indicates whether each sensitivity test passes or fails.

For more information about sensitivity tests, refer to the *BD FACSComp Software Reference Manual*.

Summary Report

When the sensitivity tests are complete, the application window automatically advances to the Summary Report view. The summary report is displayed in a scrollable window within the view.

- 1 Review the summary report.



- 2 Verify that the sensitivity tests passed.
- 3 Add a comment to the report.

IMPORTANT You cannot add comments to a summary report after leaving the Summary view.



For Automatic Tube Loading—To add a comment to the report, you must click Pause during the 5-second countdown.

- a Scroll down to the Comments section of the report.
 - b Click the comments area.
 - c Type *Operator Course practice*.
- 4 Click Stop Run.

The summary report prints automatically when leaving the Summary view.

4-Color Lyse/Wash FACSComp Report

Institution: BD
Director:
Operator: STUDENT

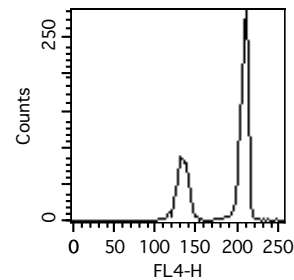
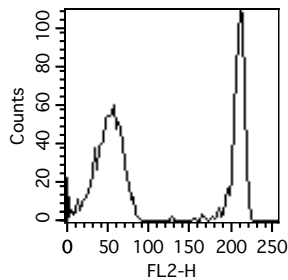
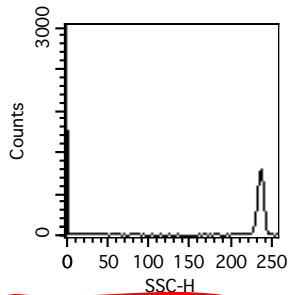
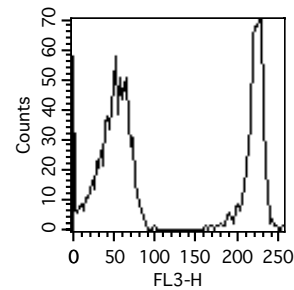
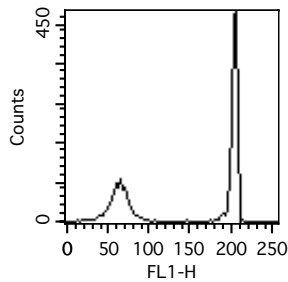
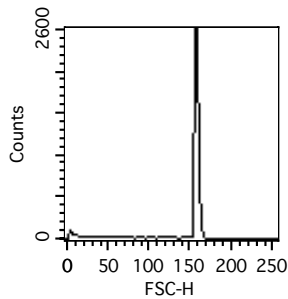
Date: Thu, Jul 10, 2008 5:35 AM
Software: FACSComp 6.0
Cytometer: FACSCalibur E0612

sensitivity
results

Parameter	High	Low	Separation	Minimum	Result	Lot ID
FSC	155	4	151	100	Pass	75414I
SSC	229	0	229	210	Pass	75414I
FL1	199	63	136	108	Pass	75212M
FL2	204	50	154	135	Pass	77566H
FL3	217	48	169	128	Pass	75248M
FL4	204	131	73	42	Pass	00131L

Parameter	Detector	Amplifier	Threshold	Blue Laser Current	Blue Laser Power
FSC	E00	2.00	52	5.70 Amps	15.05 mWatts
SSC	406	1.00			
FL1	690	Log			
FL2	727	Log			
FL3	731	Log			
FL4	758	Log			

Compensation	FL1-%FL2	FL2-%FL1	FL2-%FL3	FL3-%FL2	FL3-%FL4	FL4-%FL3
	1.2	25.5	0.0	19.1	7.1	6.9



Comments:

Time Delay Calibration Passed.
 Operator Course practice.

Ending the Run

- 1 Quit BD FACSComp software.



For Manual Tube Loading

- a Remove tube B from the cytometer.
- b Click Quit.



For Automatic Tube Loading

- a Click Quit.
The Loader lowers tube 2.
The automatic cleaning cycle begins.
Tube 39 runs for 90 seconds followed by tube 40, which runs for 90 seconds.
- b Remove the rack when the cleaning cycle is complete.

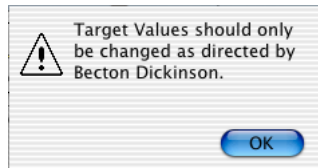
- 2 Install a tube containing no more than 1 mL of DI water on the SIP and place the cytometer in STNDBY mode.

Verifying Target Values

You will need to input new target values in BD FACSComp software when instructed by BD. Target values change when a new lot of raw bead materials is used to make BD Calibrite beads. This occurs periodically—once a year or once every two years.

- 1 Select FACSComp > Edit Target Values.

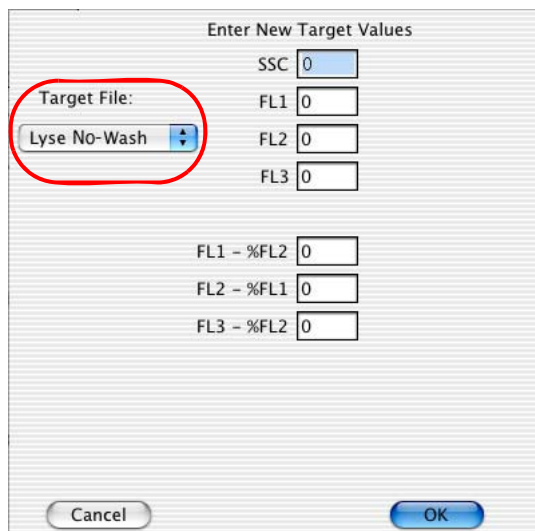
A dialog appears.



- 2 Click OK.
- 3 Compare the target values listed in the dialog that appears with those listed on the target values insert from the BD Calibrite bead kit you are using.

NOTE Target value inserts are provided in BD Calibrite bead kit only after a raw bead material change.

There are target values for Lyse/Wash and Lyse/No-Wash assays. To view target values for Lyse/No-Wash, click the Target File menu control and select Lyse/No-Wash.










- 4 Enter new values, if needed.
- 5 Click OK to close the dialog and save the settings.

BD FACSComp Software Preferences

You can change some default settings for BD FACSComp software in the BD FACSComp Preferences dialog.

Preference Overview

BD FACSComp software preferences are grouped into tabbed views as shown in the following table.

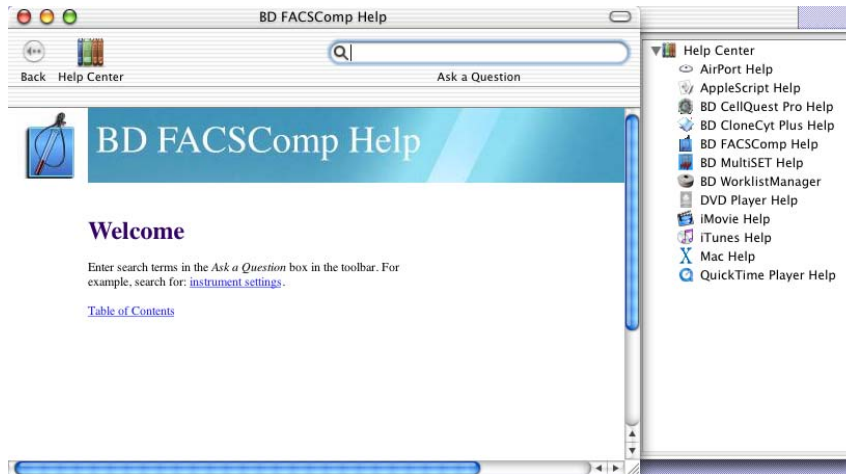
Preference Group	Description
 Optimization	In the Optimization view, you can customize several options. A default file name and optimization plot parameters are associated with each option. You can change the file name and the X and Y parameters for Optimization plots, and select the settings that are downloaded when you access the view.
 Export	Use the Export view to select a delimiter (tab or comma) for BD FACSComp and HLA-B27 Summary Report files (text versions) and Levey-Jennings data files. Delimiters are used to separate values in a text file.
 Printing	You can use the Printing view to specify if you want to automatically print Summary Reports.
 LJ Data	In the LJ Data view, you can choose the number of runs to be saved in Levey-Jennings data files: 30, 60, or None.
 Loader	Use the Loader preferences to specify whether or not you will be using a Loader for tube loading.
 Leucocount	You can use the Leucocount view to specify if you want to automatically save a Leucocount calibration file at the successful completion of any LNW assay.
 BD CBA	You can use the BD CBA view to specify if you want to automatically save a BD CBA calibration file at the successful completion of any LNW assay.

For more information about individual BD FACSComp software preferences, refer to the *BD FACSComp Software Reference Manual*.

Using BD FACSComp Software Help

When you need assistance with BD FACSComp software, use the Help viewer to quickly locate the information.

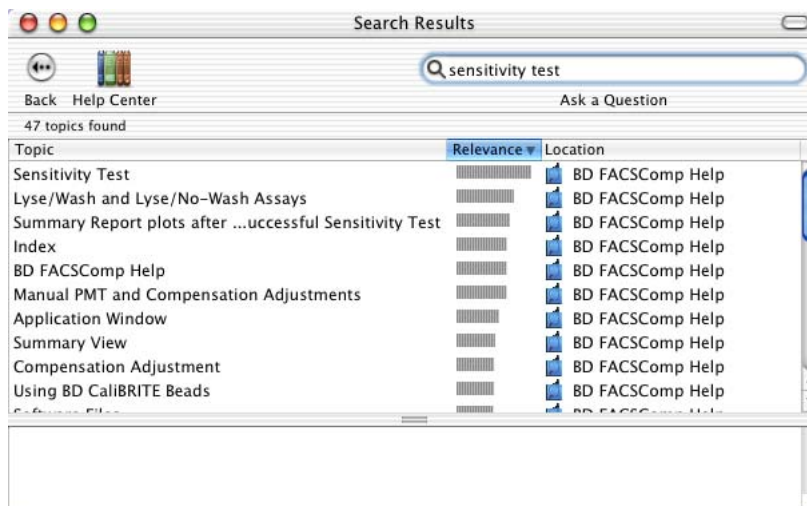
- 1 Select Help > FACSComp Help.



The fastest way to search for help is to use the Ask a Question feature. You can also search by looking through the table of contents or index.

- 2 Enter *sensitivity test* in the Ask a Question field, and press Return.

A list of topics appears in the lower portion of the Help view. Double-click a topic to display that section of Help.



- 3 When you are finished, close the Help viewer by clicking the red button in the upper-left corner of the viewer.

BD FACSComp File Naming Conventions

File Type	File Name (LW setup)	File Name (LNW setup)	Default Storage Location
Summary Report (text)	<i>DDMMYY.sum</i> <i>DDMMYY.PerCP-Cy5.5sum</i>	<i>DDMMYY.LNWsum</i> <i>DDMMYY.LNWPerCP-Cy5.5sum</i>	BD Files: BD FACSComp Files: dated folder from day assay was run
Summary Report (graphic)	<i>DDMMYY.sum.PDF</i> <i>DDMMYY.PerCP-Cy5.5sum.PDF</i>	<i>DDMMYY.LNWsum.PDF</i> <i>DDMMYY.LNWPerCP-Cy5.5sum.PDF</i>	BD Files: Instrument Settings Files
Calibration	Calib File Calib File.PerCP-Cy5.5	Calib File.LNW Calib File.LNWPerCP-Cy5.5	BD Applications: FACSComp Folder
Target	Target File	Target File.LNW	BD Applications: FACSComp Folder
Target Value Log	Target Value Log File	Target Value Log File.LNW	BD Applications: FACSComp Folder
Levey-Jennings Data	LJ Data LJ Data.PerCP-Cy5.5	LJ Data.LNW LJ Data.LNWPerCP-Cy5.5	BD Applications: FACSComp Folder

Troubleshooting

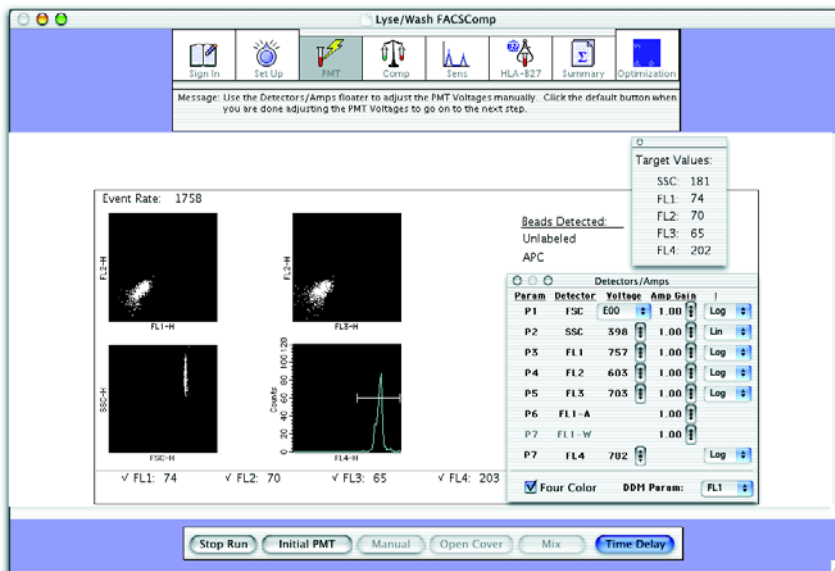
Performing Manual PMT Adjustments

During automatic PMT adjustments, BD FACSComp software will enter manual PMT adjustment mode if either:

- The sample flow rate is less than 400 events per second.
- Automatic PMT adjustments take longer than 75 seconds.

In manual PMT adjustment mode, the Target Value window appears.

- 1 Use the controls in the Detectors/Amps window to set the PMTs to ± 2 channels of the target values for the fluorescence PMTs.
- 2 For SSC, set the PMT to ± 5 channels of the target value.
- 3 If you are performing a 4-color assay, click Time Delay. Otherwise, click Next.

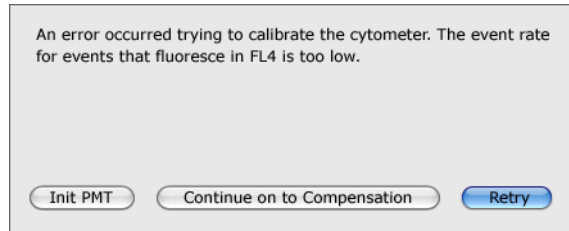


For more information about manual PMT adjustment mode, refer to the *BD FACSComp Software Reference Manual*.

Failed Time-Delay Calibration

If time-delay calibration is not successful, you have 5 seconds to choose one of the following options:

- Retry the calibration.
- Repeat initial PMT adjustment.
- Continue with compensation.



If you do not select an option within 5 seconds, the assay automatically continues with compensation.



If you continue with compensation after a failed time-delay calibration, the compensation values you obtain for FL3-%FL4 and FL4-%FL3 could be incorrect.

A failed time-delay calibration will be noted in the comments section of the Summary Report. If compensation does not appear to be correct during subsequent optimizations, troubleshoot for time-delay calibration errors. Refer to the *BD FACSComp Software Reference Manual* for more information.

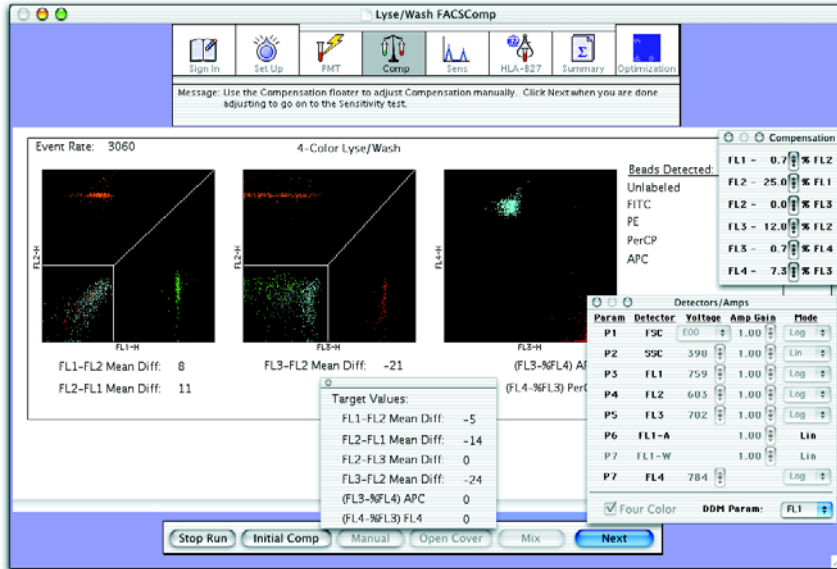
Performing Manual Compensation Adjustments

BD FACSComp software will enter manual compensation adjustment mode if any of the following conditions are met:

- The flow rate is less than 400 events per second.
- Automatic compensation adjustment takes longer than 75 seconds.
- A compensation value is greater than 50%.

In manual compensation adjustment mode, the Target Value window appears.

- 1 Use the controls in the Compensation window to set the mean difference values to within ± 2 channels of their respective target values.
- 2 Set the mean value for FL4 to within ± 2 channels of its target value.



For more information about manual compensation adjustment mode, refer to the *BD FACSComp Software Reference Manual*.

BD FACSComp Software Review

1 BD FACSComp software PMT settings should be adjusted manually if the event rate is below 400.

T F

2 What part of the BD Calibrite beads lot ID is used by BD FACSComp software? How is it used?

3 You need to enter new target values each time BD FACSComp software is run.

T F

4 Explain why time-delay calibration is performed.

BD CellQuest Pro Workflow

After completing this chapter, you will be able to:

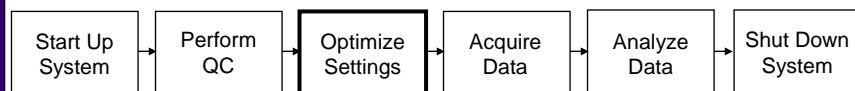
- Create a BD CellQuest Pro Experiment document.
- Optimize and save cytometer settings.
- Acquire and analyze data files.
- Use the Next Data File feature.



Optimization



Big Picture



- Set up a BD CellQuest Pro Experiment document.
- Optimize instrument settings for your sample type.
- Save optimized settings.

2



Optimization Steps

1. Adjust FSC and SSC detector settings.
2. Adjust FSC threshold setting.
3. Gate the population of interest.
4. Adjust fluorescence PMT voltages.
5. Adjust fluorescence compensation.

3



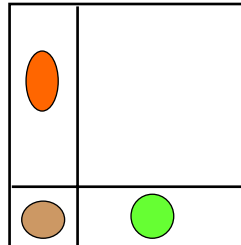
Optimization Samples

- Unlabeled Beads
- **FITC Beads**
- **PE Beads**
- **PerCP Beads**
- **APC Beads**

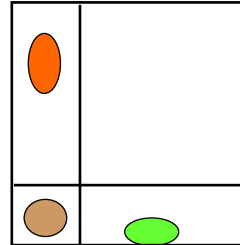
4

Overcompensation vs Undercompensation

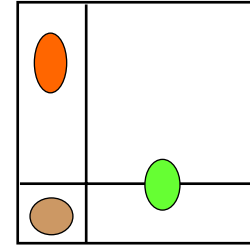
Correct Compensation



Overcompensation

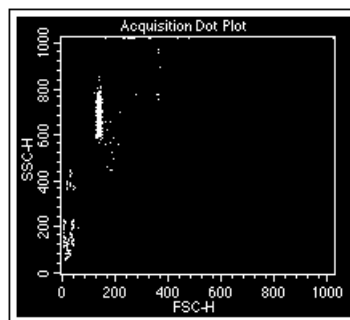


Undercompensation

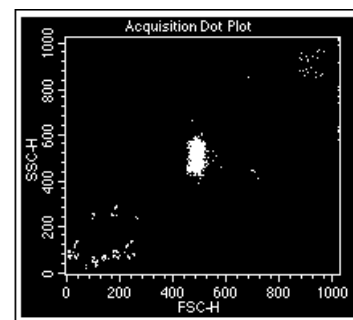


5

Adjust FSC and SSC Settings



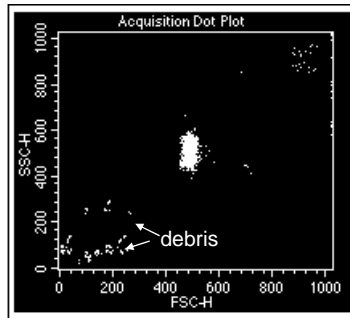
Before



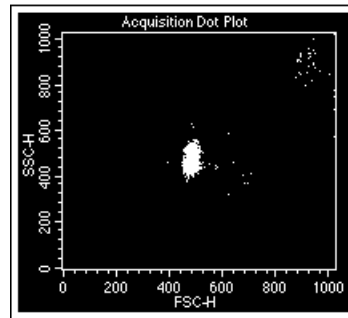
After

6

Adjust FSC Threshold



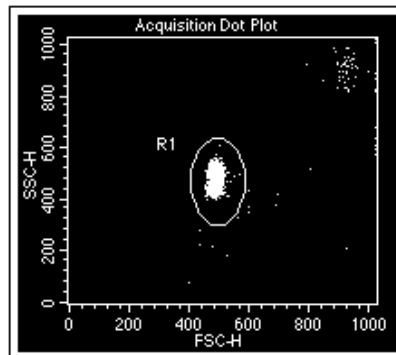
Before



After

7

Gate the Population of Interest



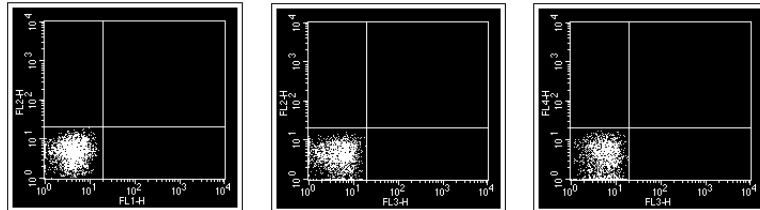
R1 encompasses the Singlet population

8



Optimize Fluorescence PMT Settings

Plots are gated to display R1 events.



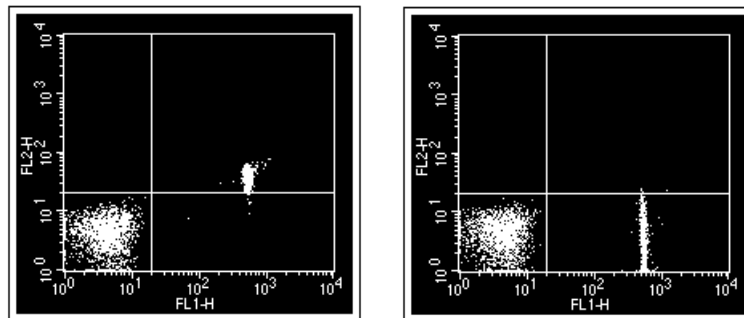
Place the negative population in the lower left quadrant.

9



Optimize Compensation – FITC Control

FL2-%FL1



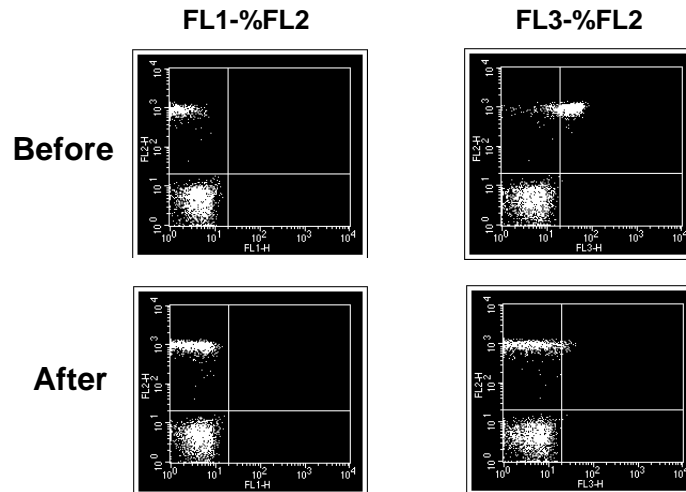
Before

After

10



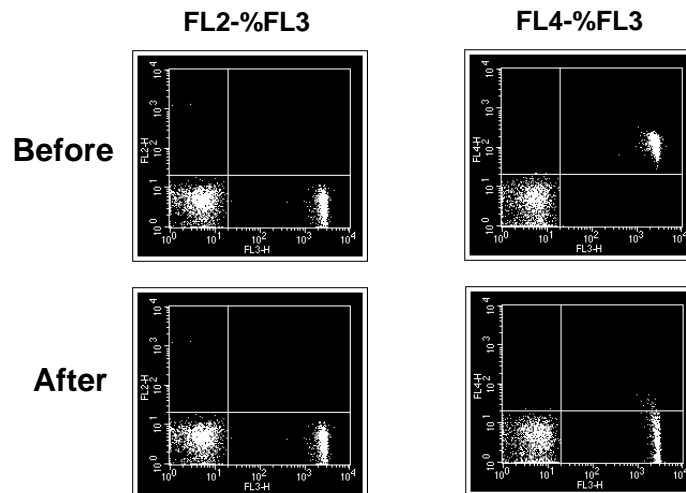
Optimize Compensation – PE Control



11



Optimize Compensation – PerCP Control

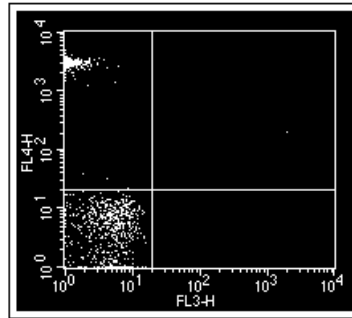


12

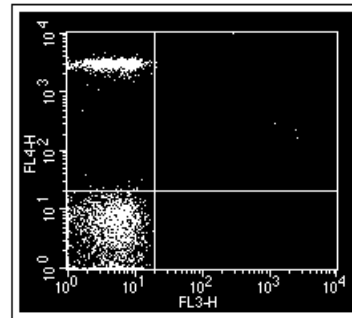


Optimize Compensation – APC Control

FL3-%FL4



Before

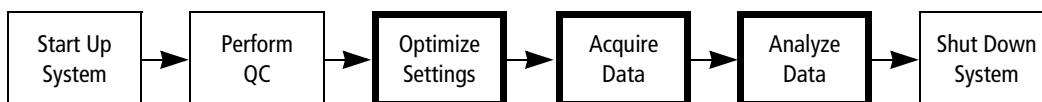


After

13

Introduction

Acquisition of flow cytometry data is a multistep process. The following figure outlines the main steps involved. This chapter focuses on optimization of instrument settings, data acquisition, and data analysis.



Materials

BD Calibrite beads will be used in the exercises in this chapter to demonstrate the data acquisition process. These same steps can be used for the immunophenotyping of many cell types including peripheral blood samples, bone marrow, cell lines, and mouse cells.

The following bead tubes will be provided by your instructor for these exercises:

- Unlabeled beads
- FITC beads
- PE beads
- PerCP beads
- APC beads
- Mixed beads 1
- Mixed beads 2
- Mixed beads 3

Before You Begin

Before beginning the exercises in this chapter, you should perform the following procedures:

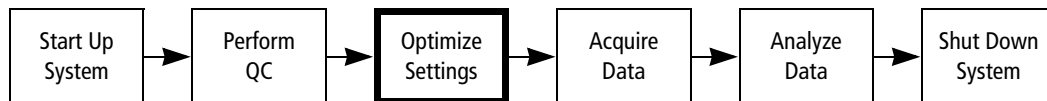
- System Startup on page 33.
- Performing QC and Instrument Setup on page 71.

Regions, Gates, Markers And Statistics

In this chapter, you will use regions, gates, and markers to define subpopulations and generate statistics for beads. These tools and methods will be described and explored in greater detail in Chapter 6, BD CellQuest Pro Software Analysis Tools.

Optimizing Settings

In this exercise, you will create an Experiment document and use it to optimize instrument settings.



Creating a Custom Experiment Document

In this section you will start BD CellQuest Pro software, add plots to a blank Experiment document, and save the customized Experiment document.

Starting BD CellQuest Pro Software

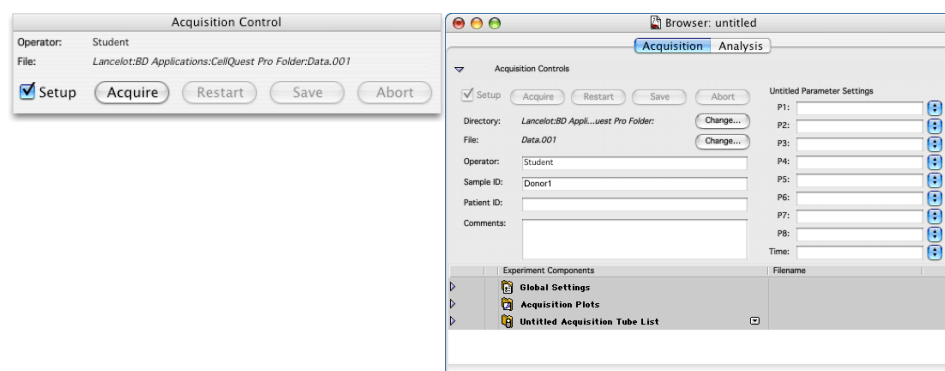
- 1 Start BD CellQuest Pro software by clicking its icon on the Dock.



A blank Experiment document opens.

- 2 Click the green button in the upper-left corner of the Experiment window to expand it to full size.
- 3 Select Acquire > Connect to Cytometer (⌘-B).

The Acquisition Control dialog and the Acquisition view of the Browser appear.




TIP When you click the Experiment document, the Browser will move to the back of the active window. If the Browser is hidden, select Windows > Show Browser.

- 4 Click the red button in the upper-left corner of the Browser to close the Browser.

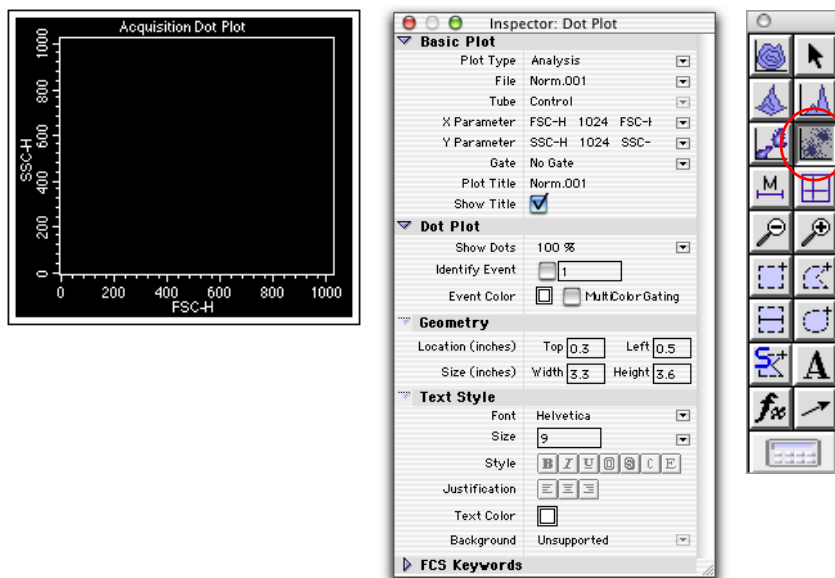
Creating Plots

You'll need to create some plots in which to display data before you can optimize cytometer settings.

Using the Dot Plot Tool

- 1 Click the Dot plot tool () on the tool palette to select it.
- 2 Click in an unused area of the Experiment document.
- 3 Drag diagonally until the plot outline is the appropriate size.

A dot plot is created on the Experiment document, and the dot plot Inspector appears.



Setting Plot Properties with the Inspector


- 1** Specify settings in the Basic Plot section.
 - a** Expand the Basic Plot section, if needed.
 - b** Click the Plot Type menu control and select Acq → Analysis.
 - c** Verify that the X parameter is FSC-H and the Y parameter is SSC-H.
 - d** Display the plot title by clicking the Show Title checkbox.
- 2** Specify settings in the Geometry section.
 - a** Expand the Geometry section, if needed.
 - b** Move the plot to the upper left corner by specifying 0.0 for both the Top and Left locations.
 - c** Make the plot square by specifying 3.0 for both the Width and Height sizes.

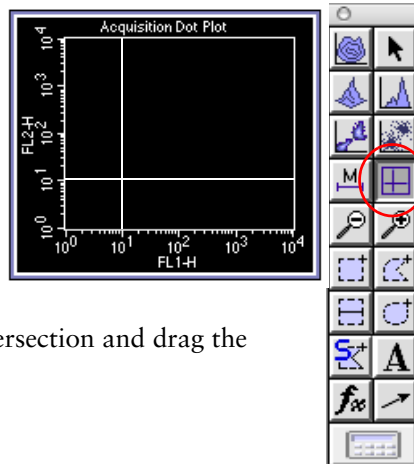
Creating Additional Plots

Now you'll create additional Acq → Analysis dot plots by duplicating the first one and then using the Inspector to make changes.

- 1** Create an FL1-H vs FL2-H plot.
 - a** Select the FSC-H vs SSC-H dot plot.
 - b** Select Edit > Duplicate. Click in the plot border to select it and drag the plot to a desired location.
 - c** Use the Inspector to change the axes to FL1-H vs FL2-H.
 - d** Use the Inspector to align the plots if desired.
- 2** Repeat step 1 to create an FL3-H vs FL2-H plot.
- 3** Repeat step 1 to create an FL3-H vs FL4-H plot.

Adding Quadrant Markers to Fluorescence Plots

- 1 Add quadrant markers to the FL1 vs FL2 plot.
 - a Select the Quadrant-Marker tool () on the tool palette to highlight it.
 - b Click in the FL1 vs FL2 plot and drag the handle of the marker so the marker is set at 10^1 in FL1 and 10^1 in FL2. Release the mouse button to set the quadrant markers.



To change the position, click the marker intersection and drag the marker to a new location.

- 2 Add quadrant markers to the FL3 vs FL2 plot.
- 3 Add quadrant markers to the FL3 vs FL4 plot.

Saving an Experiment Document

Save your Experiment document to protect your work up to this point.

- 1 Select File > Save Document.
- 2 Navigate to your student folder and name the document *4-Color Beads*.
- 3 Click Save.

Optimizing Cytometer Settings

Before collecting data, you will optimize cytometer settings for your samples using the following standard optimization sequence:

- Adjust FSC amp gain and SSC detector voltage
- Adjust FSC threshold setting
- Gate on the population of interest
- Adjust FL1, FL2, FL3, and FL4 detector voltages
- Adjust fluorescence compensation

Adjusting FSC Amp Gain and SSC Voltage

In this section, you will adjust the FSC amp gain and SSC detector voltage to place the population of interest (singlet beads) on scale.

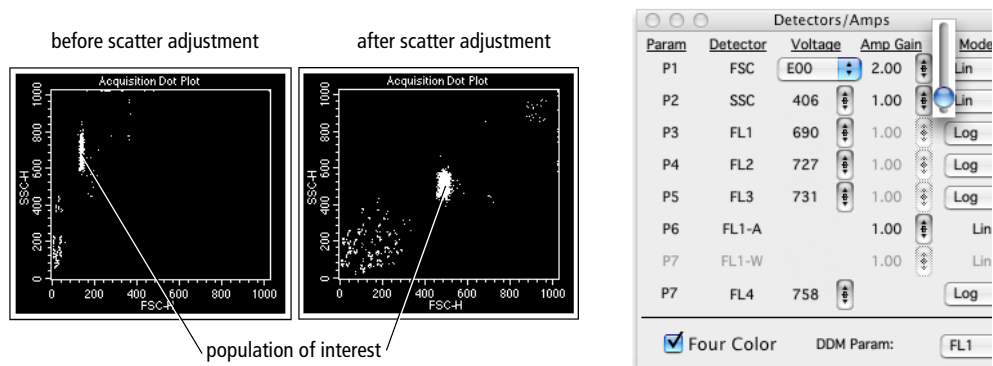
TIP You will use the Unlabeled bead tube to optimize FSC and SSC settings. However, any setup tube could be used for this step since the light scatter properties of the beads in all tubes are similar.

- 1 Place the Unlabeled bead tube on the SIP.
- 2 Set the flow rate to MED to conserve sample, and place the cytometer in RUN mode.
- 3 Confirm that the Setup checkbox is selected in the Acquisition Control dialog, and then click Acquire.

TIP When the Setup checkbox is checked, you can view events in experiment plots without saving data.

- 4 Select Cytometer > Detectors/Amps to display the detectors and amps controls.
- 5 Drag the Detectors/Amps window to a clear area of the screen.
- 6 Using the controls in the Detectors/Amps window, adjust the FSC Amp Gain and the SSC voltage to place the singlet beads near the center of the FSC vs SSC plot.

TIP Click the Pause and Restart acquisition controls periodically during the optimization process to quickly update the events displayed in the plots.



There are several ways to adjust instrument settings in the Detectors/Amps window:

- Click the up or down arrow to increase or decrease the value by 1.
- Hold down the Option key, and click the up or down arrow to increase or decrease the value by 10.
- Click and hold between the two arrows to obtain a slider bar. Drag the slider.

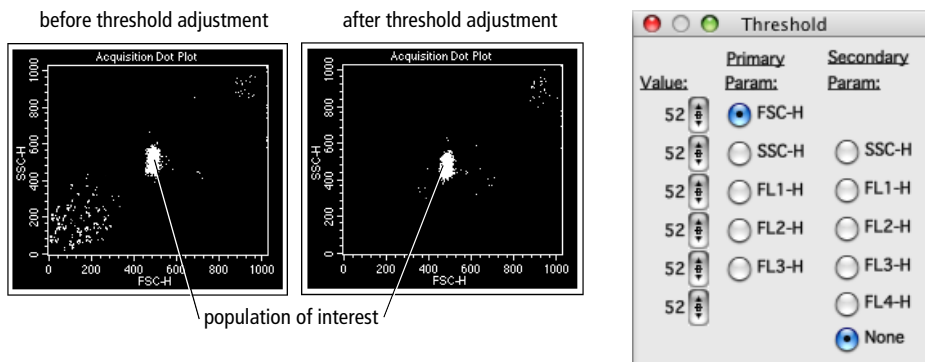
Adjusting FSC Threshold

Threshold is used to eliminate the electronic processing of unwanted events. This optimization improves the likelihood of processing events of interest. The samples you are running contain debris, which usually consists of small particles appearing in the lower end of the FSC scale. Setting the FSC threshold at a value above the debris eliminates processing of debris events.

TIP For other applications, you may need to threshold a parameter other than FSC.

- 1 Select Cytometer > Threshold to display the Threshold window.
- 2 Click and drag the Threshold window to a clear area of the screen.
- 3 Using the controls in the Threshold window, adjust the FSC threshold value until most of the debris is excluded.

IMPORTANT Avoid setting the threshold so high that it eliminates events from the population of interest.




Gating on the Population of Interest

Different cell populations can have different autofluorescent characteristics. To optimize fluorescence instrument settings for the population of interest, draw a region encompassing the singlet beads and view only those events when adjusting fluorescence PMT voltages.

TIP Clicking in a plot makes it active (colored border). When a plot is active, you can create regions in that plot. You cannot delete, move, or resize an active plot.

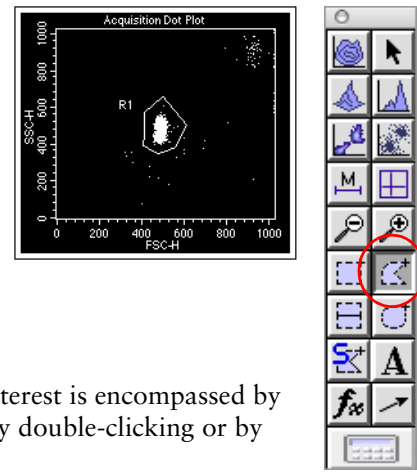
- 1 Create a polygon region encompassing the population of interest in the FSC vs SSC plot.

- a Click the Polygonal-Region tool () on the tool palette to select it.

- b Move the cursor over the plot on which you want to create the region. Note that the cursor changes to a crosshair. Click to set the first vertex.

- c Release the mouse button and move the cursor to where you want the second vertex and click again.

- d Continue this way until the population of interest is encompassed by the region. You can close the region either by double-clicking or by clicking on the first vertex.



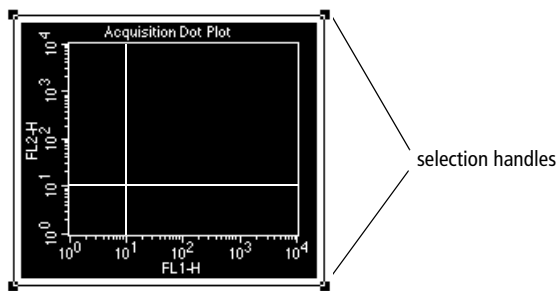
- 2 Click outside the region to deselect it, then click and drag the region label R1 to a new location on the plot away from the data.
- 3 Remove the Unlabeled bead tube from the SIP to conserve sample while you prepare to do the next optimization step.

Adjusting the Fluorescence Detector Settings

Negative populations exhibit measurable amounts of background fluorescence (non-antigen specific binding in combination with autofluorescence). Adjust the FL1, FL2, FL3, and FL4 detector settings to place negative populations in the lower-left corner of the fluorescence plots.

- 1 Select the three fluorescence plots (FL1-H vs FL2-H, FL3-H vs FL2-H, and FL3-H vs FL4-H).

TIP To select a plot, click on the border of the plot. Square selection handles appear in the corners of the plot. To select multiple plots, hold down the Shift key while clicking the border of each plot.



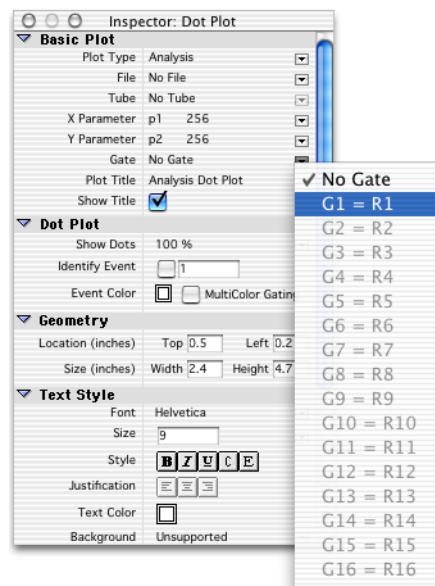
When a plot is selected, you can click on its border and move it. You can also click on a square handle and resize it. Delete a selected plot by pressing Delete or by selecting Edit > Clear.

- 2 Select Windows > Show Inspector.

The Inspector displays information about all three selected plots.

- 3 Click the Gate menu control and select G1 = R1.

The selected fluorescence plots will now display only events that are in the population gated by G1.



- 4 Verify that the mode for the FL1, FL2, FL3, and FL4 parameters are set to LOG in the Detectors/Amps window.

- 5 Install the Unlabeled bead tube on the SIP.

- 6 Click Pause, Restart in the Acquisition Controls dialog.

- 7 Adjust the FL1, FL2, FL3, and FL4 voltages in the Detectors/Amps window to place the negative population in the lower-left quadrant.

- 8 Click Pause in the Acquisition Control dialog.

- 9 Remove the Unlabeled bead tube from the SIP.

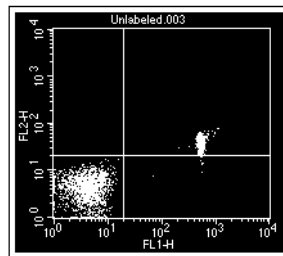
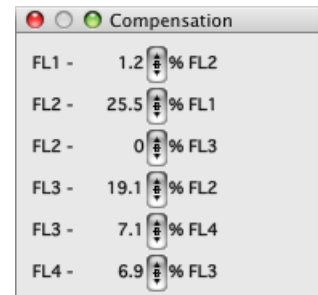
- 10 Close the Detectors/Amps and Threshold windows.

Adjusting Fluorescence Compensation

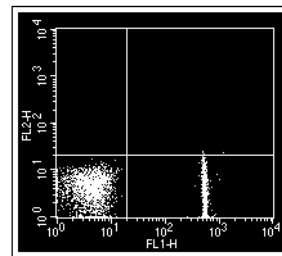
The last step in the optimization process is adjusting compensation to correct for spectral overlap in fluorochrome emissions.

IMPORTANT Compensation adjustments are dependent on the fluorescence detector voltage settings. Do NOT adjust detector voltages after compensation adjustment begins. If a fluorescence detector voltage is changed, you must redo your compensation adjustments.

- 1 Select Cytometer > Compensation to display the Compensation window.
- 2 Click and drag the Compensation window to a clear area of the screen.
- 3 Adjust FL2-%FL1 compensation.
 - a Place the FITC bead tube on the SIP.
 - b Click Restart in the Acquisition Control dialog.
 - c Adjust the FL2-%FL1 control to move the FITC⁺ population into the lower right quadrant, and align it with the negative population on the FL1-H vs FL2-H plot.



before compensation adjustment

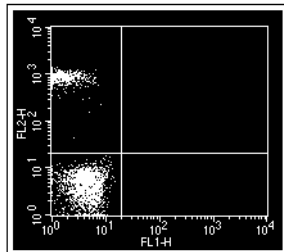


after compensation adjustment

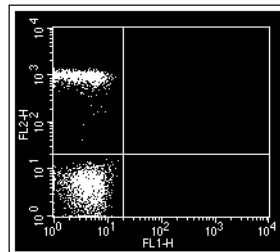
- d Remove the FITC bead tube from the SIP.

4 Adjust FL1-%FL2 and FL3-%FL2 compensation.

- a** Place the PE bead tube on the SIP.
- b** Adjust the FL1-%FL2 control to move the PE⁺ population into the upper left quadrant, and align it with the negative population on the FL1-H vs FL2-H plot.

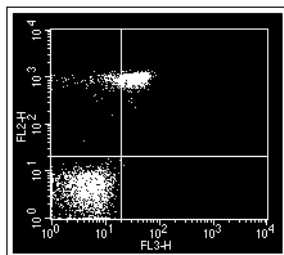


before compensation adjustment

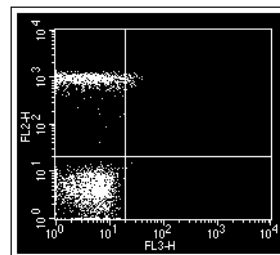


after compensation adjustment

- c** Adjust the FL3-%FL2 control to move the PE⁺ population into the upper left quadrant, and align it with the negative population on the FL1-H vs FL2-H plot.



before compensation adjustment



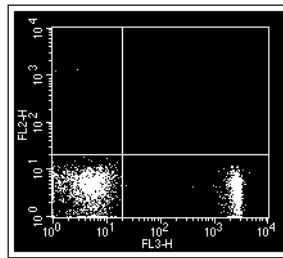
after compensation adjustment

- d** Remove the PE bead tube from the SIP.

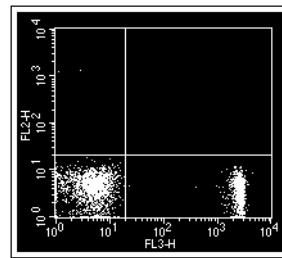
5 Adjust FL2-%FL3 and FL4-%FL3 compensation.

- a** Place the PerCP bead tube on the SIP.
- b** Adjust the FL2-%FL3 control to move the PerCP⁺ population into the lower right quadrant, and align it with the negative population on the FL3-H vs FL2-H plot.

TIP The adjusted FL2-%FL3 setting is typically near 0.0%. This is because the range of PerCP emission does not extend into the range of FL2 detection. When other fluorochromes are used with FL3, more compensation may be necessary.

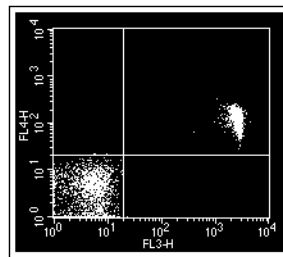


before compensation adjustment

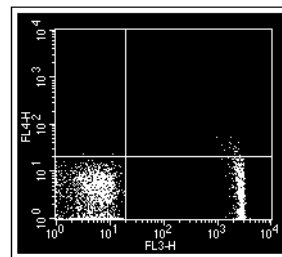


after compensation adjustment

- c** Adjust the FL4-%FL3 control to move the PerCP⁺ population into the lower right quadrant, and align it with the negative population on the FL3-H vs FL4-H plot.



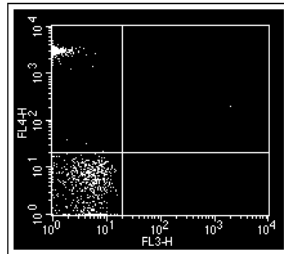
before compensation adjustment



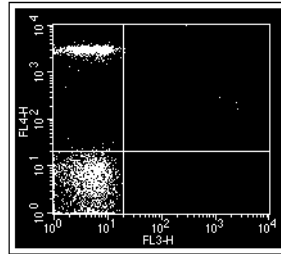
after compensation adjustment

- d** Remove the PerCP bead tube from the SIP.

- 6** Adjust FL3-%FL4 compensation.
 - a** Place the APC bead tube on the SIP.
 - b** Adjust the FL3-%FL4 control to move the APC⁺ population into the lower right quadrant, and align it with the negative population on the FL3-H vs FL4-H plot.



before compensation adjustment



after compensation adjustment

- 7** Close the Compensation window.
- 8** Click Pause, then Abort in the Acquisition Control window.
- 9** Remove the APC bead tube from the SIP.
- 10** Place a tube containing no more than 1 mL of DI water on the SIP.
- 11** Place the cytometer in STNDBY mode.
- 12** Select File > Save Document to save recent changes to your Experiment document.

Saving Instrument Settings

The optimized instrument settings can be saved for use at a later time.

- 1 Select Cytometer > Instrument Settings.

TIP If the Instrument Settings menu item is inactive, click Pause and then Abort in the Acquisition Control dialog.

- 2 Click Save in the Instrument Settings window.

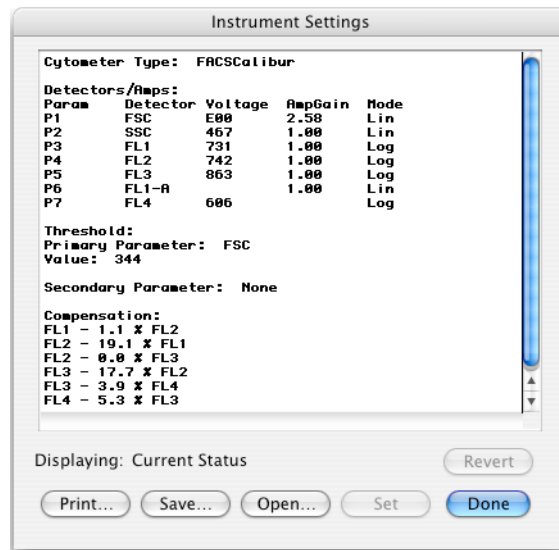


Figure 4-1 Instrument Settings dialog

- 3 Navigate to your student folder and name the instrument settings file *4-Color Beads Settings*.
- 4 Click Save.
- 5 Click Done in the Instrument Settings window.

Restoring Instrument Settings

You can restore instruments settings in one of two ways:

- From an instrument settings file.
- From an FCS data file.

In this exercise, you will restore the original Calib File instrument settings.

- 1** Select Cytometer > Instrument Settings.
- 2** Click Open.
- 3** Navigate to BD Files/Instrument Settings Files, select the Calib File, and click Open.

The Calib File settings appear in the Instrument Settings window.

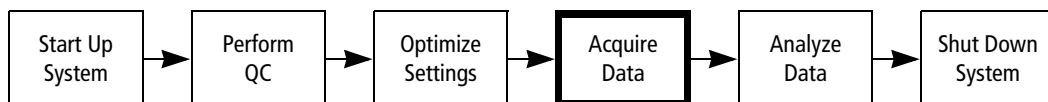
- 4** Click Set in the Instrument Settings window to send the settings to the cytometer.

IMPORTANT You must click Set to send the displayed instrument settings to the cytometer.

- 5** Click Done.

Acquiring Data

In this exercise, you will specify data collection information, and then acquire and save FCS data files for three sample tubes.



Specifying Instrument Settings

- 1 Verify that your 4-Color Beads Experiment document is open.

If needed, select File > Open Document and then navigate to your student folder, select the 4-Color Beads document, and open it.

- 2 Verify that your optimized instrument settings are set. If needed, use the procedure in Restoring Instrument Settings on page 113 to restore the optimized settings that you saved to your student folder.

Specifying Data Collection Information

Before you acquire data, you need to specify data collection information, including data file name, storage location, parameter labels, and number of events to save.

Specifying Data File Name and Location

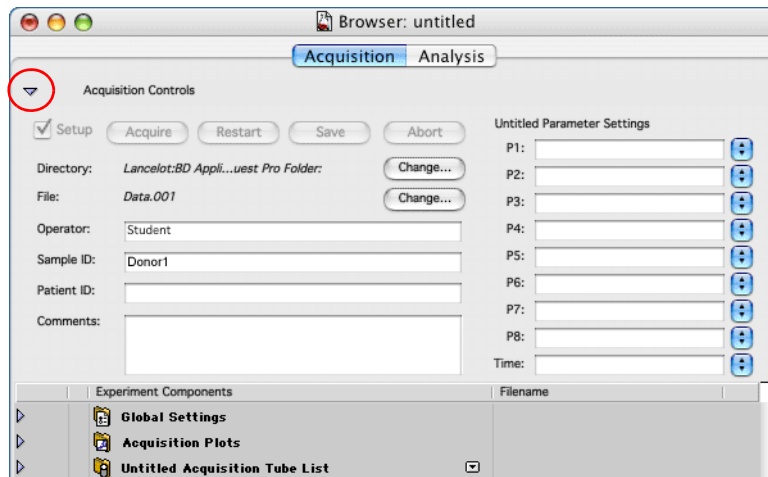
- 1 Verify that your 4-Color Beads Experiment document is open.

If needed, select File > Open Document and then navigate to your student folder, select the 4-Color Beads document, and open it.

- 2 Select Windows > Show Browser.

The Acquisition view of the Browser is displayed. File storage and parameter labels are specified in the Acquisition view of the Browser.

- 3 Click the Acquisition Controls disclosure triangle to view the controls, if needed.

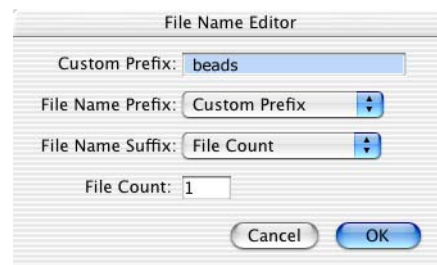


- 4 Specify a folder in which to save your FCS data files.

- a Click the Directory Change button.
- b Navigate to your student folder, and select it.
- c Click Open.

- 5 Specify file names for your FCS data files.

- a Click the File Change button.
The File Name Editor appears.
- b Type *beads* in the Custom Prefix field.
- c Verify that File Name Suffix is set to File Count.
- d Verify that File Count is set to 1.
- e Click OK to close the File Name Editor.



- 6 Type *Student* in the Operator field.
- 7 Type *Donor1* in the Sample ID field.
- 8 Save the Experiment document.

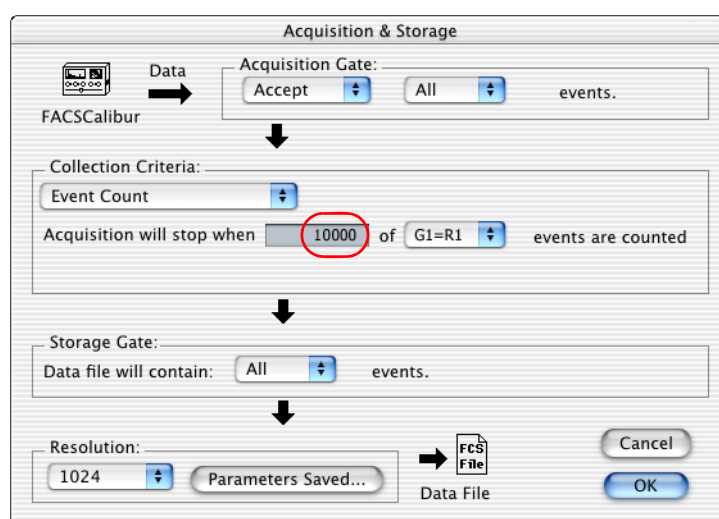
Specifying Acquisition and Storage Settings

Use global acquisition and storage settings to define the number and type of events to acquire and store for each tube. Global acquisition settings apply to all tubes in an Experiment document that do not have tube-specific settings.

NOTE There are two ways to display and set global acquisition and storage settings, the Acquisition & Storage dialog and the Acquisition & Storage Inspector. In this exercise you will use the Acquisition & Storage dialog. For more information on acquisition and storage settings, refer to the *BD CellQuest Pro Software Reference Manual*.

- 1 Select Acquire > Acquisition & Storage.

The Acquisition & Storage dialog appears.



- 2 Specify settings in the Acquisition & Storage dialog.
 - a Verify that Acquisition Gate is set to the defaults Accept and All.
 - b Verify that Collection Criteria is set to Event Count.
 - c Type 10000 in the *Acquisition will stop when* field and select G1 = R1 (as the stopping gate).
 - d Verify that Storage Gate is set to the default All.
 - e Verify that Resolution is set to the default 1024.

With these settings, data collection will stop when 10,000 events is reached for the population defined by gate G1. However, all events acquired to that point are saved.

- 3 Click OK to close the Acquisition & Storage window, if needed.
- 4 Save the Experiment document.

Specifying Parameter Labels

Parameter labels allow you to identify parameters with reagent-specific names. There are two ways to specify parameter labels:

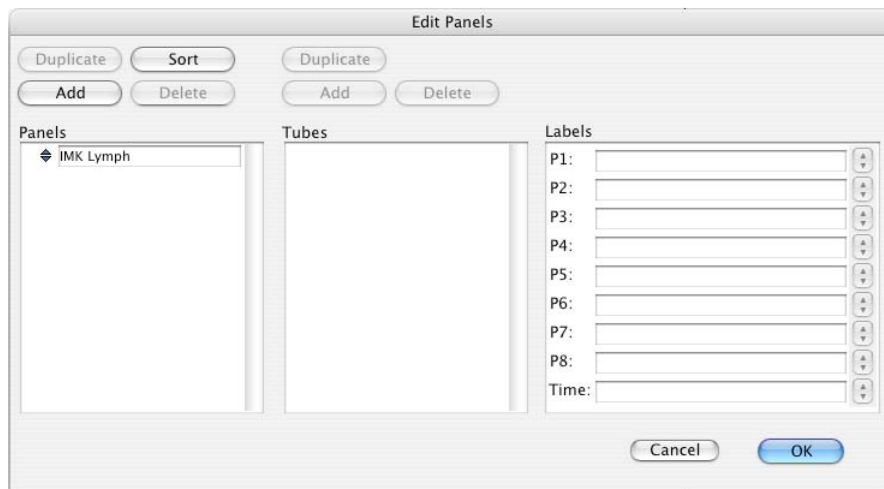
- Create a predefined list of labels and save it as a reagent panel that can be reused. Use reagent panels if you are acquiring the same reagent combinations (one or more tubes) for many experiments or donors. In this exercise, you will define a one-tube reagent panel for mixed beads. In a later exercise, Creating a Reagent Panel on page 141, you will create a multiple-tube reagent panel for immunophenotyping.
- Use the browser to enter labels for each tube. Use this method when you are acquiring a small number of tubes, or tubes stained with a unique reagent combination. You will practice this method in Specifying a Tube List on page 134.

Creating a Reagent Panel

In this section, you will create a single-tube panel. The tube will have the following BD Calibrite beads:

- FITC
- PE
- PerCP
- APC

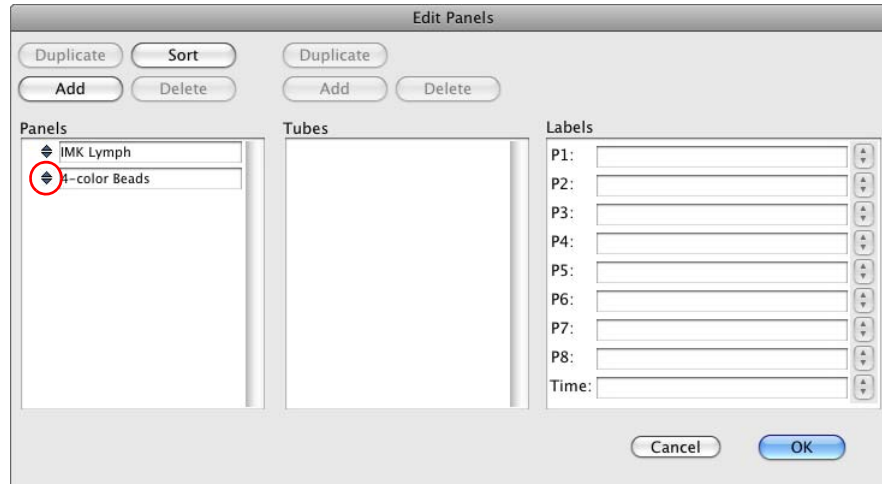
- 1 Select Acquire > Edit Panels to display the Edit Panels dialog.



- 2 Click Add (above the Panels list).

A new panel appears in the Panels list.

- 3 Type *4-color Beads* for the panel name.



- 4 Click the selection icon (to the left of 4-color Beads) to select the panel.

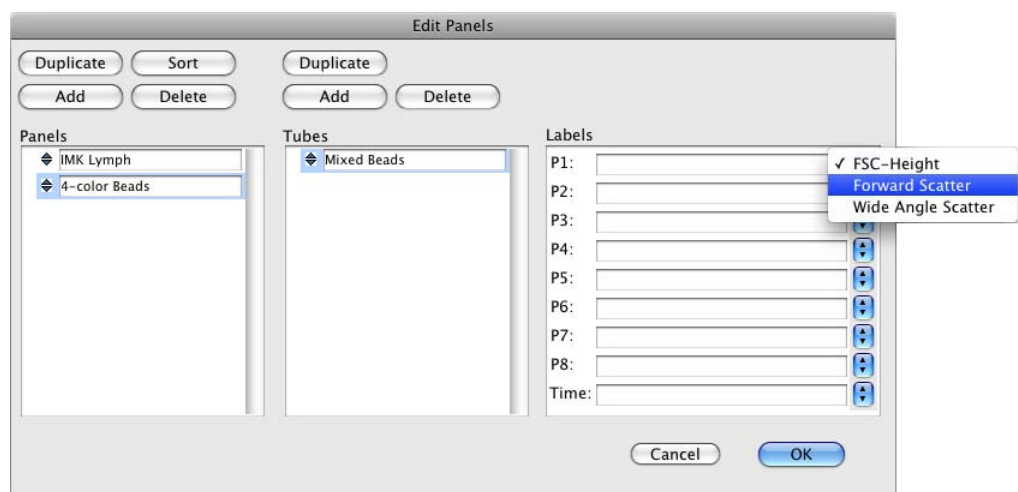
- 5 Click Add above the Tubes list.

A new tube appears in the Tubes list with the default name *tube #1*.

- 6 Change the tube name to *Mixed Beads*.

- 7 Click the selection icon to the left of Mixed Beads to select the tube.

- 8 Click the menu control to the right of the P1 label field and select Forward Scatter.



- 9 Click the menu control to the right of the P2 label field and select Side Scatter.
- 10 Specify fluorescence parameter labels for the Mixed Beads tube by typing the labels in the following table for parameters P3, P4, P5, and P7.

IMPORTANT Parameter P6 is not used. When entering the parameter labels, skip P6 and enter APC for parameter P7.

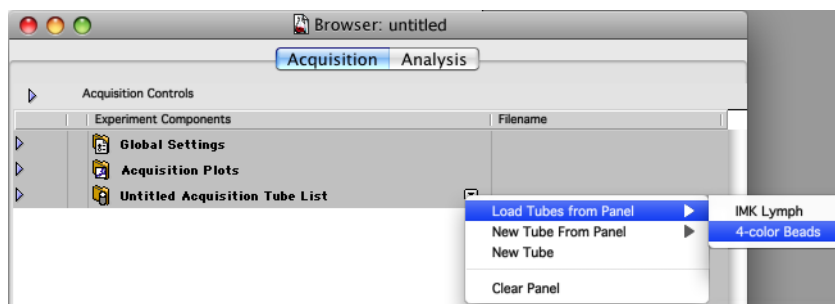
Parameter	Label
P3	FITC
P4	PE
P5	PerCP
P7	APC

- 11 Click OK to close the Edit Panels dialog.

Using a Reagent Panel

In this section you will load your new 4-color Beads panel into the Browser tube list.

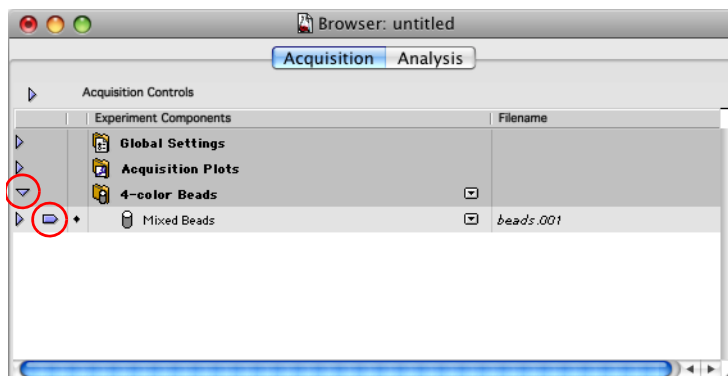
- 1 If needed, select Windows > Show Browser.
- 2 In the Browser, click the menu control next to Untitled Acquisition Tube List and select Load Tubes from Panel > 4-color Beads.



The name of the tube list changes to 4-color Beads.

- 3 If needed, click the 4-color Beads disclosure triangle to display its tubes.

A pointer indicates that parameter labels for the Mixed Beads tube will be used for the next acquisition.



- 4 Select File > Save Document As.
- 5 Save a copy of the Experiment document named *4-color Beads* in your student folder.

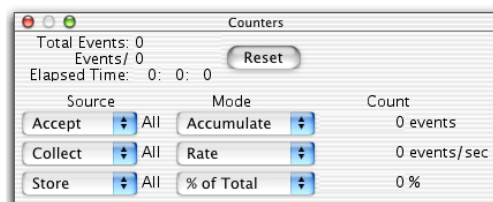
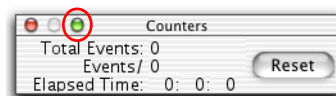
The next time you open the 4-color Beads Experiment, the 4-color Beads panel will already be loaded in the Browser tube list.

Acquiring and Saving Data

At this point, the instrument settings are optimized for your samples, the number of events to acquire and a storage gate have been established, and file location and naming have been specified. Data for the three sample tubes can now be collected and saved.

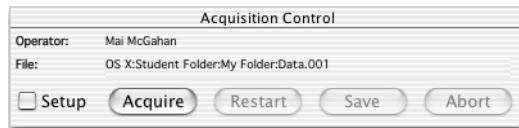
- 1 Select Acquire > Counters to display the Counters window, if needed.

The Counters window has two sizes. Click the green Zoom button to obtain the enlarged window if you are acquiring gated data.



- 2 Move the Counters window to a clear area of your screen.

- 3 Clear the Setup checkbox in the Acquisition Control window.



- 4 Verify in the Browser that the 4-color Beads panel has been loaded, the acquisition pointer is pointing to the only tube in the panel, and the parameter labels are correct.

NOTE When using a multiple tube panel or a manual tube list, you should also verify that:

- The tubes in the tube list are in the correct order.
- The acquisition pointer is pointing to the first tube to be acquired.

- 5 Place the cytometer in RUN mode.

- 6 Install the first sample tube on the SIP (immediately place the support arm under the tube).

IMPORTANT There is a brief period during which the sample tube is being pressurized. Wait approximately 5 seconds for the pressure in the sample tube to stabilize before clicking Acquire.

- 7 Click Acquire.

Live quadrant statistics will be displayed as the data is acquired. When acquisition for the tube is complete, the system beeps and in the Browser, the file name to the right of the tube increments to beads.002.

- 8 Remove the tube after the file name increments to beads.002.

TIP When there is more than one tube in the tube list and acquisition for a tube completes, a green check appears next to the tube, and the pointer moves to the next tube in the list. Since you are using a one tube panel, the green check does not appear.

- 9 Verify that the acquisition pointer is still pointing to the only tube in the 4-color Beads panel, and the parameter labels are correct.

- 10 Place the next sample tube on the SIP.

- 11 Wait approximately 5 seconds and then click Acquire.

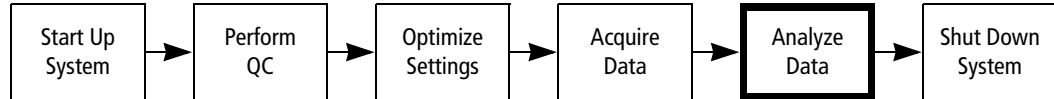
- 12 Remove the tube after the file name increments in the Browser.

- 13 Repeat steps 9 through 12 for the last tube.

- 14 Place a tube containing no more than 1 mL of DI water on the SIP and place the cytometer in STNDBY mode.

Analyzing Data

In this exercise, you will analyze data by creating plots for displaying data, and creating gates for generating statistics. In addition you will learn how to perform a batch analysis.



This exercise uses BD Calibrite beads to demonstrate the analysis features of BD CellQuest Pro software. You can apply the same principles to analyze peripheral blood and many other sample types.

Displaying Data

In this exercise, you will create dot plots for displaying and analyzing data. See Chapter 6, BD CellQuest Pro Software Analysis Tools, and refer to the *BD CellQuest Pro Software Reference Manual*, for information about using other plot types for data analysis.


TIP You can export plots in a variety of formats and save Experiment documents as PDF files, if needed. Refer to the *BD CellQuest Pro Software Reference Manual* for details.

- 1 Start BD CellQuest Pro software, if necessary.

If BD CellQuest Pro software is already open, select File > New Document.

- 2 Expand the Experiment window to full size by clicking the zoom button.

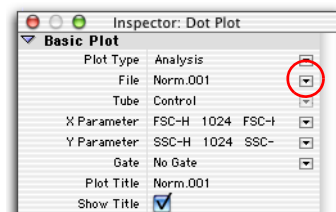
Displaying Data in a Dot Plot

- 1 Select the Dot-Plot tool () from the tool palette.

The Dot-Plot tool becomes highlighted.

- 2 Click in a clear area of the Experiment window and drag diagonally until the plot outline is an appropriate size.

When you release the mouse button, a dot plot and the Dot Plot Inspector appear.

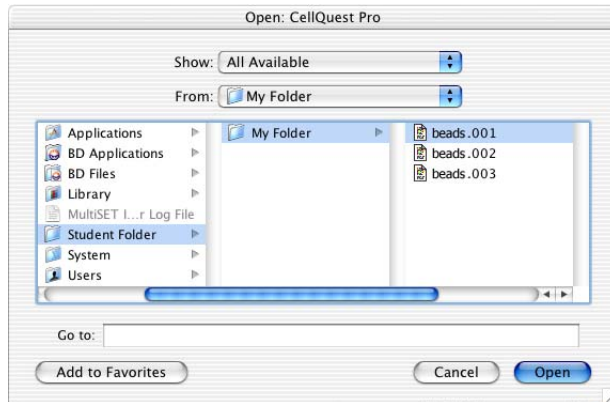


- 3 Click the File menu control and select Select File.

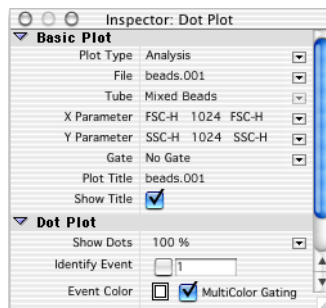
The Open a Data File dialog appears.

TIP To display FCS data files, you must first create an analysis plot and then select Select File from the Inspector. You cannot open an FCS data file in BD CellQuest Pro software by double-clicking its icon.

- 4 Navigate to the data files you acquired in the previous exercise, select the first tube's data file, and then click Open.



- 5 Select Forward Scatter as the X parameter and Side Scatter as the Y parameter.
- 6 Click the MultiColor Gating checkbox under Dot Plot in the Inspector.
- 7 Use the Geometry section of the Inspector to resize the plot to 3 inches square.



- 8** Create a second dot plot by duplicating the first.
 - a** Select the dot plot and select Edit > Duplicate.

A duplicate dot plot appears and the Inspector now references this new plot.
 - b** Move the second plot below the first.
 - c** Change the X parameter to FITC
 - d** Change the Y parameter to PE.
- 9** Create a third dot plot by duplicating the second.
 - a** Select the second dot plot (if needed) and select Edit > Duplicate.

A duplicate dot plot appears and the Inspector now references this new plot.
 - b** Move the third plot below the second.
 - c** Change the X parameter to PerCP
 - d** Change the Y parameter to APC.
- 10** Use the Geometry section of the Dot Plot Inspector to align the plots.
 - a** Shift+click to select all three plots.
 - b** In the Inspector, type 0.2 in the Left text box.


Creating Gates and Displaying Statistics

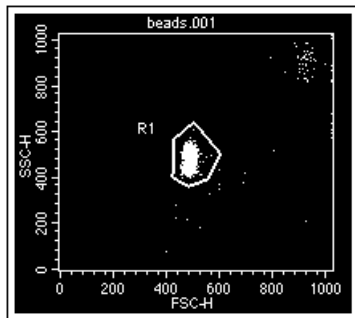
In this section, you will create a gate for the population of interest. You will also create quadrant markers to define negative and positive populations, and display quadrant statistics.

The Snap-To-Region tool will be used to create a region. See Chapter 6, *BD CellQuest Pro Software Analysis Tools*, and refer to the *BD CellQuest Pro Software Reference Manual* for information about using other region types in an analysis.

TIP Snap-To regions cannot be created in Acquisition only plots. Also, you cannot use a Snap-To region as an acquisition gate.

Creating a Snap-To Region

- 1 Draw a region encompassing the population of interest in the FSC vs SSC plot using the Snap-To-Region tool.
 - a Click the Snap-To-Region tool () on the tool palette to select it.
 - b Click on the population. The region is automatically drawn.



If the cluster is too small or diffuse for the Snap-To-Region tool to recognize, the system beeps and no region is drawn. In this case, try again, or use the polygon region tool to manually draw the region.

- 2 Click anywhere outside the region to deselect it.
- 3 Drag the R1 symbol off to the side of the region.

TIP To delete a region, select the region and select Edit > Cut, or press Delete.


Gating the Data in the Fluorescence Plots

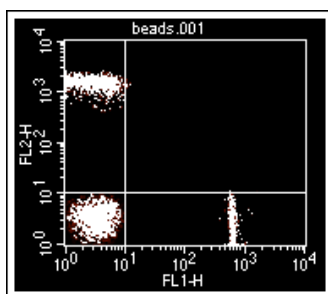
- 1 Shift+click to select the FITC vs PE and PerCP vs APC dot plots.
- 2 In the Dot Plot Inspector, click the Gate menu control and select G1=R1.

The selected plots are gated to show only the events included in the G1 gate.

Creating Quadrant Markers

In this section, you will set quadrant markers on gated data in the FITC vs PE and PerCP vs APC dot plots.

- 1 Select the FITC vs PE dot plot.
- 2 Draw quadrant markers on the FITC vs PE dot plot.
 - a Click the quadrant-marker tool () on the tool palette to select it.
 - b Click in the plot and drag the handle of the marker so the marker is set at 10^1 in FITC and 10^1 in PE. Release the mouse button to set the quadrant markers.



- 3 Repeat steps 1 and 2 for the PerCP vs APC dot plot.

Displaying Quadrant Statistics

- 1 Click the FITC vs PE dot plot to make it active.
- 2 Select Stats > Quadrant Stats.

The Quadrant Statistics view for the FITC vs PE dot plot appears.

Quadrant Statistics							
File: beads.001				Log Data Units: Linear Values			
Sample ID:				Patient ID:			
Tube: Mixed Beads 1				Panel:			
Acquisition Date: 30-Feb-04				Gate: G1			
Gated Events: 2000				Total Events: 2380			
X Parameter: FITC (Log)				Y Parameter: PE (Log)			
Quad Location: 11, 10							
Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	634	31.70	26.64	7.54	7.30	1757.85	196.79
UR	23	1.15	0.96	170.04	71.55	707.49	143.04
LL	748	37.40	31.42	6.67	6.13	3.87	3.42
LR	595	29.75	25.00	549.56	24.82	5.70	5.23

The Quadrant Statistics view gives statistics for each of the four quadrants defined by quadrant markers. The meaning of the quadrant abbreviations in the Quad column, and the fluorescence populations specified are as follows.

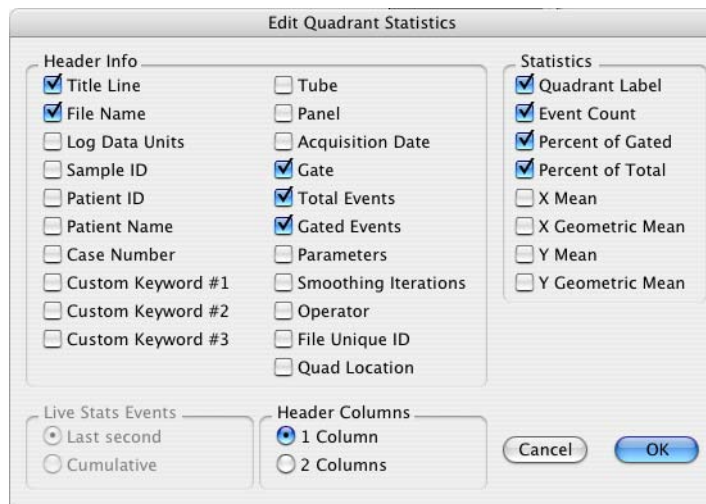
Quad	Quadrant	Population
UL	Upper Left	FITC ⁻ PE ⁺
UR	Upper Right	FITC ⁺ PE ⁺
LL	Lower Left	FITC ⁻ PE ⁻
LR	Lower Right	FITC ⁺ PE ⁻

3 Edit the type of statistics displayed.

- a Select the Quadrant Statistics view.
- b Select Stats > Edit Quadrants Stats.

The Edit Quadrant Statistics dialog appears.

- c Modify the dialog selections as needed to match those in the following figure.



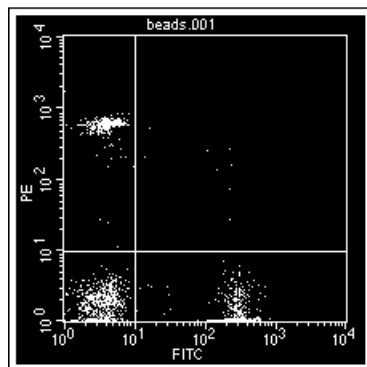
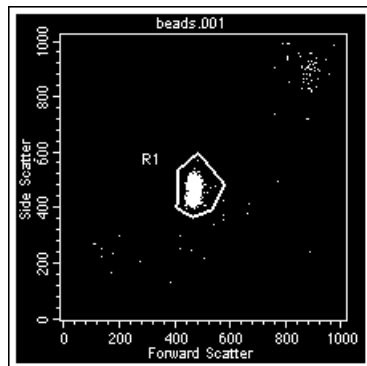
- d Click OK to accept the changes and close the dialog.

TIP When you export statistics, only the statistics that appear in the statistics view are exported.

4 Create a Quadrant Statistics view for the PerCP vs APC dot plot.

5 Resize and position the Quadrant Statistics views as in the following figure.

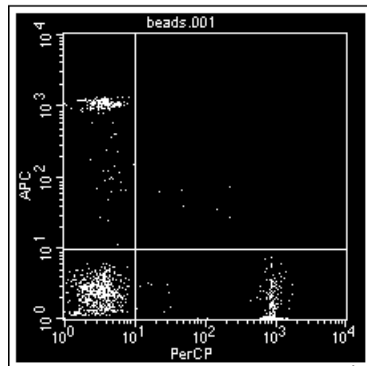
HINT Use the Geometry section of the Inspector to align plots and views.



Quadrant Statistics

File: beads.001
Gate: G1
Gated Events: 2000
Total Events: 2380

Quad	Events	% Gated	% Total
UL	0	31.70	26.64
UR	0	1.15	0.96
LL	0	37.40	31.42
LR	0	29.75	25.00



Quadrant Statistics

File: beads.001
Gate: G1
Gated Events: 2000
Total Events: 2380

Quad	Events	% Gated	% Total
UL	0	29.40	25.64
UR	0	1.75	0.96
LL	0	31.15	26.42
LR	0	37.70	31.00

Analyzing the Remaining Data Files

In this section, you will print your analysis results and use the Next Data File feature to analyze the remaining data files.

- 1** Select File > Print.
- 2** In the Print dialog, click Print.
- 3** Shift+click to select all plots.
- 4** Select Plots > Next Data File.

The next data file in your student folder, beads002, is displayed in the plots. The Snap-To singlet bead gate is redrawn. The statistics view recalculates its values.

TIP Command (**⌘**)+(right bracket) is a keyboard shortcut for Next Data File.

- 5** Print the Experiment document with the beads002 results.
- 6** Use Next Data File to display the beads003 results.
- 7** Print the Experiment document with the beads003 results.

Reviewing Analysis Results

Use your analysis printouts to record answers to the following.

- 1 From the first data file, report the %Gated events in the Lower Left (LL) quadrant of the FITC vs PE plot.

% LL _____

- 2 From the first data file, report the %Gated events in the Lower Left (LL) quadrant of the PerCP vs APC plot.

% LL _____

- 3 Which tube has the most PE-positive singlet beads?

- 4 Why are the %Total and %Gated values different for the PE-positive population? How are they calculated?

- 5 From the first data file, what percentage of singlets beads are PE-positive?

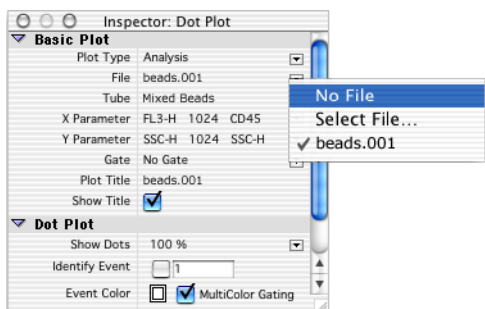
Saving an Experiment Document as a Template

A BD CellQuest Pro software document can be saved as a generic template so it can be used for routine acquisition or analysis work. All plots, regions, markers, statistics, and so on (except data files) can be defined and saved as a template.

- 1 Select all plots in the Experiment document.

To select multiple plots, Shift+click the border of each plot. Square handles appear on all selected plots.

- 2 Click the File menu control in the Inspector and select No File.



Each plot is now empty. The next time you open the Experiment document, the plots remain empty until data are read in.

- 3 Select File > Save Document As.
- 4 Name the document *4-color Beads Template* and save it in your student folder.
- 5 Make sure an appropriate destination folder is displayed in the dialog and click Save.

TIP To protect a template, you can lock it or make it a stationary pad. See Locking and Using Documents as Templates on page 323.

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BD CellQuest Pro Software Acquisition Tools

This chapter provides exercises on some acquisition topics that supplement the material in Chapter 4, BD CellQuest Pro Workflow. After completing the exercises in this chapter, you will be able to:

- Specify a tube list and parameter labels.
- Specify tube-specific settings.
- Create and use a Reagent Panel.

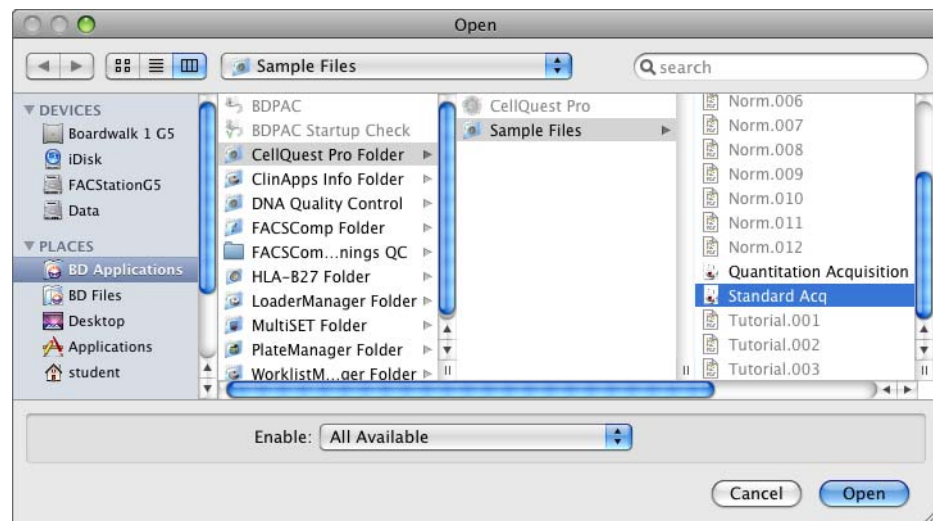
Specifying a Tube List

The Browser's Acquisition Tube List determines the order in which tubes are acquired, and can be used to specify parameter labels and tube specific settings. There are two ways to specify a tube list.

- Use the Browser to manually add tubes. Use this method if you are acquiring tubes stained with a unique reagent combination. In this exercise, you will use the Browser to manually specify a tube list.
- Create a predefined list of tubes and save it as a Reagent Panel that can be reused. Use this method if you are acquiring the same reagent combination for many experiments or donors. You will create a reagent panel in the next exercise (see Reagent Panels on page 141).

Getting Started

- 1 If you have not already completed a system startup today, perform the System Startup procedure on page 33.
- 2 Start BD CellQuest Pro software, if needed.
- 3 Close any Experiment documents.
- 4 Select File > Open Document. Navigate to the Standard Acq document and click Open.



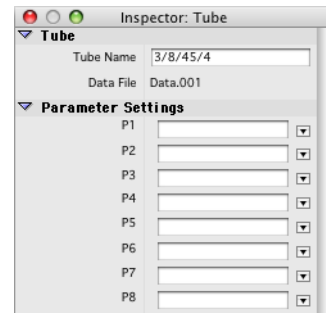
Adding Tubes Manually

- 1 Select Windows > Show Browser.
- 2 In the Browser, if needed:
 - a Click the Acquisition tab.
 - b Click the Acquisition Controls disclosure triangle to open that portion of the Browser.
 - c Click the Untitled Acquisition Tube List disclosure triangle to display its tubes.

The tube list initially contains a single tube named Untitled.

- 3 Double-click the Untitled tube icon to select the tube and display the Tube Inspector.
- 4 Enter 3/8/45/4 in the Tube Name field.
- 5 Under Parameter Settings, click the P1 menu control and select Forward Scatter.

TIP If the parameter label you want does not appear in the menu, you can type it into the text field.

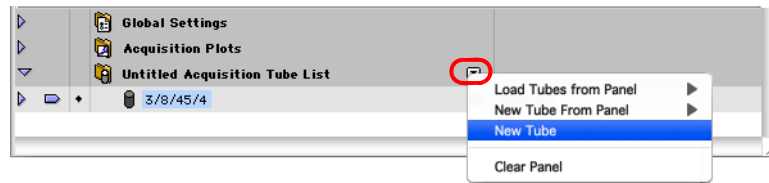


Labels can be added or deleted from the parameter label menu by selecting Acquire > Edit Reagent List. Refer to the *BD CellQuest Pro Software Reference Manual* for details.

- 6 Specify labels for the remaining parameters as shown in the following table.

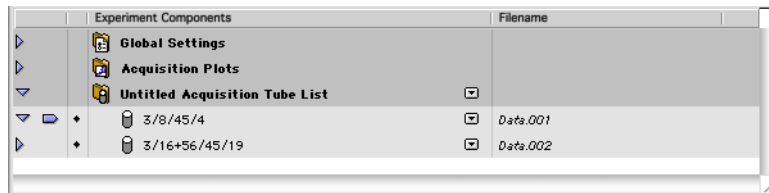
Parameter	Label
P1	Forward Scatter
P2	Side Scatter
P3	CD3 FITC
P4	CD8 PE
P5	CD45 PerCP
P7	CD4 APC

- Click the menu control next to Untitled Acquisition Tube List and select New Tube.



A new tube is added to the tube list.

- Click the icon for the new tube to select it and use the Inspector to name the tube 3/16+56/45/19.



- Use the Inspector to specify labels for the 3/16+56/45/19 tube as shown in the following table.

Parameter	Label
P1	Forward Scatter
P2	Side Scatter
P3	CD3 FITC
P4	CD16+CD56 PE
P5	CD45 PerCP
P7	CD19 APC

- Save a copy of the Experiment document in your student folder. Name it *Manual 4-color TBNK*.

The next time you open the Manual 4-color TBNK document, it will contain the tubes and parameter labels you specified in this exercise.

Specifying Tube-Specific Settings

In BD CellQuest Pro software you can specify tube-specific acquisition and storage settings and instrument settings. In this exercise, you will modify the Manual 4-color TBNK Experiment document that you saved in the previous section.

NOTE You will not acquire data in this exercise, but the cytometer must be powered on.

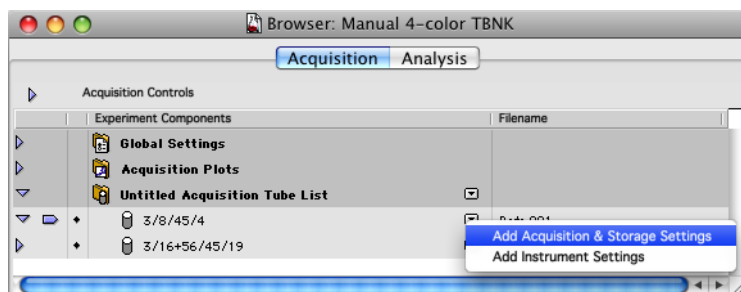
Getting Started

- 1 If you have not already completed the Specifying a Tube List exercise on page 134, do so now. Otherwise, open the Manual 4-color TBNK Experiment you saved in the Specifying a Tube List exercise, if needed.
- 2 If needed, select Acquire > Connect to Cytometer to display the Acquisition Control dialog and the Acquisition view of the Browser appear.

Specifying Tube-Specific Acquisition and Storage Settings

In this section, you will specify the number of events to acquire for a particular tube.

- 1 Click the menu control next to the 3/8/45/4 tube in the tube list.
- 2 Select Add Acquisition & Storage Settings from the menu that appears.

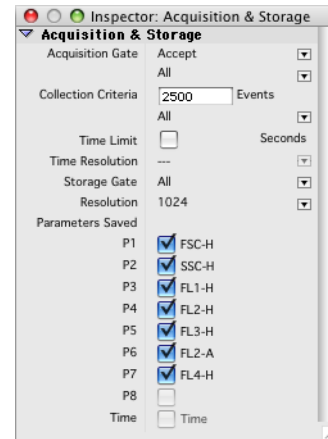
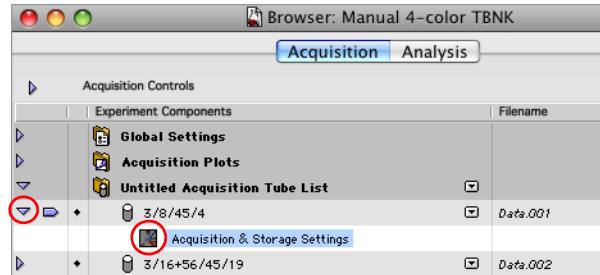


An Acquisition & Storage Settings object appears in the Browser, directly under the tube. The settings stored by this object are specific to the tube.

TIP Click the disclosure triangle next to the tube name if the Acquisition & Storage Settings object is hidden.

- 3 In the Browser, double-click the Acquisition & Storage icon under the 3/8/45/4 tube to display the Acquisition & Storage Inspector.

The Inspector displays the current acquisition and storage settings.

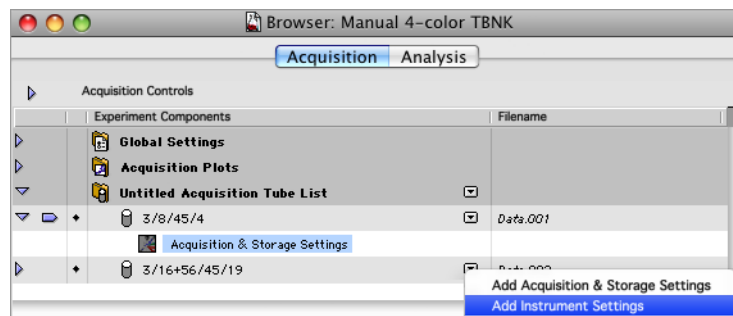


- 4 Change the Collection Criteria to 2500 events.

Specifying Tube-Specific Instrument Settings

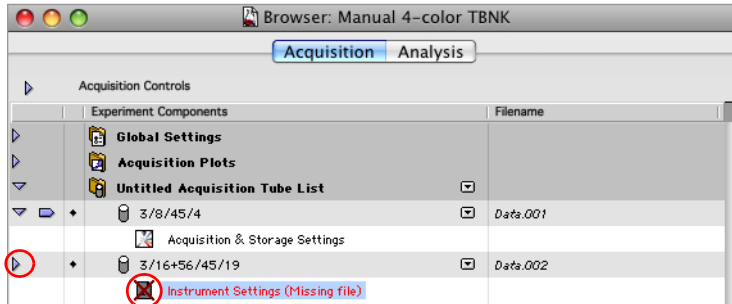
In this section, you will learn how to load instrument settings for a specific tube.

- 1 Click the menu control next to 3/16+56/45/19 in the tube list and select Add Instrument Settings.

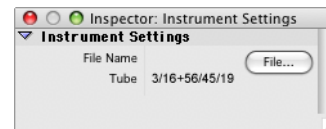


An Instrument Settings object appears in the Browser, under the tube. The browser display indicates that at this point the Instrument Settings are unspecified.

TIP Click the disclosure triangle next to the tube name if the Instrument Settings object is hidden.



2 Double-click the Instrument Settings icon to display the Instrument Settings Inspector.

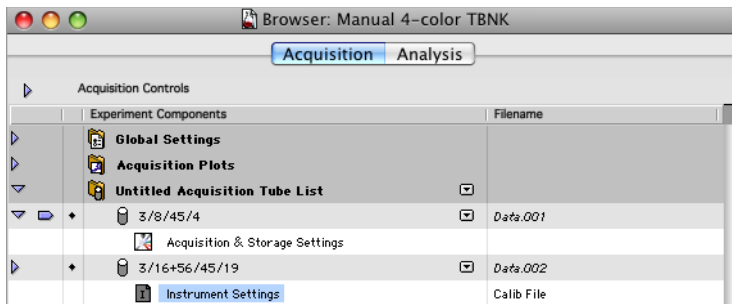


3 Click File.

A file location dialog appears.

4 Navigate to BD Files/Instrument Settings Files and select the Calib File.

The file name is displayed in the Inspector and the Browser is updated.



Removing Tube-Specific Settings

1 Click the 3/8/45/4 Acquisition & Storage Settings icon to select it.

2 Select Edit > Clear.

The 3/8/45/4 Acquisition & Storage Settings object is deleted.

3 Click the 3/16+56/45/19 Instrument Settings icon to select it.

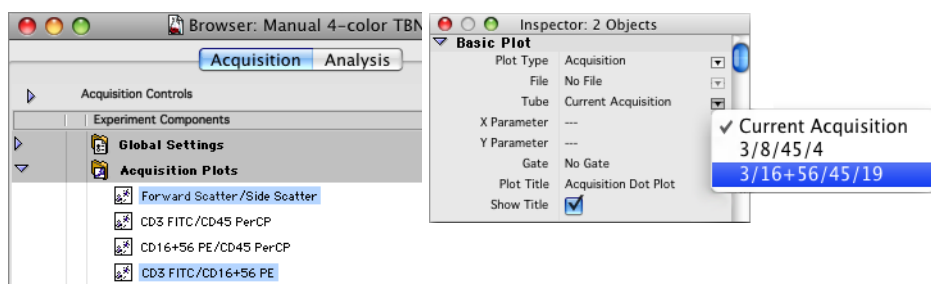
4 Select Edit > Clear.

The 3/16+56/45/19 Instrument Settings object is deleted.

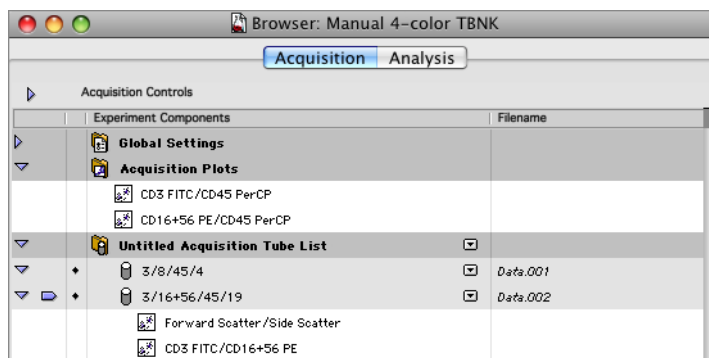
Associating Plots With Tubes

Plots can be associated with specific tubes. During acquisition, these plots will only display data from the designated tube. If they are Acq → Analysis plots, data will remain on display from the designated tube while the remaining tubes are acquired.

- 1 Associate the FSC vs SSC and FL1 vs FL2 plots with the 3/16+56/45/19 tube.
 - a Drag the tube pointer to the 3/16+56/45/19 tube, if needed.
 - b Open the Acquisition Plots list by clicking its disclosure triangle.
 - c Command (⌘)+click to select both the FSC vs SSC and FL1 vs FL2 plots.
 - d Click the Tube menu control in the Inspector and select 3/16+56/45/19.



The Browser now displays the plots under the 3/16+56/45/19 tube.



- 2 Remove the FSC vs SSC and FL1 vs FL2 plot associations.
 - a Shift+click to select both the FSC vs SSC and FL1 vs FL2 plots.
 - b Click the Tube menu control in the Inspector and select Current Acquisition.

Reagent Panels

A panel is a list of tubes with predefined parameter labels. In this exercise you will create a reagent panel and use it to specify tubes and parameter labels for an acquisition. You will also create and save an Experiment document with the panel loaded into the Acquisition Tube List.

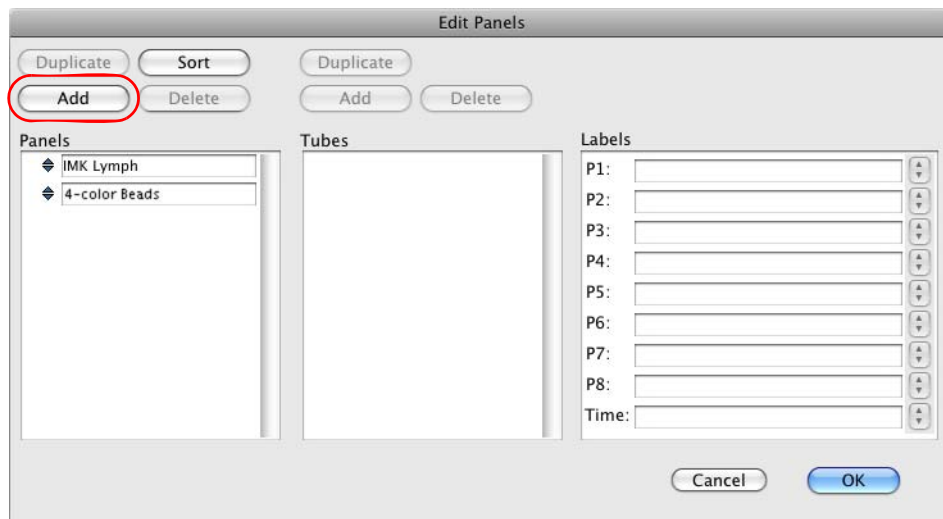
NOTE You will not acquire data in this exercise, but you might use this panel to acquire data in later exercises (see Lab Practice—Lyse/Wash Manual Tube Loading on page 349 and Lab Practice—Lyse/Wash Automatic Tube Loading on page 353).

Creating a Reagent Panel

In this section, you will create the following two-tube panel.

- CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC
- 1 Start BD CellQuest Pro software, if needed.
 - 2 Select Acquire > Edit Panels.

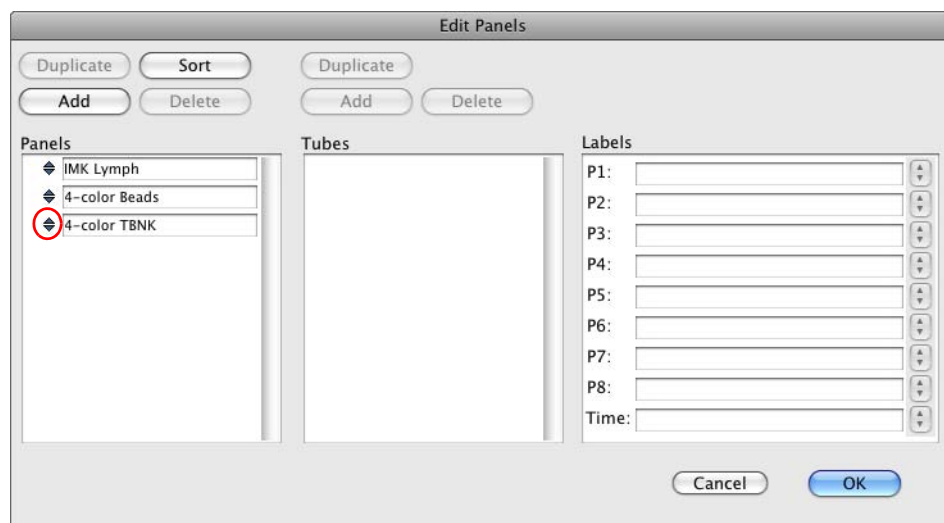
The Edit panels dialog appears.



- 3 Click Add above the Panels list.

A new panel appears in the Panels list.

- 4 Type *4-color TBNK* for the panel name.



- 5 Click the selection icon (to the left of 4-color TBNK) to select the panel.
- 6 Click Add above the Tubes list.

A new tube appears in the Tubes list with the default name *tube #1*.
- 7 Change the tube name to *3/8/45/4*.
- 8 Click the selection icon to the left of the *3/8/45/4* tube.
- 9 Use the parameter label menus to specify Forward Scatter as the label for P1 and Side Scatter as the label for P2.
- 10 Specify fluorescence parameter labels for the *3/8/45/4* tube as shown in the following table.

HINT You can select the label from the parameter label menus or type new labels.

Parameter	Label
P3	CD3 FITC
P4	CD8 PE
P5	CD45 PerCP
P7	CD4 APC

- 11 Add a tube named 3/16+56/45/19 to the panel. Give it the following parameter labels:

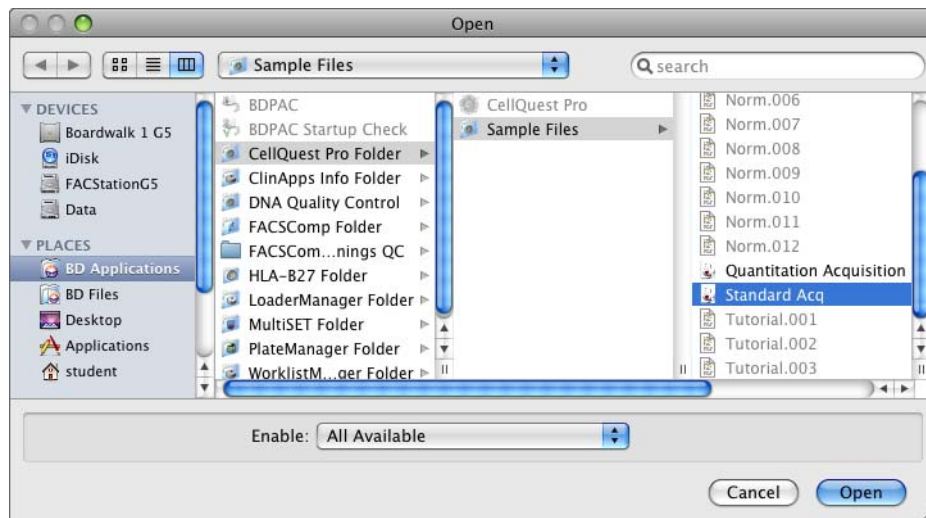
Parameter	Label
P1	Forward Scatter
P2	Side Scatter
P3	CD3 FITC
P4	CD16+CD56 PE
P5	CD45 PerCP
P7	CD19 APC

- 12 Click OK to save the 4-color TBNK panel and close the Edit Panel dialog.

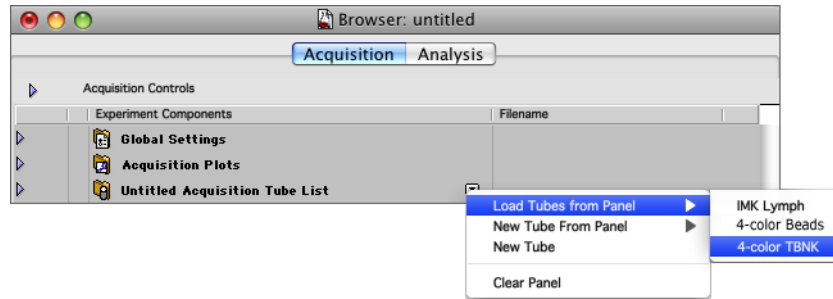
Using a Reagent Panel

In this section you will create a copy of the Standard Acq document and load the 4-color TBNK panel into its Acquisition Tube List.

- 1 Close any Experiment documents.
- 2 Select File > Open Document. Navigate to the Standard Acq document and click Open.

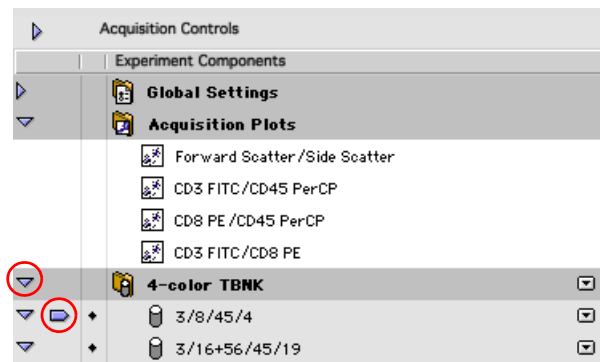


- 3 Select Windows > Show Browser.
- 4 If needed, click the Acquisition tab, and open the Acquisition Controls portion of the Browser.
- 5 In the Browser, click the menu control next to Untitled Acquisition Tube List and select Load Tubes from Panel > 4-color TBNK.



- 6 If needed, click the 4-color TBNK disclosure triangle to display its tubes.

A pointer indicates that parameter labels for the first tube in the panel will be used for the next acquisition.



- 7 Select File > Save Document As.
- 8 Save a copy of the Experiment document named *4-color TBNK* in your student folder.

The next time you open the 4-color TBNK Experiment, the 4-color TBNK panel will already be loaded in the Browser's Acquisition Tube List.

BD CellQuest Pro Software Analysis Tools

After completing the exercises in this chapter, you will be able to:

- Analyze immunophenotyping data.
- Generate population statistics using regions and gates.
- Generate population statistics using the expression editor.
- Perform a batch analysis.
- Use overlaid histograms to analyze data.

Introduction—Regions, Gates, Markers, and Statistics

In this chapter, you will use regions, gates, and markers to define subpopulations and generate statistics for immunophenotyping data.

Regions

A **region** is a area on a 2D plot (or interval on a histogram) typically defined by drawing its boundary. BD CellQuest Pro software provides tools for creating several types of regions on a plot: rectangular, polygonal, histogram, elliptical, and snap-to.

A **snap-to region** automatically encompasses populations in dot, density, or contour plots, and adjusts when new data is read into a plot.

Gates

A **gate** is composed of one or more regions on one or more plots, and can be used to identify populations by color, and also to restrict the data displayed in a plot.

Markers

A **histogram marker** specifies an interval on a histogram. A **quadrant marker** divides a 2D plot into four quadrants.

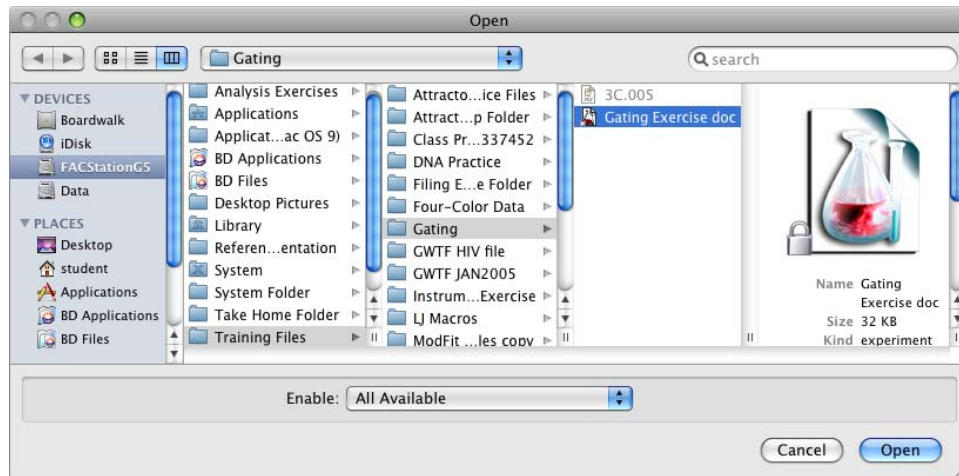
Statistics

Statistics views can be generated for a plot. There are several types of statistics views: histogram, quadrant, region, gate, and Kolmogorov-Smirnov.


Additional information about markers, regions, gates, and statistics can be found in the *BD CellQuest Pro Software Reference Manual*.

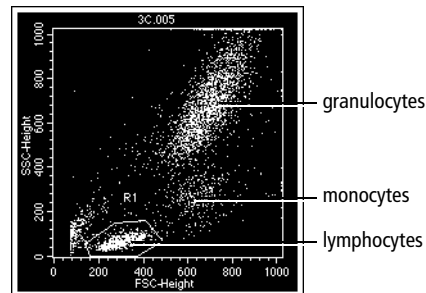
Working With Regions

- 1 Use the Finder to navigate to and open *Gating Exercise doc* as shown in the figure.



Gating Exercise doc is a stationery pad document, so a copy of the *Gating Exercise doc* Experiment document will open in BD CellQuest Pro software. The Experiment document opens with three analysis dot plots displaying the data file 3C.005.

- 2 Use the Polygon-Region tool () to draw a region (R1) encompassing the lymphocyte population on the FSC vs SSC dot plot.
- 3 Move the R1 label off to the side of the region.
 - a Click outside the region to deselect it.
 - b Click and drag the label to a position where it is not obscured by the data.



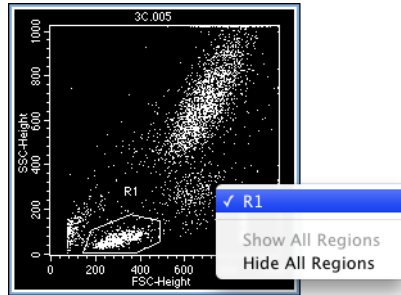
Modifying a Region

You can modify a region by resizing, repositioning, or rotating it. With polygonal regions, you can reposition individual vertices. You can also choose to display or hide a region.

TIP The same techniques you will practice here for polygonal regions can be used to resize, reposition, and rotate rectangular and elliptical regions.

Hiding a Region

- 1 Command (⌘)+click the plot, and select R1.



The region and its label disappear.

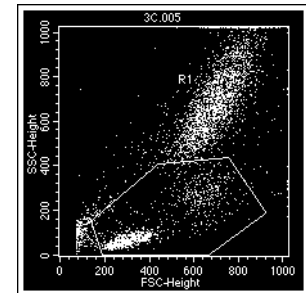
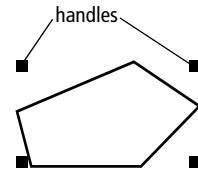
- 2 Redisplay the region by repeating step 1.

Resizing a Region

- 1 Click the boundary of region R1.

Four handles will appear, one at each corner of the region. You can click and drag these handles to stretch the region along the x- and y-axes.

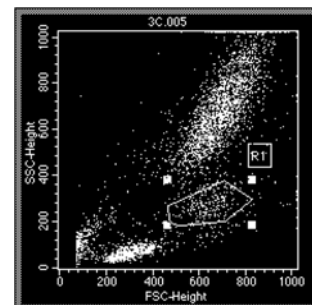
- 2 Drag the upper right handle to stretch the region so that it also encompasses the monocyte cluster.
- 3 Return the region to its original position by clicking and dragging the upper right handle.



Repositioning a Region

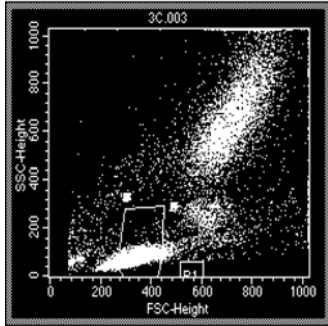
While a region is selected, you can drag anywhere on its border to reposition it.

- 1 Select region R1.
- 2 Drag the region border to position it over the monocyte cluster.
IMPORTANT Do not drag the handles to reposition a region. Drag the region border.
- 3 Return region R1 to its position encompassing the lymphocytes.



Rotating a Region

- 1 Select region R1.
- 2 Select Gates > Rotate Region.
- 3 Click and drag a handle to rotate the region 90° counterclockwise.

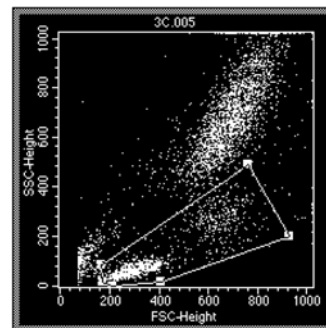
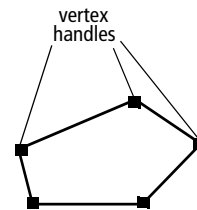


- 4 When finished, click on the plot anywhere outside the region.
- 5 Return region R1 to its original orientation.

TIP You can also return the region to its original position by selecting Undo from the Edit menu.

Repositioning Vertices


- 1 Double-click the border of region R1.
Handles appear at the vertices of the region.
- 2 Drag handles to reposition the vertices so that region R1 also encompasses the monocyte cluster.
- 3 When finished, click on the plot anywhere outside the region.
- 4 Return the region R1 vertices to their original positions.



Using Snap-To Regions

You can use the Snap-To-Region tool to automatically create regions that encompass populations in dot, density, or contour plots. When the data displayed in a plot changes, a Snap-To region automatically adjusts to the new data.

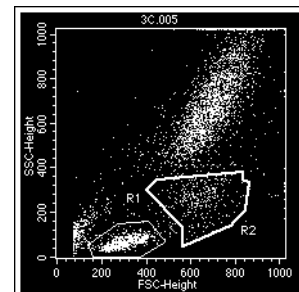
- 1 Create a Snap-To region that encompasses the monocyte population in the FSC vs SSC dot plot.

- a Click the Snap-To-Region tool () on the tool palette.

- b Click on the monocyte population in the FSC vs SSC dot plot.

A region is automatically drawn. The region boundary is thicker than that of other regions to help distinguish it as a Snap-To region.

If the population is too small or diffuse, the system beeps and no region is drawn. In this case, use another region tool to draw the region.



- c Drag the R2 label to a position where it is not obscured by the data.

TIP A Snap-To region can be modified with the same techniques used with polygonal regions.

- 2 Read a new data file into the FSC vs SSC plot.

- a Select the FSC vs SSC plot.
- b Select Windows > Show Inspector to display the Plot Inspector, if needed.
- c Click the File menu control and select Select File.

The Open a Data File dialog appears.

- d Navigate to BD Applications/CellQuest Pro/Sample Files and open the data file Norm.001.

The new data is displayed in the plot. The R2 Snap-To region is redrawn to encompass the new monocyte population. If the population is too small or diffuse, the system beeps and the region is redrawn in its original position.

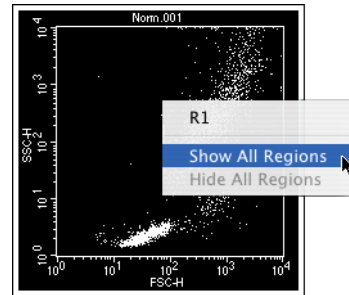
3 Display R2 on a duplicate FSC vs SSC plot.

- a** Select the FSC vs SSC plot and select Edit > Duplicate.

A duplicate FSC vs SSC plot appears. It does not display the R1 and R2 regions.

- b** Option+click anywhere inside the duplicate plot and select Show All Regions.

The R1 and R2 regions are now displayed in the duplicate FSC vs SSC plot.

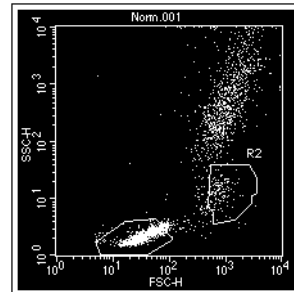


4 Reposition the R2 Snap-To region in the duplicate FSC vs SSC plot so that the region excludes part of the monocyte population.

The R2 region is also repositioned in the original FSC vs SSC plot.

5 Select only the duplicate FSC vs SSC plot, and use the Inspector to load the data file Norm.002.

The R2 Snap-To region is NOT redrawn. Why?



6 Load the data file Norm.002 into the original FSC vs SSC plot.

The R2 Snap-To region is redrawn to encompass the monocytes in Norm.002.

7 Select only the duplicate plot and press Delete to delete it.

Using Additional Snap-To Region Features (Optional)

You can control how a Snap-To region is redrawn by adjusting its Auto Movement and Auto Size settings.

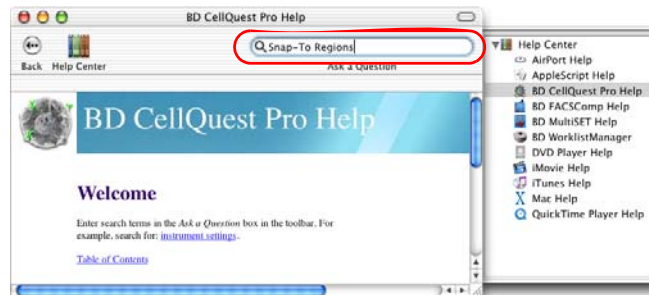
- The Auto Movement setting specifies how far a Snap-To region can move when redrawn. Larger Auto Movement values allow a Snap-To region to move farther.
- The Auto Size setting specifies how tight a Snap-To region will be. Larger Auto Size values cause a Snap-To region to encompass more outlying events.


A Snap-To region can be tethered to other region types. This is useful when a diffuse population maintains a spatial relationship to a stronger population with a Snap-To region. When the Snap-To region is redrawn, the tethered regions move with the Snap-To region.

You can practice these Snap-To region features by following tutorials contained in BD CellQuest Pro Help.

- 1 Select File > New Document to create a new Experiment document for the tutorials.
- 2 Select Help > CellQuest Pro Help.

The BD CellQuest Pro Help window appears.
- 3 Enter Snap-To Regions in the search field and press Return.




- 4 In the search results, double-click *Becoming Familiar With Snap-To Regions*.
- 5 When you finish with the first tutorial, click the right arrow on the navigation bar to continue with the next tutorial, *Tethering Snap-To Regions*.

- 6 Close the Experiment document you used in the tutorials. Do not save changes.

Using Additional Region Features

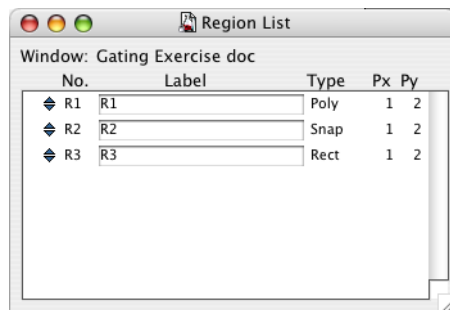
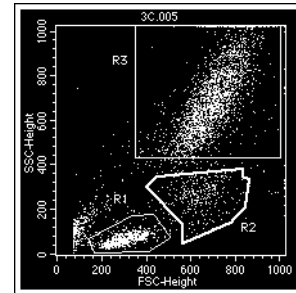
In this section, you will return to the *Gating Exercise* Experiment document for more practice with regions.

Hiding, Showing, and Deleting Regions

You can hide a region so that its boundary and its label are not displayed in a plot. A hidden region remains in the Region list, where it can be copied and pasted into other plots.

- 1 Verify that the *Gating Exercise* Experiment document is opened.
- 2 Display the data file 3C.005 in all plots.
 - a Select all plots, click the File menu control in the Inspector, and select Select File.
 - b Navigate to Training Files/Gating and open 3C.005.
- 3 Click the Rectangular–Region tool () on the tool palette, and create a rectangular gate that encompasses the granulocyte population on the FSC vs SSC plot.
- 4 Select Gates > Region List.

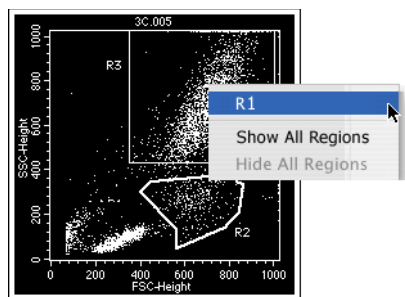
The Region List appears. The Region List can be used to change the default region labels to descriptive ones. You will not use descriptive region labels in this exercise.



- 5 Select region R1 on the FSC vs SSC plot if it is not already selected.
- 6 Press Option-Delete to hide region R1.

The R1 region is still shown in the Region List, but no longer appears in the FSC vs SSC plot.

- 7 Command (⌘)+click the FSC vs SSC plot and select R1.



The R1 region is now displayed in the plot.

- 8 Select region R2 if it is not already selected, and then press Delete.


The region is removed from the plot and the Region List.

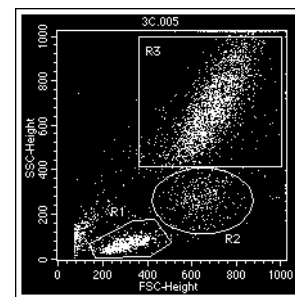
TIP To delete multiple regions in a plot, Shift+click each region to be deleted, then press Delete.

- 9 Close the Region List.

Using Region Statistics to Analyze Data

In this section you will use region statistics to obtain population statistics.

- 1 Using the Elliptical–Region tool () , create an elliptical region (R2) that encompasses the monocyte population on the FSC vs SSC plot.
- 2 Move the R2 label so that it is not obscured by the data.
- 3 Use region modification techniques to adjust the regions so that they conform to their populations.
- 4 Select the FSC vs SSC plot and select Stats > Region Stats.



A region statistics view appears.

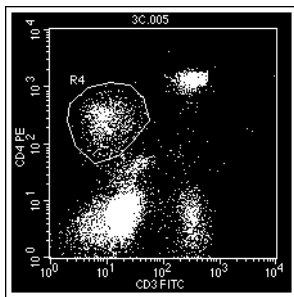
Region Statistics									
File: 3C.005					Log Data Units: Linear Values				
Sample ID:					Patient ID:				
Patient Name:					Case Number:				
Acquisition Date: 28-Feb-95					Gate: No Gate				
Gated Events: 10000					Total Events: 10000				
X Parameter: FSC-Height (Linear)					Y Parameter: SSC-Height (Linear)				
Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px	Py
R1	1891	18.91	18.91	303.38	297.03	67.06	62.80	1, 2	
R2	832	8.32	8.32	650.35	646.23	270.99	263.42	1, 2	
R3	5744	57.44	57.44	694.77	687.60	683.26	669.53	1, 2	
R4	971	9.71	9.71	659.52	645.68	298.92	279.86	3, 4	

- 5 Record the %Total statistics for R1, R2, and R3.

Region	Population	%Total
R1	lymphocytes	
R2	monocytes	
R3	granulocytes	

- 6 Why are the %Gated and %Total statistics identical?

- 7 Draw a polygonal region (R4) that encompasses the CD3⁻CD4⁺ population on the CD3 FITC vs CD4 PE plot.



- 8 Create a Region statistics view for the CD3 FITC vs CD4 PE plot to determine the %Total CD3⁻CD4⁺.

Record the %Total for CD3⁻CD4⁺: _____

- 9 Delete all statistic views from the Experiment document.
- Shift+click each view to be deleted.
 - Press Delete.

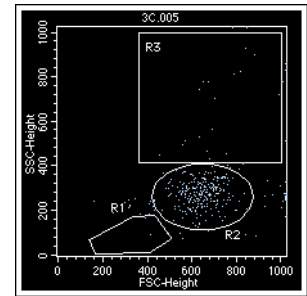
Working With Gates

A gate operates on the data in a region, or in a logical combination of one or more regions. By default, BD CellQuest Pro software assigns gate 1 to region 1 (G1 = R1), gate 2 to region 2 (G2 = R2), and so on. You can change these assignments as needed.

Backgating (Fluorescence Gating)

Gates can be used to determine where a population of interest is located on a plot. In backgating, a population defined by a region in a fluorescence plot, is displayed on another plot (usually FSC vs SSC).

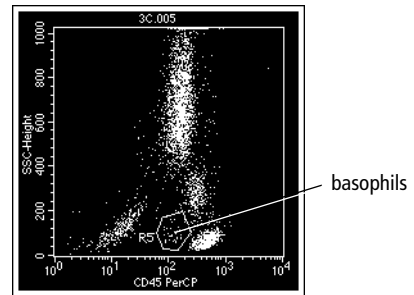
- 1 Draw a region (R4) that encompasses the CD3⁻CD4⁺ population on the CD3 FITC vs CD4 PE plot, if you have not already done so.
- 2 Gate the FSC vs SSC plot using G4 = R4.
 - a Select the FSC vs SSC plot.
 - b Select Windows > Show Inspector to display the Inspector, if needed.
 - c Click the Gate menu control and select G4 = R4.



You are now viewing the scatter properties of events that fell within the R4 region.

- 3 Cells in the CD3⁻CD4⁺ population are mostly _____.
- 4 Use backgating to determine the position of the basophils (see figure) on the FSC vs SSC plot.

Where are the basophils located on the FSC vs SSC plot?



Using Multicolor Gates

With multicolor gating, you can assign a color to the events in a gate, which can help you to locate populations on a plot without gating the data.

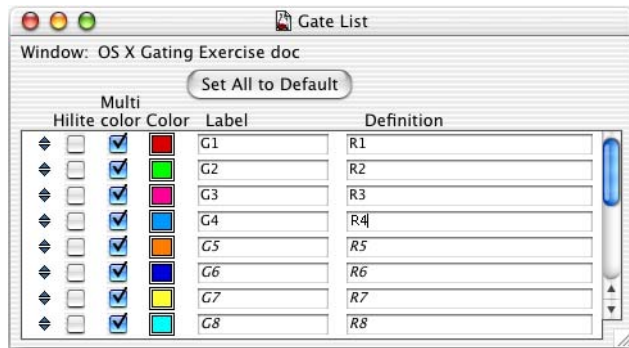
NOTE Colors are associated with gates, not regions.

- 1 Activate multicolor gating for all plots.
 - a Select all plots in the Experiment document.
 - b Display the Inspector, if needed.
 - c Select the Multicolor Gating checkbox under Dot Plot.
 - d Click the Gate menu control and select No Gate.

The plots now display red lymphocyte events, green monocyte events, and magenta granulocyte events.

- 2 Select Gates > Gate List.

The Gate List appears.

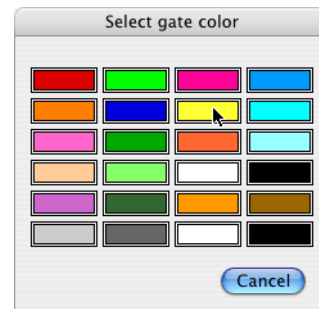


- 3 Click the red Color box next to the G1 label.

The *Select gate color* dialog appears.

- 4 Click the yellow rectangle.

The *Select gate color* dialog closes. The Gate List G1 Color box is now yellow. Lymphocyte events in all plots are now colored yellow.



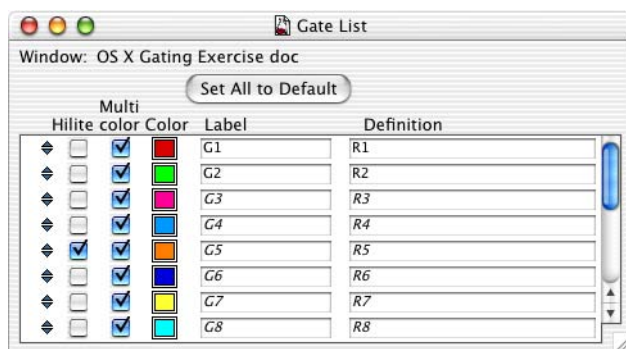
- 5 Return the color of G1 to red.

Highlighting a Population

To help locate a rare population or differentiate overlapping populations, use the Hilite feature. When the Hilite checkbox is selected for a gate in the Gate List window, events in that gate are drawn larger throughout the Experiment document, making them more prominent and easier to find.

NOTE Highlighting does not affect population statistics.

- 1 Draw a region (R5) that encompasses the basophils on the CD45 PerCP vs SSC plot, if you have not already done so.
- 2 Select Gates > Gate List to display the Gate List.
- 3 Select the G5 Hilite checkbox.



- 4 Find the basophils on the FSC vs SSC plot.
- 5 Clear the G5 Hilite checkbox in the Gate List.

Reordering the Gate List

Events in gates are colored in the order the gates appear in the Gate List. As a result, events from gates lower in the Gate List can be difficult to locate. You can change the gate order to help locate hidden populations.

- 1** The color for G5 = R5 is orange, but the basophils are colored red on the CD45 PerCP vs SSC plot. Why?

- 2** Display the basophils as orange colored by moving G5 to the top of the Gate List.
 - a** Select Gates > Gate List to display the Gate List, if needed.
 - b** Click the G5 selection icon and drag the G5 row to the top of the list.

The basophil events are now orange.

The events in G5 are colored first. These events will not be colored red even though they fall in G1 because they have already been colored with the G5 color.

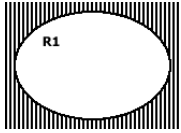
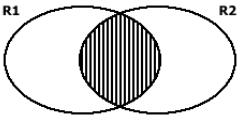
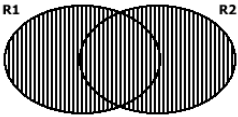
On the FSC vs SSC plot, it is difficult to distinguish the orange events from the red events because the colors are similar.

- 3** What can you do to better visualize the basophils against the lymphocyte population on the FSC vs SSC plot? Try it.

- 4** Click Set All to Default on the Gate List to restore the default gate order and colors.

Using Combined Gates

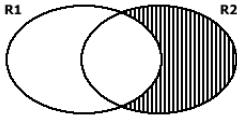
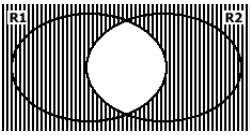
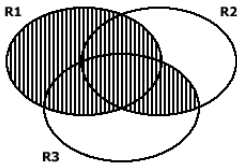
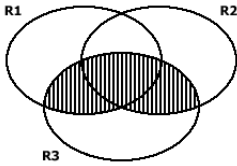
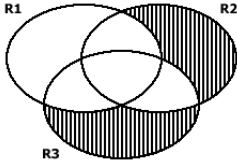
Regions and gates can be combined with logical operators (AND, OR, and NOT) to create sophisticated gates for analysis and sorting. Symbols can be used in place of operator names in combined gate expressions as shown in the following table.

Operator Symbol	Examples	Meaning
NOT -	$G3 = \text{NOT } R1$ $G3 = - R1$	G3 consists of all events that are not in R1. 
AND *	$G1 = R1 \text{ AND } R2$ $G1 = R1 * R2$	G1 consists of all events that are in both R1 and R2.  IMPORTANT Avoid confusion with common uses of the word AND. The AND operator specifies the intersection of regions. Use OR if you want to specify union.
OR +	$G2 = R1 \text{ OR } R2$ $G2 = R1 + R2$	G2 consists of all events that are in either R1 or R2 (including those in both R1 and R2).  IMPORTANT Avoid confusion with common uses of the word OR. The OR operator specifies the (inclusive) union of regions.

Combined Gate Expression Guidelines

- Operators are applied in the order: **NOT, AND, OR**.
- Use parentheses to override order. Operations inside parenthesis are applied first.
- If NOT appears in the middle of the expression, it must be preceded by an operator ($R1 \text{ AND NOT } R2$ is a valid expression, but $R1 \text{ NOT } R2$ is invalid).
- Leave a space between region designations and operators.
- You can use a combined gate designation as a shortcut in another combined gate specification.
- You can use region and combined gate designations multiple times in combined gate specifications.

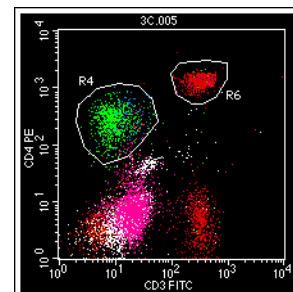
Combined Gates Examples

<p>$G1 = \text{NOT } R1 \text{ AND } R2$</p> <p>The NOT operator is applied to R1 first, so this is the same as: $G1 = (\text{NOT } R1) \text{ AND } R2$</p>	
<p>$G2 = \text{NOT } (R1 \text{ AND } R2)$</p>	
<p>$G3 = R1 \text{ OR } R2 \text{ AND } R3$</p> <p>The AND operator is applied to R2 and R3 first, so this is the same as: $G3 = R1 \text{ OR } (R2 \text{ AND } R3)$</p>	
<p>$G4 = (R1 \text{ OR } R2) \text{ AND } R3$</p>	
<p>$G5 = \text{NOT } G3 \text{ AND } (R2 \text{ OR } R3)$</p> <p>The OR operator is applied to R2 and R3 first, then the NOT operator is applied to G3 (recall, $G3 = R1 \text{ OR } R2 \text{ AND } R3$), so this is the same as: $G5 = (\text{NOT } (R1 \text{ OR } R2 \text{ AND } R3)) \text{ AND } (R2 \text{ OR } R3)$</p>	

Practicing Combined Gates

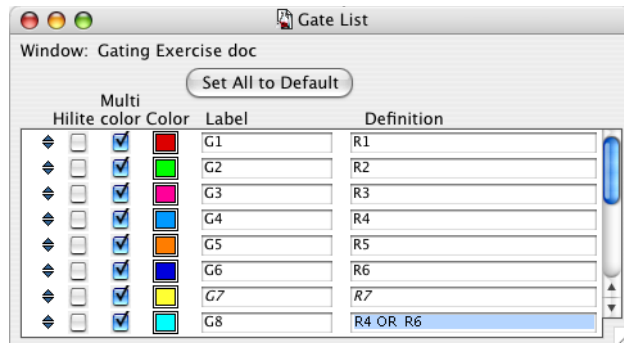
Analyzing CD4⁺ Events

- 1 Verify that all plots are ungated (in the Plot Inspector, the value of Gate is No Gate).
- 2 Draw a region (R6) on the CD3 FITC vs CD4 PE plot that encompasses the CD3⁺CD4⁺ population.
- 3 Select Gates > Gate List to display the Gates List, if needed.



- 4 Change the definition of G8 to **R4 OR R6**.
 - a Click in the Definition field for G8 and type *R4 OR R6*.
 - b **IMPORTANT** Press Return to submit the definition for validation.

If the gate definition validates successfully, it is displayed in plain text. If the definition has syntax problems, or refers to regions that do not exist, the definition is displayed in italics.

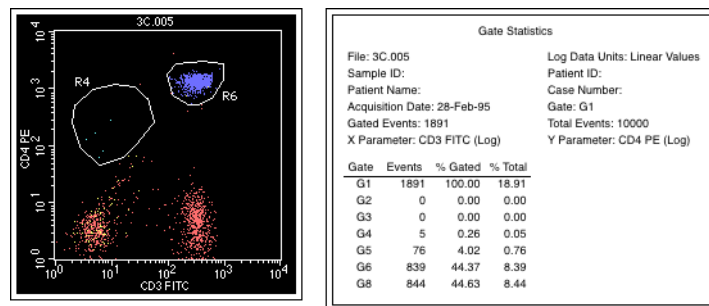


- 5 Display a Gate Statistics view for the CD3 FITC vs CD4 PE plot.
- 6 Record the %Total for G8: _____.

G8 consists of all the CD4⁺ cells (both monocytes and lymphocytes). However, you can gate the data to obtain statistics for CD4⁺ lymphocytes.

- 7 Gate the CD3 FITC vs CD4 PE plot using G1 = R1.

The CD3 FITC vs CD4 PE plot now displays only lymphocytes. The %Gated statistics are now different from the %Total statistics.



- The %Gated statistic for G8 is the percentage of lymphocytes that are CD4⁺. It is calculated by dividing the number of events in G8 that are in the G1 gate (**G8 AND G1**), by the number of events in G1.
- The %Total statistic for G8 is the percentage of all events that are CD4⁺. It is calculated by dividing the number of events in G8 by the total number of events in the data.

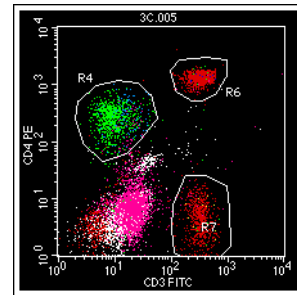
- 8 Record the %Gated for G8: _____.
- 9 Change the definition of G8 to **R4 AND R6** (press Return to submit the definition for validation).
- 10 Display a Gate Statistics view for the CD3 FITC vs CD4 PE plot.
- 11 The %Total for G8 is 0. Why?

- 12 Delete the Gate Statistics view.

Analyzing CD3⁺ Events

- 1 Change the CD3 FITC vs CD4 PE plot Gate setting back to No Gate.
- 2 Draw a region (R7) on the CD3 FITC vs CD4 PE plot that encompasses the CD3⁺CD4⁻ population.

TIP Use region modification techniques to extend R7 to the baseline so you do not miss any events.



- 3 Change the definition of G8 to **R1 AND NOT (R5 OR R6 OR R7)**.

G8 now consists of lymphocytes that are not CD3⁺ (non-T lymphocytes).

- 4 Record the %Total for G8: _____.
The %Total for G8 is the percentage of all cells that are non-T lymphocytes.
- 5 Delete the Gate Statistics view.

Combined Gates Review

- 1** How would you view the non-T lymphocytes in comparison with all lymphocytes on the scatter plot? Try it.

- 2** Write a gate specification for all non-granulocytes that are CD4⁺. Enter your specification as the definition for G8.

- 3** Gate the FSC vs SSC plot using G8.

The plot should show no granulocytes, some lymphocytes, and all monocytes.

- 4** Write a gate specification for all non-lymphocytes that are CD4⁺. Enter your specification as the definition for G8.

- 5** Use backgating to locate the G8 events located on the FSC vs SSC plot. What kind of cell are the majority of G8 events?

- 6** Display a Gate Statistics view for the FSC vs SSC plot.

- 7** The %Gated for G8 is 100.0. Why?

- 8** Record the %Total for G8: _____.

- 9** What does the %Total for G8 statistic represent? (choose one):

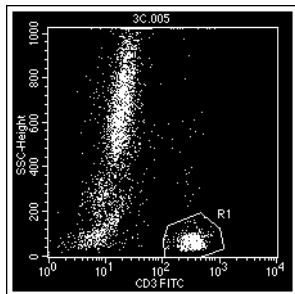
- a The percentage of monocytes that are CD4⁺.
- b The percentage of all cells that are CD4⁺.
- c neither a nor b.

- 10** Delete the Gate Statistics view.

Using Histograms

In this exercise, you will create a histogram plot and use histogram markers to generate population statistics.

- 1 Delete all regions from the Experiment document.
- 2 Display the Gate List window and click Set All to Default.
- 3 Change all plots to display ungated data.
 - a Select all plots in the Experiment document.
 - b Display the Inspector, if needed.
 - c Click the Gate menu control and select No Gate.
- 4 Verify that all plots display the 3C.005 data file.
- 5 Change the X parameter of the FSC vs SSC plot from FSC to CD3 FITC, using either of the following methods:
 - Move the cursor over the parameter label to be changed. The cursor becomes a menu control icon. Click and select CD3 FITC.
 - Select the FSC vs SSC plot. In the Inspector, click the X Parameter menu control and select CD3 FITC.
- 6 Create a polygonal region (R1) encompassing the CD3⁺ cells on the CD3 FITC vs SSC plot.



Enlarging the Experiment Document

Each new Experiment document is set to the default 10.19 x 8-inch page size. The view area is set to this page size. To increase the view area, you can increase the document size.

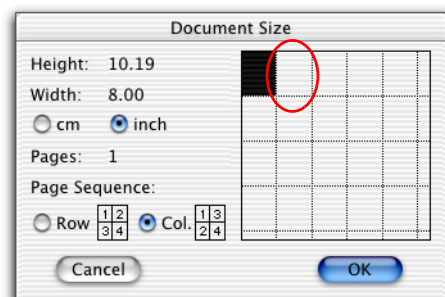
- 1 Click on the Experiment document to make it the active window.
- 2 Select File > Document Size.

The Document Size dialog appears.

- 3 Click the rectangle to the right of the current page selection to add an additional page.


The Width and Pages values are updated.

- 4 Click OK to close the dialog.



The view area increases to a two-page width. Page breaks are indicated by a dotted line. Do not place Experiment document objects on the dotted line representing the page break. Use the scroll bar to navigate within the enlarged view area.

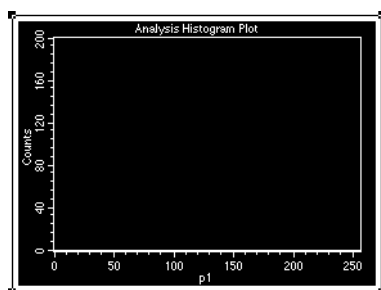
Displaying Data in a Histogram Plot

- 1 Select the Histogram-Plot tool () from the tool palette.

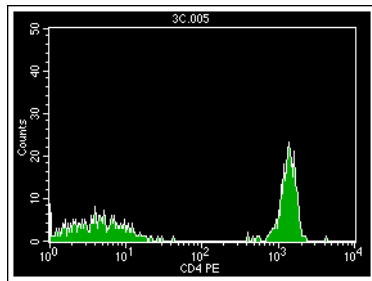
The Histogram-Plot tool becomes highlighted.


- 2 Click in a clear area of the Experiment window and drag diagonally until the plot outline is an appropriate size.

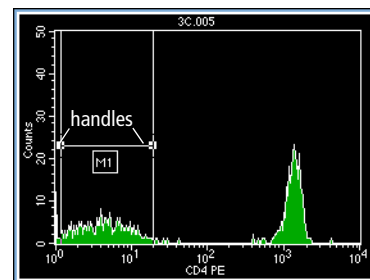
When you release the mouse button, a histogram plot appears, and the Inspector changes to the Histogram Plot Inspector.



- 3** Use the Inspector to to:
- Click the Plot Type menu control and select Analysis.
 - Click the File menu control and select file 3C.005.
 - Click the X parameter menu control and select CD4 PE.
 - Click the Gate menu control and select G1=R1.
 - Verify that the Manual Scale checkbox is selected and set Manual Scale to 50.
 - Resize the histogram plot to Width 4.00 and Height 3.00.



- 4** Create histogram markers for the CD4⁻ and CD4⁺ populations.
- Click the Histogram Marker tool () on the tool palette to select it.
 - Click on the histogram plot on the left edge of the CD4⁻ population and drag to the right edge of the population.
 - Adjust the histogram marker (M1) as needed to encompass the CD4⁻ population.
 - Click the marker to obtain handles.
 - Drag a handle left or right to position that boundary.
 - Drag a handle up or down to position the horizontal bar.
 - Click outside the marker to deselect it. Then drag the marker label to position it.
 - Repeat steps a through d to create a histogram marker (M2) for the CD4⁺ population.



Analyzing Histogram Statistics

- 1** Click in the CD4 PE histogram to activate it and select Stats > Histogram Stats.
- 2** Record the %Gated for M2 (CD4⁺): _____.
- 3** Record the %Total for M2 (CD4⁺): _____.
- 4** Which statistic specifies the percentage of CD3⁺ cells that are CD4⁺? (choose one)
 - a %Gated for M2.
 - b %Total for M2.
 - c neither a nor b.
- 5** Record the %Gated for M1 (CD4⁻): _____.
- 6** Does %Gated for M1 + %Gated for M2 = 100%? If not, try to modify the histogram markers to make it so.
- 7** Which statistic in the histogram statistics view specifies the percentage of all cells that are CD3⁺? (choose one)
 - a %Total for M2.
 - b %Total for All.
 - c neither a nor b.

Batch Analysis

In this section, you will use batch analysis with your Experiment document to analyze additional data files.

Once you set up an Experiment document with plots, gates, markers, and statistics, BD CellQuest Pro software can automate the same analysis for subsequent files. Batch analysis options include:

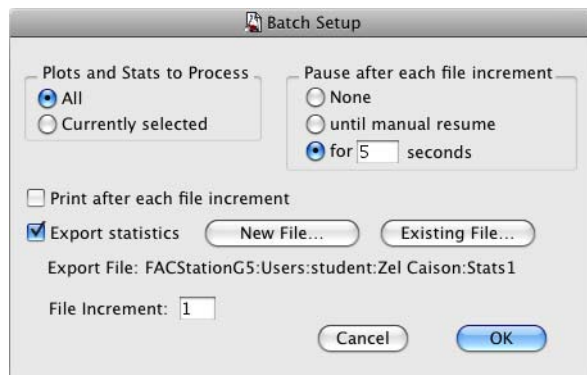
- Automatic pause after each file so that you can adjust markers or regions.
- Automatic printing of the Experiment document.
- Automatic export of statistics to an export file.

For details on these and other batch analysis features, refer to the *BD CellQuest Pro Software Reference Manual*.

- 1 Display data file NORM.001 in all plots.
 - a Shift+click to select all plots.
 - b In the Inspector, click the File menu control and select Select File.
 - c Navigate to BD Applications/CellQuest Pro/Sample Files and select NORM.001.
- 2 Select Batch > Setup.

The Batch Setup dialog appears.

- 3 Make the following selections.



- 4 Click the New File button.

A Save dialog appears.

- 5 Type Stats1 in the Save as: field.
- 6 For folder destination, specify your student folder.
- 7 Click Save.

These Batch Setup selections specify that:

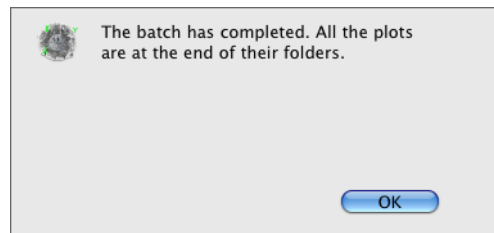
- Data will be processed through all plots in the Experiment document.
- The batch will pause for 5 seconds after each data file is processed.
- All statistics from the Experiment document are exported to the specified file.
- The file suffix is incremented by one each time to determine the next file to analyze (no files are skipped).

- 8 Click OK to accept the selections and close the dialog.
- 9 Select Batch > Run to start the run.

A Batch Control floater appears. Use these controls at any time during the run to interrupt batch analysis.



A completion dialog appears at the end of the batch.



Importing Statistics into a Spreadsheet

In this exercise, you will import the export file you saved during your data analysis into a third-party spreadsheet application.

- 1 Start Microsoft Excel by clicking its icon on the Dock.
A blank worksheet appears on the screen.
- 2 Select File > Open.
A dialog appears.
- 3 Navigate to the export file in your student folder and open it.
The file opens and displays the statistics in a spreadsheet format.
- 4 Click Finish on the Text Import Wizard window.
- 5 View the results.

File Name	Log Data	Uni Sample ID	Patient ID	Patient Name	Case Number	Acquisition Date	Gated Events	Total Events	X Parameter Label	Left	Right	Events	% Gated	% Total	X Me
7 Norm 001	Linear Values Sample 1					8-Sep-00 G1	3	6000	Mouse I5G2AM1	1	9910	3	100	0.05	
8 Norm 001	Linear Values Sample 1					8-Sep-00 G1	3	6000	Mouse I5G2AM2	1	28	2	66.67	0.03	
9 Norm 001	Linear Values Sample 1					8-Sep-00 G1	3	6000	Mouse I5G2AM2	29	9910	1	33.33	0.02	
5 Norm 002	Linear Values Sample 1					8-Sep-00 G1	2219	6000	CD19 PE (Log)AI	1	9910	2219	100	36.98	
6 Norm 002	Linear Values Sample 1					8-Sep-00 G1	2219	6000	CD19 PE (Log)M1	1	28	2214	99.77	36.9	
7 Norm 002	Linear Values Sample 1					8-Sep-00 G1	2219	6000	CD19 PE (Log)M2	29	9910	9	0.23	0.08	
8 Norm 002	Linear Values Sample 1					8-Sep-00 G1	2271	6000	CD4 PE (Log)AI	1	9910	2271	100	37.85	
9 Norm 003	Linear Values Sample 1					8-Sep-00 G1	2271	6000	CD4 PE (Log)M1	1	28	1020	44.91	17	
10 Norm 003	Linear Values Sample 1					8-Sep-00 G1	2271	6000	CD4 PE (Log)M2	29	9910	1251	55.09	20.85	
11 Norm 004	Linear Values Sample 1					8-Sep-00 G1	2269	6000	CD8 PE (Log)AI	1	9910	2269	100	37.82	
12 Norm 004	Linear Values Sample 1					8-Sep-00 G1	2269	6000	CD8 PE (Log)M1	1	28	1264	57.03	21.57	
13 Norm 004	Linear Values Sample 1					8-Sep-00 G1	2269	6000	CD8 PE (Log)M2	1	9910	973	42.88	18.22	
14 Norm 005	Linear Values Sample 2					8-Sep-00 G1	9	6000	Mouse I5G2AM1	1	9910	9	100	0.15	
15 Norm 005	Linear Values Sample 2					8-Sep-00 G1	9	6000	Mouse I5G2AM1	1	28	9	100	0.15	
16 Norm 005	Linear Values Sample 2					8-Sep-00 G1	9	6000	Mouse I5G2AM2	29	9910	0	0	0	
17 Norm 006	Linear Values Sample 2					8-Sep-00 G1	1528	6000	CD19 PE (Log)AI	1	9910	1528	100	25.47	
18 Norm 006	Linear Values Sample 2					8-Sep-00 G1	1528	6000	CD19 PE (Log)M1	1	28	1524	99.74	25.4	
19 Norm 006	Linear Values Sample 2					8-Sep-00 G1	1528	6000	CD19 PE (Log)M2	29	9910	4	0.26	0.07	
20 Norm 007	Linear Values Sample 2					8-Sep-00 G1	1649	6000	CD4 PE (Log)AI	1	9910	1649	100	27.48	
21 Norm 007	Linear Values Sample 2					8-Sep-00 G1	1649	6000	CD4 PE (Log)M1	1	28	915	55.49	15.25	
22 Norm 007	Linear Values Sample 2					8-Sep-00 G1	1649	6000	CD4 PE (Log)M2	29	9910	734	44.51	12.23	
23 Norm 008	Linear Values Sample 2					8-Sep-00 G1	1651	6000	CD8 PE (Log)AI	1	9910	1651	100	27.52	
24 Norm 008	Linear Values Sample 2					8-Sep-00 G1	1651	6000	CD8 PE (Log)M1	1	28	958	58.03	15.97	
25 Norm 008	Linear Values Sample 2					8-Sep-00 G1	1651	6000	CD8 PE (Log)M2	29	9910	691	41.85	11.52	
26 Norm 009	Linear Values Sample 3					8-Sep-00 G1	3	6000	Mouse I5G2AM1	1	9910	3	100	0.05	
27 Norm 009	Linear Values Sample 3					8-Sep-00 G1	3	6000	Mouse I5G2AM1	1	28	2	66.67	0.03	
28 Norm 009	Linear Values Sample 3					8-Sep-00 G1	3	6000	Mouse I5G2AM2	29	9910	1	33.33	0.02	
29 Norm 010	Linear Values Sample 3					8-Sep-00 G1	2220	6000	CD19 PE (Log)AI	1	9910	2220	100	37	
30 Norm 010	Linear Values Sample 3					8-Sep-00 G1	2220	6000	CD19 PE (Log)M1	1	28	2215	99.77	36.92	
31 Norm 010	Linear Values Sample 3					8-Sep-00 G1	2220	6000	CD19 PE (Log)M2	29	9910	2	0.23	0.08	
32 Norm 011	Linear Values Sample 3					8-Sep-00 G1	2271	6000	CD4 PE (Log)AI	1	9910	2271	100	37.85	
33 Norm 011	Linear Values Sample 3					8-Sep-00 G1	2271	6000	CD4 PE (Log)M1	1	28	1020	44.91	17	
34 Norm 011	Linear Values Sample 3					8-Sep-00 G1	2271	6000	CD4 PE (Log)M2	29	9910	1251	55.09	20.85	
35 Norm 012	Linear Values Sample 3					8-Sep-00 G1	2272	6000	CD8 PE (Log)AI	1	9910	2272	100	37.87	
36 Norm 012	Linear Values Sample 3					8-Sep-00 G1	2272	6000	CD8 PE (Log)M1	1	28	1280	57.04	21.6	
37 Norm 012	Linear Values Sample 3					8-Sep-00 G1	2272	6000	CD8 PE (Log)M2	29	9910	974	42.87	16.23	
38 Tutorial 001	Linear Values					8-Aug-01 G1	2	50672	CD69 PE (Log)AI	1	9910	2	100	0	
39 Tutorial 001	Linear Values					8-Aug-01 G1	2	50672	CD69 PE (Log)M1	1	28	0	0	0	
40 Tutorial 001	Linear Values					8-Aug-01 G1	2	50672	CD69 PE (Log)M2	29	9910	2	100	0	
41 Tutorial 002	Linear Values					8-Aug-01 G1	40	52883	CD69 PE (Log)AI	1	9910	40	100	0.08	
42 Tutorial 002	Linear Values					8-Aug-01 G1	40	52883	CD69 PE (Log)M1	1	28	1	2.5	0	
43 Tutorial 002	Linear Values					8-Aug-01 G1	40	52883	CD69 PE (Log)M2	29	9910	39	97.5	0.07	
44 Tutorial 003	Linear Values Donor 935					28-Mar-01 G1	619	190452	CD69 PE (Log)AI	1	9910	619	100	0.33	
45 Tutorial 003	Linear Values Donor 935					28-Mar-01 G1	619	190452	CD69 PE (Log)M1	1	28	24	3.88	0.01	
46 Tutorial 003	Linear Values Donor 935					28-Mar-01 G1	619	190452	CD69 PE (Log)M2	29	9910	593	95.8	0.31	

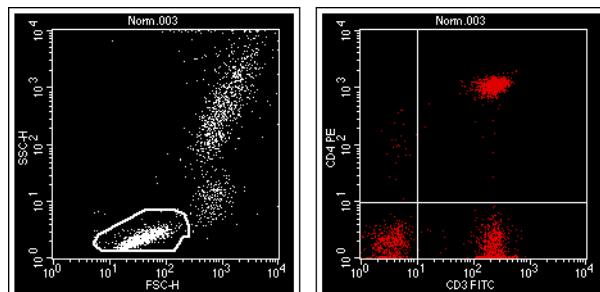
- 6 After you have finished viewing the statistics, select File > Quit.

Using the Expression Editor

In this exercise, you will use the Expression Editor to calculate statistics for CD3⁺ and CD4⁺ cells. Expression views update automatically as new data is loaded into plots.

Setting Up the Experiment Document

- 1 Delete the CD4 PE histogram plot and the CD45 PerCP vs SSC dot plot.
- 2 Delete all regions on the remaining plots.
- 3 Change the CD3 FITC vs SSC plot back to FSC vs SSC.
- 4 Display the data file BD Applications/CellQuest Pro/Sample Files/Norm.003 in both plots.
- 5 Create a Snap-To region (R1) for the lymphocytes on the FSC vs SSC plot.
- 6 Gate the CD3 FITC vs CD4 PE plot using G1 = R1.
- 7 Set a quadrant marker on the CD3 FITC vs CD4 PE plot. Set the marker to encompass the double-negative population in the lower left quadrant.
- 8 Display Quadrant Statistics for the CD3 FITC vs CD4 PE plot.



Quadrant Statistics

File: Norm.003 Log Data Units: Linear Values
Sample ID: Sample 1 Patient ID:
Patient Name: Case Number:
Acquisition Date: 08-Sep-00 Gate: G1
Gated Events: 2869 Total Events: 6000
X Parameter: CD3 FITC (Log) Y Parameter: CD4 PE (Log)
Quad Location: 10, 9

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	24	0.84	0.40	4.87	4.65	134.26	97.26
UR	1271	44.30	21.18	248.96	236.16	1097.98	1073.71
LL	530	18.47	8.83	3.65	3.34	1.93	1.77
LR	1044	36.39	17.40	242.52	226.39	1.55	1.39

Creating an Expression

- 1 Click the Expression Editor tool () on the tool palette.

The Expression Editor appears.

- 2 Click in the Label field and type *%Lymphocyte CD3+ cells =*

TIP Type a space after the = to improve readability of the label with expression value.

- 3 Construct the expression.

- a Click in the expression box.
- b Click the UR %Gated value in the quadrant statistics view.

The UR %Gated value appears in the expression box.

- c Click the plus button () in the Expression Editor.

The + sign appears next to the UR %Gated value in the expression box.

- d Click the LR %Gated value.

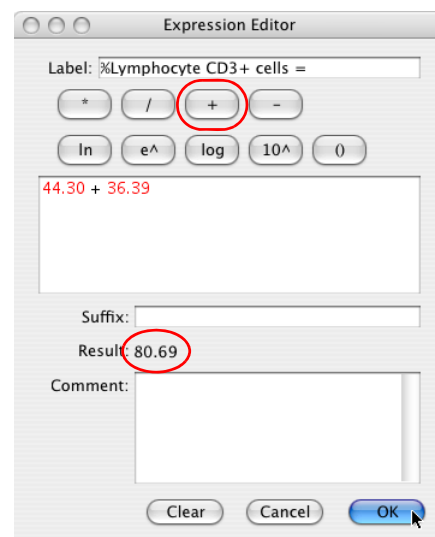
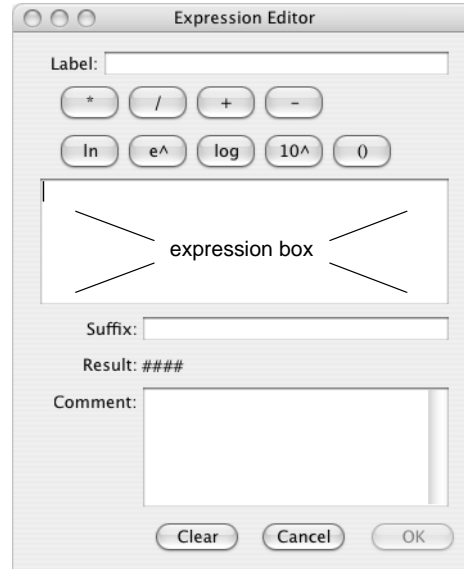
The LR %Gated value appears after the + sign in the expression box. The calculated result is displayed in the Result field.

- 4 Click OK to close the editor.

Quadrant Statistics							
File: Norm.003	Log Data Units: Linear Values						
Sample ID: Sample 1	Patient ID:						
Patient Name:	Case Number:						
Acquisition Date: 08-Sep-00	Gate: G1						
Gated Events: 2869	Total Events: 6000						
X Parameter: CD3 FITC (Log)	Y Parameter: CD4 PE (Log)						
Quad Location: 10, 9							
Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	24	0.84	0.40	4.87	4.65	134.26	97.26
UR	1271	44.30	21.18	248.96	236.16	1097.98	1073.71
LL	530	18.47	8.83	3.65	3.34	1.93	1.77
LR	1044	36.39	17.40	242.52	226.39	1.55	1.39

The expression view is displayed in the Experiment document.

`%Lymphocyte CD3+ cells = 80.69`



- 5 Expand the expression view as needed. See Adjusting an Expression on page 174.

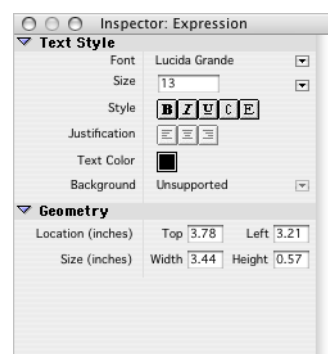
Adjusting an Expression

You can resize, reposition, and modify several properties of an expression similar to the way you adjust plots and statistics views.

- Drag an expression to reposition it.
- Click the border of an expression to select it.



- A selected expression has a handle in each corner. Click a selected expression handle and drag to resize the expression.
- Properties of a selected expression can be modified in the Inspector. Expression properties include:
 - font
 - text size, style, color, and justification
 - location and size







TIP The double lines of the expression are displayed on screen but not in print.


Creating a Complex Expression

The Expression editor can create very complex expressions. Refer to the *BD CellQuest Pro Software Reference Manual* for additional information about expressions.

In this section, you will create an expression for the % of LymphocyteCD3⁺ cells that are CD4⁺.

- 1 Click the Expression Editor tool () to display the Expression Editor.
- 2 Click in the Label field and type *%LymphocyteCD3+ CD4+ cells =*
- 3 Click in the expression box and type 100 followed by a space, click the multiply button () , type a space, click the UR %Gated value in the quadrant statistics view, type a space, click the divide button () , type a space, and click the parentheses button () .

100 * UR %Gated value / () is displayed in the expression box.

- 4 Click to position the entry cursor between the parentheses and click the UR %Gated value in the quadrant statistics view, click the plus button (), and click the LR %Gated value in the quadrant statistics view.

$100 * UR \%Gated\ value / (UR \%Gated\ value + LR \%Gated\ value)$ is displayed in the expression box. The calculated result is displayed in the Result field.

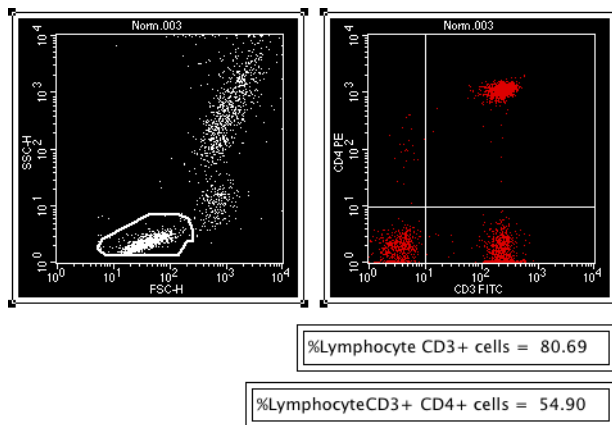
- 5 Click OK to close the editor.

The expression view is displayed in the Experiment document.

- 6 Resize and reposition the expressions as needed.

Analyzing Data Files

- 1 Shift+click to select both plots.



- 2 Select Plots > Next Data File.

The next data file in the BD Applications/CellQuest Pro/Sample Files folder, Norm.004, is displayed in the plots. The Snap-To lymphocyte gate is redrawn. The expressions recalculate their values.

TIP Command (⌘)+(right bracket) is a keyboard shortcut for Next Data File, and Command (⌘)+(left bracket) is a keyboard shortcut for Previous Data File.

- 3 Use the keyboard shortcuts to step forward and back through the data files in BD Applications/CellQuest Pro/Sample Files.

Overlaid Histogram Analysis

In this exercise, you will use the histogram analysis features in BD CellQuest Pro software to analyze data. After completing this exercise, you will be able to:


- Create overlay histograms.
- Create and edit histogram statistics.
- Change overlaid histogram plots by formatting and using histogram tools.
- Create statistics and Kolmogorov-Smirnov (K-S) statistics for overlaid histograms.

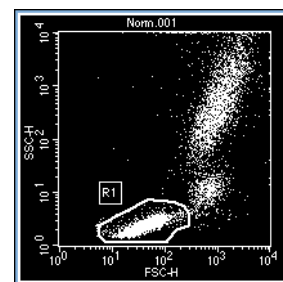
Creating a Lymphocyte Gate

In this section, you will create a scatter plot, display the data from a control file, and draw a region encompassing the lymphocyte events.


- 1 If BD CellQuest Pro software is already running, save and close any open Experiment documents, then select File > New Document.

Otherwise, start BD CellQuest Pro software.

- 2 Expand the Experiment window to full size by clicking the zoom box in the upper-right corner of the window.
- 3 Increase the Experiment document size to two pages wide (see Enlarging the Experiment Document on page 166 for detailed instructions).
- 4 Use the Dot plot tool () to create a new dot plot.
- 5 Use the Inspector to:
 - a Resize the plot to 3.0 inches Width by 3.0 inches Height.
 - b Display the data file BD Applications/CellQuest Pro/Sample Files/NORM.001.
- 6 Use the Snap-To-Region tool to create a region encompassing the lymphocytes.
- 7 Click outside the region to deselect it. Drag the region label R1 to a new location on the plot away from the data.



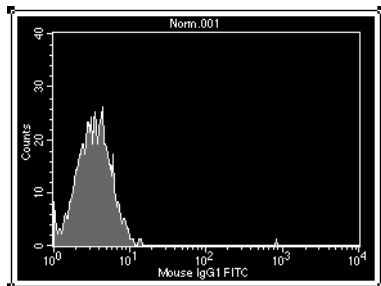
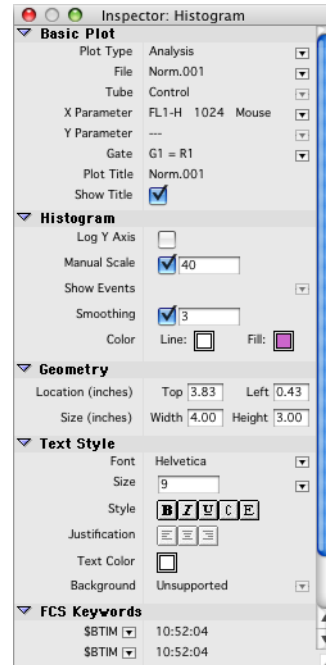
Creating an FL1 Histogram

- 1 Use the Histogram-Plot tool () to create a histogram plot.
- 2 Use the Inspector to:
 - a Resize the plot to 4.0 inches Width by 3.0 inches Height.
 - b Display the data from NORM.001.
 - c Specify FL1 as the X Parameter.
 - d Gate the data with G1 = R1.
 - e Select the Manual Scale checkbox and specify 40 as the scale value.
 - f Select the Smoothing checkbox and specify 3 as the value.

Smoothing values range from 0 (no smoothing) to 5 (maximum smoothing).

NOTE Histogram and K-S statistics are not affected by smoothing. Smoothing affects only the histogram display.

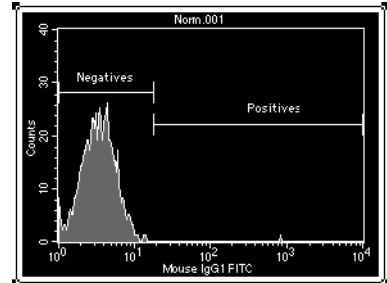
- g Click the Line Color box.
- h Click a color to change the histogram outline and close the dialog.
- i Click the Fill Color box to change the histogram fill color.



Creating Histogram Markers

In this section you will create a histogram marker for the gated control events. This marker will encompass the FL1-negative population. You will set a second marker to encompass the FL1-positive population.

- 1 Use the Histogram Marker tool to draw a marker from the left edge of the histogram plot and encompassing the negative control events.
- 2 Create a second histogram marker M2 from the right edge of M1 to the right edge of the plot.
- 3 Verify that the histogram is selected.
- 4 Select Gates > Marker List.



The Marker List appears.

- 5 Click in the M1 label text box and type *Negatives*.
- 6 Click in the M2 label text box and type *Positives*.
- 7 Close the Marker List.

No.	Label	Low	High
M1	Negatives	1	18
M2	Positives	18	9910

The Marker List can also be used to delete, copy, paste, and convert markers to regions. For more information about markers and the Marker List, refer to the *BD CellQuest Pro Software Reference Manual*.

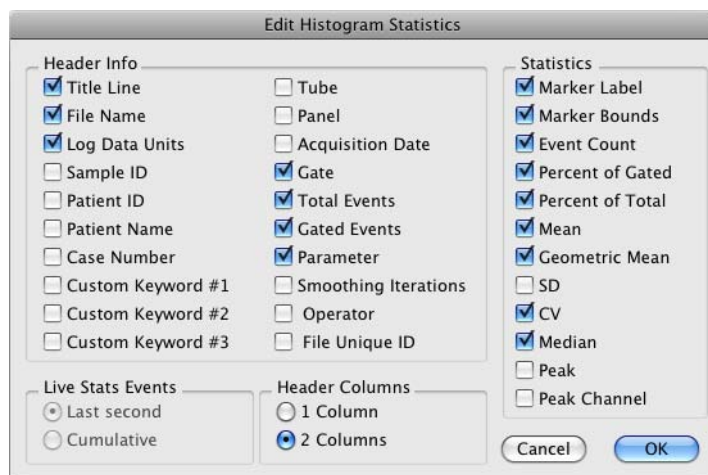
Histogram Statistics

- 1 Select Stats > Histogram Stats.

A Histogram statistics view appears. The view displays descriptive and statistical information about the histogram plot. Undefined values are indicated by ***.

Histogram Statistics										
File: Norm.001					Log Data Units: Linear Values					
Sample ID: Sample 1					Patient ID:					
Tube: Control					Panel: 2 Color Panel					
Acquisition Date: 08-Sep-00					Gate: G1					
Gated Events: 2943					Total Events: 6000					
X Parameter: Mouse IgG1 FITC (Log)										
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch	
All	1, 9910	2943	100.00	49.05	4.72	3.34	627.65	3.37		3
Negatives	1, 18	2938	99.83	48.97	3.70	3.31	49.48	3.34		3
Positives	18, 9910	5	0.17	0.08	602.98	376.51	73.31	784.99		40

- 2 Drag the Histogram Statistics view to a clear area of the Experiment window.
- 3 Verify that the histogram is selected.
- 4 Select Stats > Edit Histogram Stats to display the Edit Histogram Statistics dialog.
- 5 Specify selections as shown in the following figure.

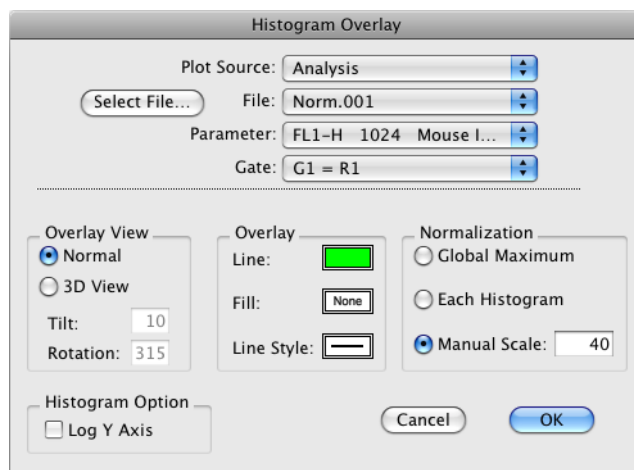


- 6 Click OK.

Creating Overlaid Histograms

An overlaid histogram plot contains additional overlaid histograms displayed in front of an initial histogram.

- 1 Select the FL1 histogram.
- 2 Select Plots > Overlay to display the Histogram Overlay dialog.



3 Click Select File.

The Open a Data File dialog appears.

4 Open NORM.002.

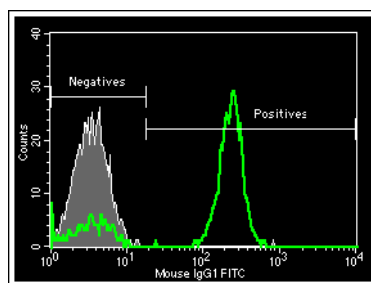
The Histogram Overlay dialog includes additional controls for formatting the overlaid histogram. These controls will also be available in the Formatting Histogram Overlay dialog (see Modifying Overlaid Histograms on page 181).

- **Overlay View**—Normal displays a two-dimensional view. 3D View displays a three-dimensional view. Tilt and Rotation values can be specified for 3D View. Markers, regions and axis scales do not appear in 3D View.
- **Overlay**—Click the Line or Fill boxes to specify colors for the overlaid histogram. Click the Line Style box to specify the line style. Line styles can help differentiate overlaid histograms, especially when printing in black and white.
- **Normalization**—Global Maximum normalizes the vertical scale to the highest count found in any of the histograms. Each Histogram normalizes the vertical scale of each histogram to the highest count found in that histogram. Manual Scale specifies a maximum vertical scale. Normalization does not affect statistics.
- **Histogram Option**—Select the Log Y axis checkbox to change the Y axis scale from linear to log.

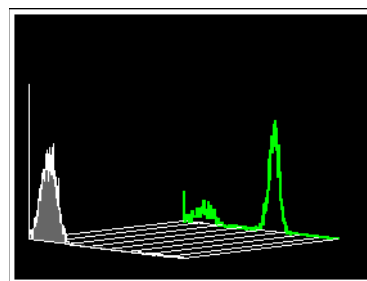
5 Click OK.

The Histogram Overlay dialog closes and the overlaid histograms are displayed in the histogram plot in the Normal view.

Both the normal view and 3D views are shown in the following figure.



Normal view





3D view

Displaying Histogram Legends

- 1 Select the overlaid histogram plot.
- 2 Select Plots > Legend.

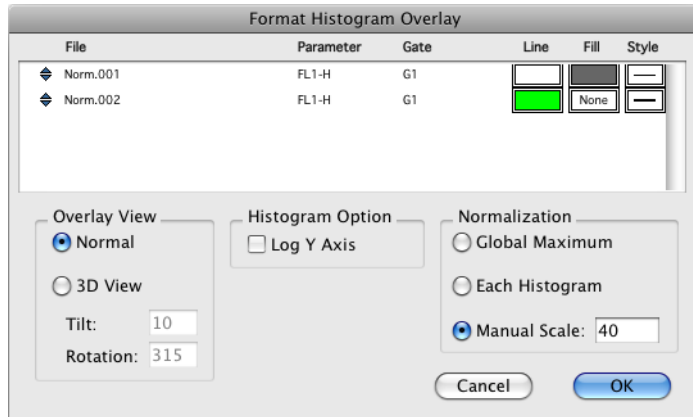
A Histogram Legend appears with information on the overlaid histogram plot. The Histogram Legend helps you to identify the histograms in an overlaid histogram plot.

Key	Name	Parameter	Gate
	Norm.001	FL1-H	G1
	Norm.002	FL1-H	G1

- 3 Drag the Histogram Legend to position it near the overlaid histogram plot.

Modifying Overlaid Histograms

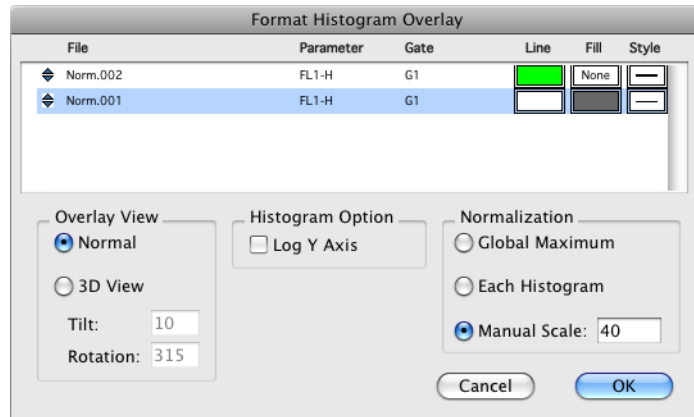
- 1 Select the overlaid histogram plot.
- 2 Select Plots > Format Histogram Overlay to display the Format Histogram Overlay dialog.



You can use the Format Histogram Overlay dialog to specify color and line styles for each of the histograms, as well as their front to back ordering. You can also modify the Overlay View, Histogram Option, and Normalization settings for the overlay histogram plot.

- Click the selection icon (🔍) for Norm.001 and drag it under Norm.002.

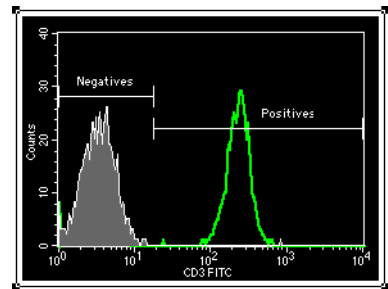
Changing the order of the histograms in the list will change the front to back ordering of the histograms in the overlay. The lower histograms in the list are displayed in front of the upper histograms.



- Click OK.

The overlaid histogram plot appears with the new histogram order.

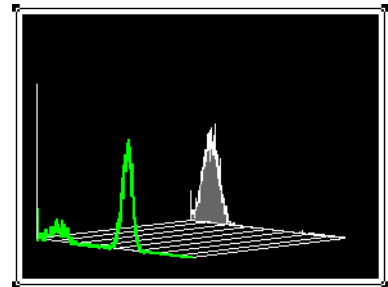
- Select Plots > Format Histogram Overlay to display the Format Histogram Overlay dialog again.



- Select 3D View in the Overlay View section.

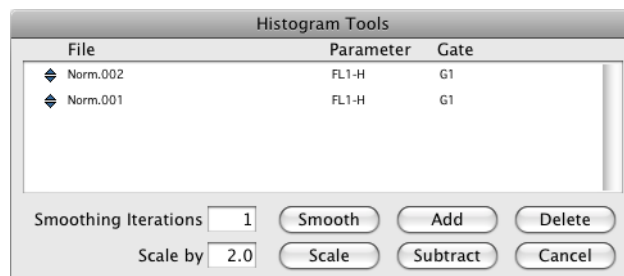
- Click OK.

The overlaid histogram plot appears in 3D view.



Using Histogram Tools

- Select Plots > Histogram Tools to display the Histogram Tools dialog.



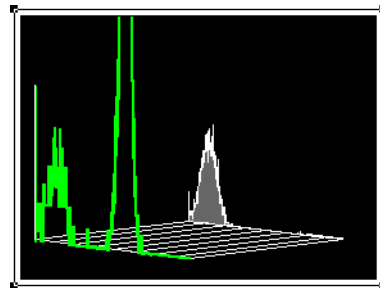
- 2 Select Norm.002 by clicking its selection icon.
- 3 Click the Scale by text box and type 5.0.

TIP You can specify Scale by values from 0.1 to 99.

- 4 Click Scale.

The Norm.002 histogram is scaled by 5.

Scaling can be used to normalize files of different sizes. For example, if Norm.001 contained 10,000 events and Norm.002 contained only 2,000 events, you could normalize the number of events to 10,000 by scaling Norm.002 by 5.



IMPORTANT Histogram and K-S statistics are affected by scaling.

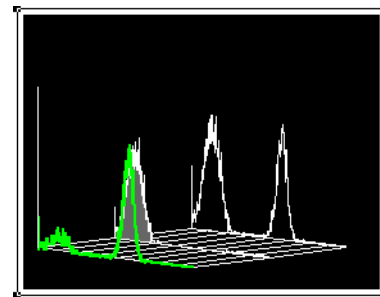
- 5 Use Histogram Tools to return the scale of Norm.002 to 1.
- 6 Create a new histogram with channel counts that are the sum of the channel counts of Norm.002 and Norm.001:
 - a Open the Histogram Tools dialog again and command (⌘)+click both the Norm.002 and the Norm.001 selection icons to select both histograms.

- b Click Add.

A Save Histogram dialog appears.

- c Save the histogram file in your student folder with the default name *Norm.002 + Norm.001*.

The NORM.001 and NORM.002 data files are unchanged. The new histogram is displayed in the overlaid histogram plot.



- 7 Delete the Norm.002 + Norm.001 summed channel count histogram:
 - a Open the Histogram Tools dialog again and select the Norm.002 + Norm.001 histogram by clicking its selection icon.
 - b Click Delete.

The histogram is removed from the plot. The saved histogram file is not deleted.

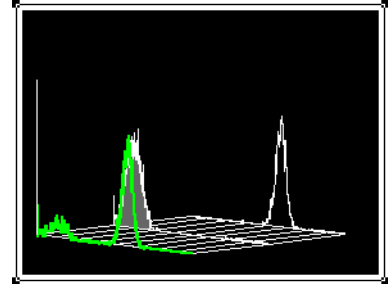
8 Create a new histogram with channel counts that are the difference of the channel counts of Norm.002 and Norm.001.

a Open the Histogram Tools dialog again and command (\oplus)+click both the Norm.002 and the Norm.001 selection icons to select both histograms.

b Click Subtract.

A Save Histogram dialog appears.

c Save the histogram file in your student folder with the default name.



The subtracted histogram is displayed in the overlaid histogram plot. Subtraction can be used to visualize the differences between similar histograms. The lower histogram in the Histogram Tools list is always subtracted from the one above it. Negative channel counts are reset to zero.

By subtracting the control histogram from the CD3 histogram, you have created a histogram with only CD3-positive events.

Overlaid Histogram Statistics

Overlaid histograms have two additional statistical options:

- Expressing the number of events in a marker of one histogram as a percentage of a second histogram. This feature is commonly used with subtracted histograms. In this section, you will use the subtracted histogram created in the previous section to practice this type of overlaid histogram statistic.
- Kolmogorov-Smirnov (K-S) statistics, which calculate the probability that two histograms are different. This is useful when trying to determine if two cell populations are distinct. See Kolmogorov-Smirnov Statistics on page 187.

Percent Gated and Percent Total Calculations

The percent gated and the percent total are important standard histogram statistics.

The percent gated is calculated by taking the number of events within a marker and dividing by the number of events in the gate that was applied to the data.

$$\text{percent gated} = \frac{\# \text{ events in marker}}{\# \text{ gated events}} \times 100$$

The percent total is calculated by taking the number of events within a marker and dividing by the total number of events in the data.

$$\text{percent total} = \frac{\text{\# events in marker}}{\text{\# total events}} \times 100$$

In overlaid histogram statistics, you can do the same calculations for percent gated and percent total by using the number of gated and total events from a different histogram as the denominators in the equations.

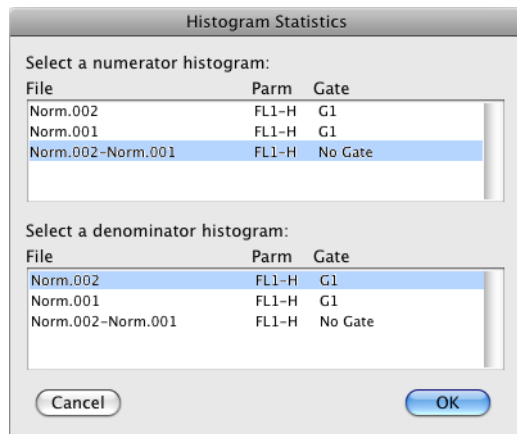
$$\text{percent gated} = \frac{\text{\# events in marker}}{\text{\# gated events in denominator histogram}} \times 100$$

$$\text{percent total} = \frac{\text{\# events in marker}}{\text{\# total events in denominator histogram}} \times 100$$

- 1 Select the overlaid histogram plot.
- 2 Select Stats > Histogram Stats.

The Histogram Statistics dialog appears.

- 3 Select Norm.002 – Norm.001 as the numerator histogram and Norm.002 as the denominator histogram, as shown in the following figure.



- 4 Click OK.

A Histogram Statistics view appears.

- 5 Drag the view to a clear area of the Experiment document.

Histogram Statistics									
* Numerator Histogram *					Log Data Units: Linear Values				
File: Norm.002-Norm.001					Patient ID:				
Sample ID:					Panel:				
Tube: CD3/CD19					Gate: No Gate				
Acquisition Date: 15-Nov-07					Total Events: 2265				
Gated Events: 2265									
X Parameter: FL1-H (Log)									
* Denominator Histogram *					Log Data Units: Linear Values				
File: Norm.002					Patient ID:				
Sample ID: Sample 1					Panel: 2 Color Panel				
Tube: CD3/CD19					Gate: G1				
Acquisition Date: 08-Sep-00					Total Events: 6000				
Gated Events: 2893									
X Parameter: CD3 FITC (Log)									
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	2265	78.13	37.75	227.62	215.88	32.49	220.67	232
M1	1, 14	0	0.00	0.00	***	***	***	***	***
M2	14, 9910	2265	78.13	37.75	227.62	215.88	32.49	220.67	232

The percent gated and percent total for M2 are calculated as follows:

$$\begin{aligned} \text{percent gated} &= \frac{\text{\# events in marker}}{\text{\# gated events in denominator histogram}} \times 100 \\ &= \frac{2265}{2893} \times 100 = 78.13\% \end{aligned}$$

$$\begin{aligned} \text{percent total} &= \frac{\text{\# total events in marker}}{\text{\# total events in denominator histogram}} \times 100 \\ &= \frac{2265}{6000} \times 100 = 37.75\% \end{aligned}$$

Only the statistics of Norm.001 appeared in the original Histogram Statistics view as you overlaid histograms. The overlaid histogram statistics appeared as a new Histogram Statistics view.

To obtain standard histogram statistics on another histogram in the overlay, create a new Histogram Statistics view and select that histogram as both the numerator and the denominator.

- 6 Open the Histogram Stats dialog again and select Norm.002 – Norm.001 as both the numerator histogram and the denominator histogram.

- 7 Click OK.

A new histogram statistics view appears. Compare the statistics from this view to the previous statistics view. The selection of the denominator should affect only the percent gated and percent total calculations.

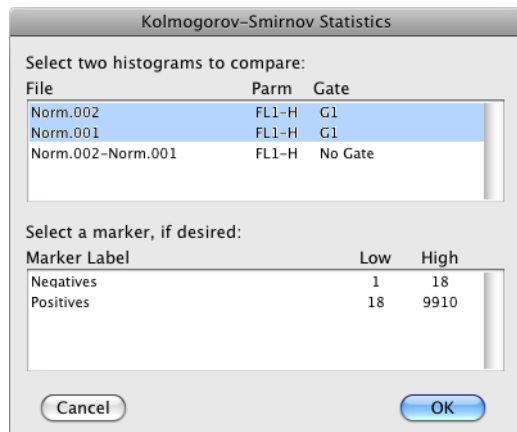
- 8 Drag the view to a clear area of the Experiment document.

Kolmogorov-Smirnov Statistics

The Kolmogorov-Smirnov (K-S) two-sample test is used to test whether two selected histograms in an overlaid histogram are distinct. K-S statistics can be calculated for the whole histogram or for a portion of it by setting a marker.

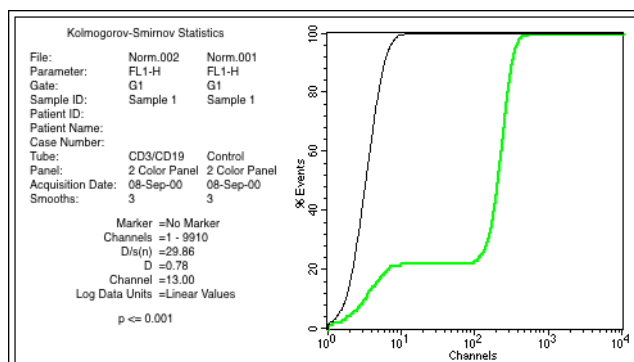
- 1 Select the overlaid histogram plot.
- 2 Select Stats > K-S Stats.
- 3 The K-S Stats dialog appears. The first two histograms, Norm.001 and Norm.002, are selected by default.

A marker can also be selected to restrict the range over which the statistics are calculated.



- 4 Click OK.

The K-S Statistics view appears.



- 5 Click the frame of the Kolmogorov-Smirnov Statistics view and drag it to a clear area of the Experiment document.

The following statistics are reported:

Channels—the portion of the histogram used in the analysis

D/s(n)—an index of similarity for the two curves

If $D/s(n) = 0$, the curves are identical. In this equation, $s(n)$ equals the square root of $(n1 + n2)/(n1 * n2)$, when $n1$ is the number of events in the first histogram and $n2$ is the number of events in the second histogram.

D—the Kolmogorov-Smirnov statistic, in other words, the greatest difference between the two curves

Channel—channel number of D

P value—the probability of D being as large as it is given that the two selected histograms are the same

For a complete discussion of these statistics, see IT Young's article in the Journal of Histochemistry and Cytochemistry. 1977:25:935-941.

- 6 Print the document, if you want.

Select File > Print and click Print in the dialog that appears.

BD Multiset Software

After completing this chapter, you will be able to:

- Optimize instrument settings using the BD Multiset Acquisition view.
- Use BD Multiset software to acquire and analyze 4-color lyse/no-wash samples prepared in BD Trucount™ tubes.



BD Multiset™ Software



Reagents

BD Multitest™

Lyse/No-Wash IVD

CD3/CD8/CD45/CD4

CD3/CD16+56/CD45/CD19

BD Tritest™

Lyse/No-Wash IVD

IgG1/IgG1/45 **CQ only**

CD3/CD4/CD45

CD3/CD8/CD45

CD3/CD16+56/CD45

CD3/CD19/CD45



BD Trucount™ Absolute Count Tubes

- Used with BD Tritest or BD Multitest reagents to determine absolute counts in whole blood.
- Contains a lyophilized pellet of a known number of fluorescent-labeled beads.

3



Process Control

- A normal sample prepared and run with patient samples.
- Controls for antibody staining, red cell lysis, instrument setup and performance, and data analysis.
- Sources:
 - Healthy subject
 - BD Multi-check control
 - BD Multi-check CD4 low control

4



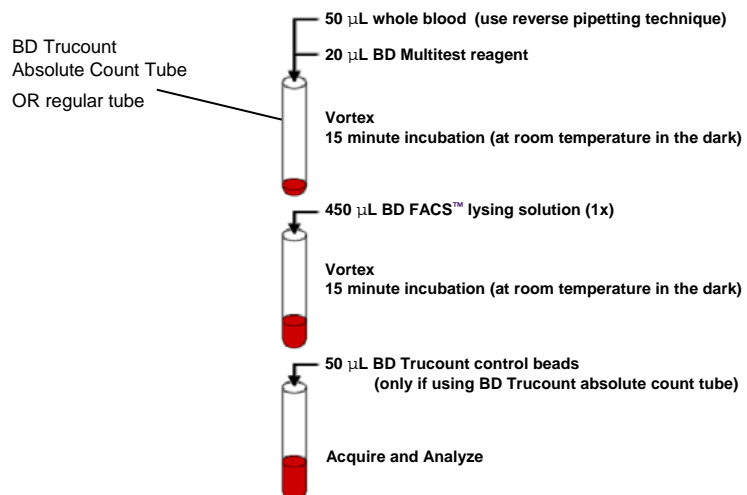
BD Trucount Controls

- Control for elements of the absolute counting process including:
 - Pipetting
 - BD Trucount Absolute Count Bead performance.
- Contains vials of Low, Medium, and High concentrations of beads.
- Provided with expected target value ranges.
- Added to process control sample.

5



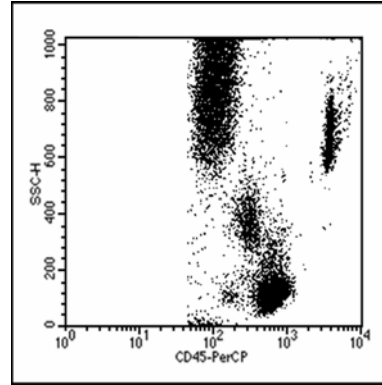
Sample Preparation



6

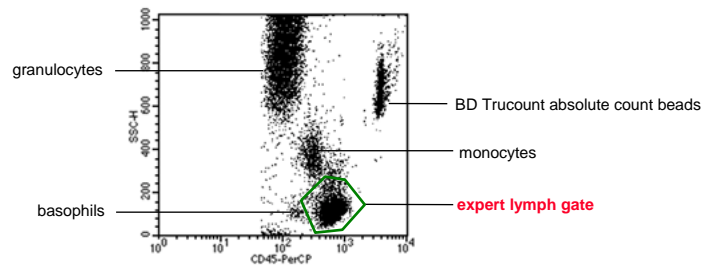
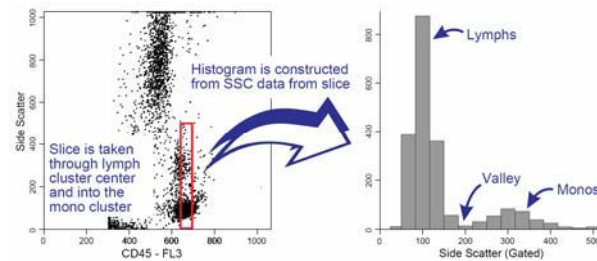
Lyse/No-Wash Optimization

- If needed, adjust the SSC PMT to leave a clear gap below the lymphs.
- If needed, raise the FL3-H threshold until most, but not all, of the debris is removed.



7

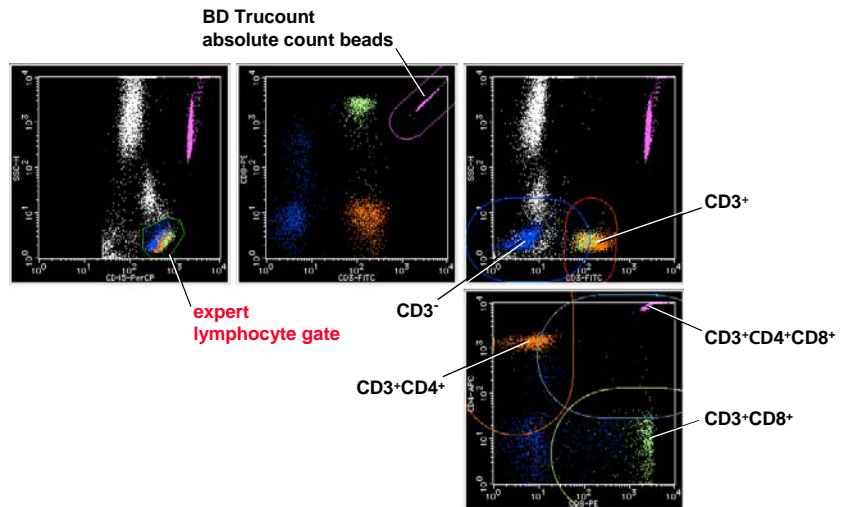
Expert Lymphocyte Gating



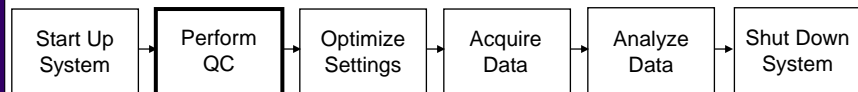
8



Analysis

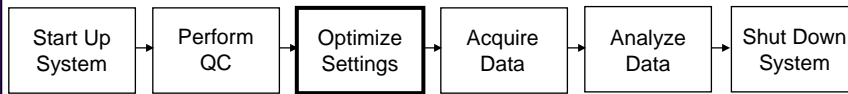


9

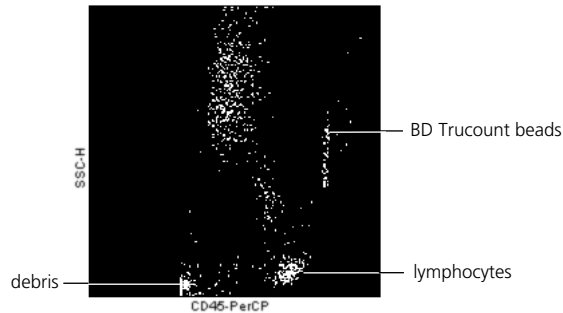


Run Lyse/No-Wash assay in BD FACSCComp software.

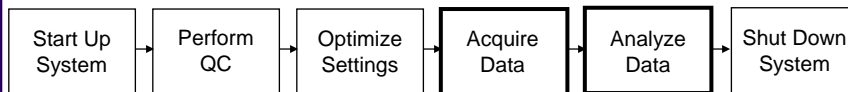
10



- Place populations on scale in SSC (if needed).
- Adjust the FL3 threshold to get rid of most of the debris (if needed).
- Save optimized settings.



11



- Specify data collection information, including:
 - file name
 - storage location
 - panels
- Click Acquire.
- Gate manually, if needed.
- Print or export results, if needed.


12




Start Up System → Perform QC → Optimize Settings → Acquire Data → Analyze Data → Shut Down System



13



Start Up System → Perform QC → Optimize Settings → Acquire Data → Analyze Data → Shut Down System



14



BD Multiset Exercise

3 samples x 2 tubes per sample = 6 tubes

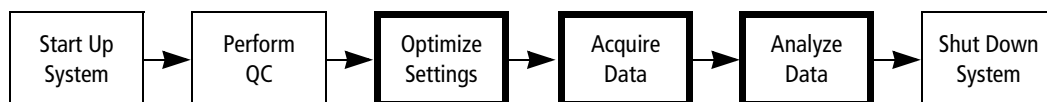
All samples are prepared in BD Trucount tubes.

- **BD Multi-check control**
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC
- **BD Multi-check CD4 Low control**
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC
- **Test sample**
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC

15

Introduction

Acquisition of flow cytometry data using BD Multiset software is a multistep process. The following figure outlines the main steps involved. This chapter will focus on optimization of instrument settings, data acquisition, and data analysis.



Materials

You will run BD Multi-check controls and a peripheral blood sample stained with BD Multitest™ reagents prepared in BD Trucount™ tubes. The sample provided is stained with the following 4-color panel:

- CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
- CD3 FITC/CD16+CD56 PE/CD45 PerCP/CD19 APC

BD Multi-check controls are a complete process control for immunophenotyping by flow cytometry. BD Multi-check controls should be handled with the same care and precautions as whole blood. Refer to the BD Multi-check control package insert for details.

BD Trucount tubes can be used to determine absolute counts of lymphocyte subpopulations. Each tube contains a known number of beads in a lyophilized bead pellet. Refer to the BD Trucount tube package insert for details.

Before You Begin

Before beginning the exercises in this chapter:

- Perform the procedure in System Startup on page 33.
- Run the Lyse/No-Wash (LNW) assay in BD FACSCComp software.

The instructions in Performing QC and Instrument Setup on page 71 are for running the BD FACSCComp software Lyse/Wash (LW) 4-color assay. The same procedure can be used to run the Lyse/No-Wash (LNW) 4-color assay by making appropriate entries at the Set Up view:

- Verify that only the Lyse/No-Wash (LNW) option is selected under Assay Selections.
- Under CaliBRITE Bead Lot IDs, verify the bead lot IDs for the FITC, PE, PerCP, APC, and Unlabeled beads.
- Under Automatic Saving Options, select the Lyse/No-Wash Summary Report checkbox.

Preparing to Run Samples

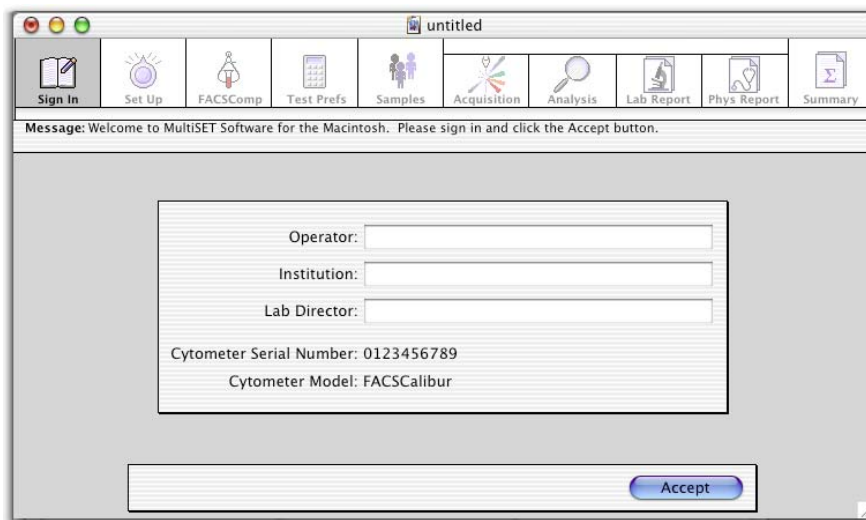
Before running samples in BD Multiset software, you will make selections and enter information at the Sign In, Set Up, FACSCComp, Test Prefs, and Samples views. You can also specify additional settings in the BD Multiset Preferences dialog. BD Multiset software will retain these settings from the previous session, so you will not always need to reenter them.

Sign In View

- 1 Start BD Multiset software by clicking its icon on the dock.



A startup screen appears, and then the application window opens at the Sign In view.



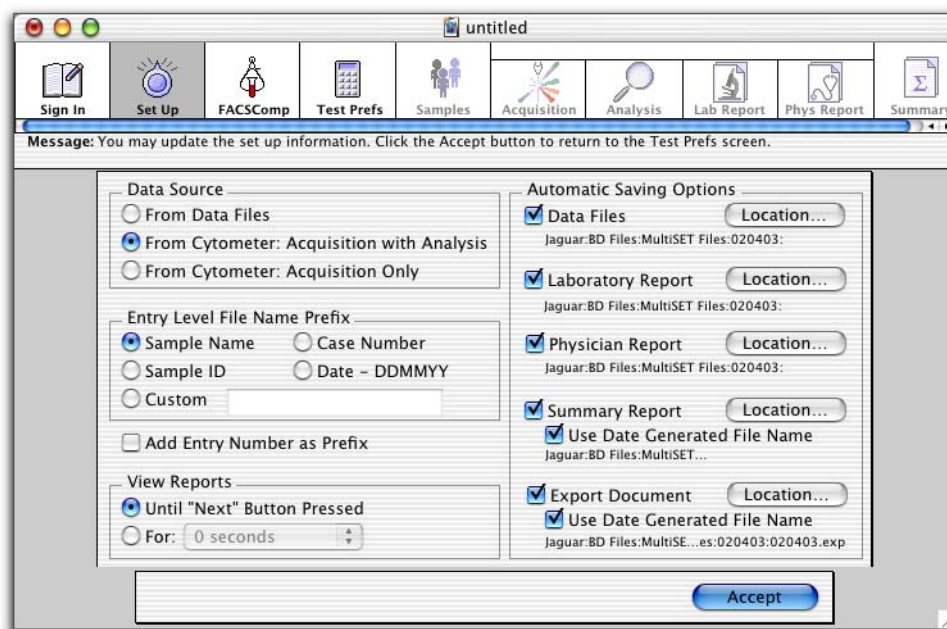
- 2 Type your name in the Operator field.
At the Sign In view, only the operator field is required.
- 3 (Optional) Fill in the Institution and Lab Director fields.
- 4 Click Accept or press Return.

The application window advances to the Set Up view.

Set Up View

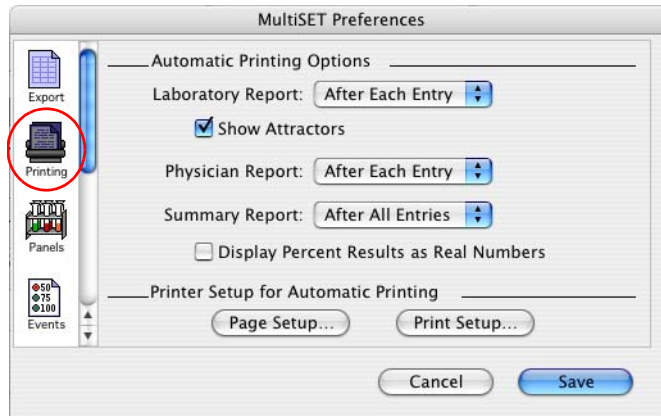
At the Set Up view, you will specify selections for Data Source, Entry Level File Name Prefix, View Reports, and Automatic Savings Options.

- 1 Verify that the following are selected in the Set Up view. Optional—change the Locations for Data Files, the Laboratory, Physician, and Summary Reports, and Export Files to your student folder.



- When the Data Source—**From Cytometer: Acquisition with Analysis** radio button is selected, data is automatically analyzed after each samples is acquired.
- When the Entry Level File Name Prefix—**Sample Name** radio button is selected, file name prefixes for FCS data files, Laboratory Report files, and Physician Report files are based on Sample Name entries. You must enter a Sample Name for each sample (see Samples View on page 204).
- When the View Reports—**Until “Next” Button Pressed** radio button is selected, you can view the analysis display for as long as you like.
- When an **Automatic Saving Options** box is selected, the corresponding file is automatically saved. The current file path appears below each file type. The default location for each file type is a dated folder (DDMMYY) in the BD Files/ MultiSet Files folder.

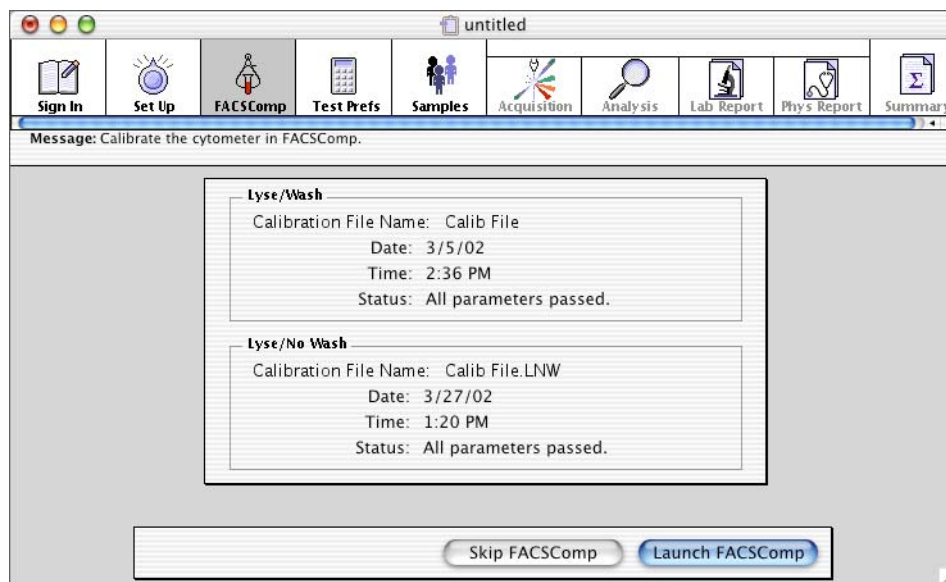
- 2 Specify automatic printing options for reports in the BD Multiset Preferences dialog.
 - a Select MultiSET > Preferences.
 - b Click the Printing icon to display the automatic printing preferences.
 - c Verify that the Automatic Printing Options are set as in the following figure.



- d Click Save to accept changes and close the preferences dialog.
- 3 At the Set Up view, click Accept or press Return.

The application window advances to the FACSComp view.

FACSComp View



The FACSComp view displays the name, date, time, and status information for the most recent Lyse/Wash and Lyse/No-Wash Calib files.



The listed Calib file settings are NOT automatically downloaded to the cytometer. See Loading Instrument Settings on page 206.

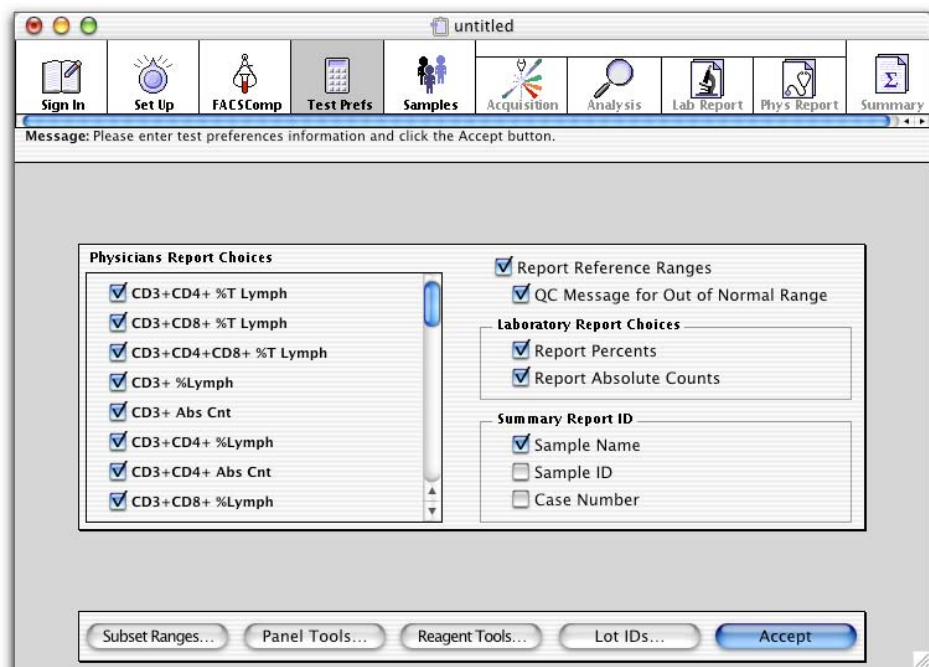
- 1 Verify that the Lyse/No-Wash Calib file listed is the one that you just created in BD FACSComp software and that the Status is *All parameters passed*.
- 2 If the Lyse/No-Wash Calib file is okay, click Skip FACSComp to advance to the Test Prefs view.

Otherwise, click Launch FACSComp, and run the Lyse/No-Wash 4-color assay. When done, quit BD FACSComp software to return to the BD Multiset software FACSComp view, and then click Continue to advance to the Test Prefs view.

Test Prefs View

At the Test Prefs view, you will specify the results to be displayed on the Laboratory, Physician, and Summary reports. You will also enter information for the BD Trucount tubes.

- 1 Verify that the following are selected in the Test Prefs view.



- All possible subsets for BD-defined reagents are listed in the **Physicians Report Choices**. A selected subset is included for an entry on the Physicians Report if reagents producing that subset are included in the panel for that entry.
- Reference ranges appear on the Physicians Report when the **Report Reference Ranges** checkbox is selected.

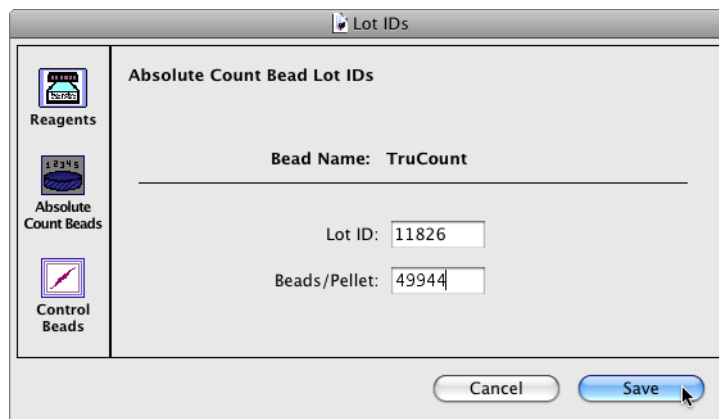
TIP Reference ranges are defined using the Subset Ranges dialog. Ranges to be reported for a panel are selected in Panel Tools.

- Percentages are reported on the Laboratory Report when the Laboratory Report Choices—**Report Percents** checkbox is selected.
- Absolute counts are reported on the Laboratory Report when the Laboratory Report Choices—**Report Absolute Counts** checkbox is selected.
- A sample name appears for each entry on the Summary Report when the Summary Report ID—**Sample Name** checkbox is selected.

2 Click Lot IDs.

The Lot IDs dialog is displayed.

3 Click the Absolute Count Beads icon.



4 Enter the Lot ID and Beads/Pellet information found on the BD Trucount tube foil pouch label.

This information is critical for calculating absolute counts. Only one lot ID can be entered per sample run.

5 Click Save to close the Lot IDs dialog.

6 Click Accept or press Return.

The application window advances to the Samples view.

Samples View

At the Samples view, you specify information for each of the samples to be run. The required fields are

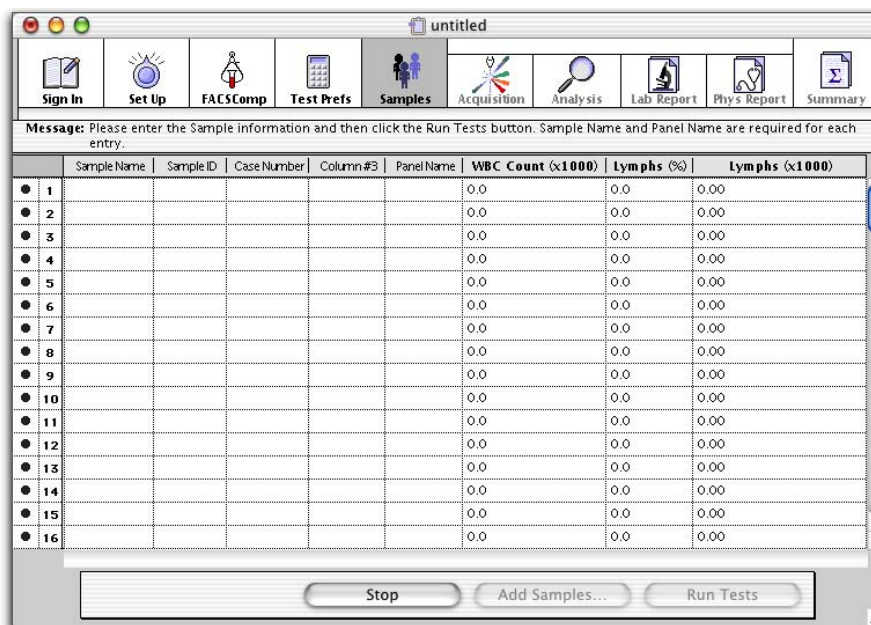
- The identifier selected at the Set Up view for the entry-level file name prefix.
- The identifier selected at the Test Preferences view for the Summary Report ID.
- Panel Name.

In this exercise, you selected Sample Name for both the entry-level file name prefix and the Summary Report ID, so Sample Name and Panel Name are the only required fields.

TIP The Message box at the top of the Sample view indicates which fields must be provided for each entry.

TIP When using BD Trucount tubes, do not enter information in the WBC Count (x1000), Lymphs (%), and Lymphs (x1000) fields. When not using BD Trucount tubes, you can enter either a WBC Count (x1000) and Lymphs (%) obtained from a hematology instrument, or a Lymphs (x1000) (absolute lymphocyte count) obtained from a hematology instrument. You cannot enter both.

- 1 Click the Sample Name field in row 1, type *Multi-check Control*, and press Return.



- 2 Click the Panel Name column in row 1 to obtain a panel menu and select 4C TBNK + TruC Panel.

- 3** Create another entry in row 2, with *Multi-check Low* for the Sample Name and 4C TBNK + TruC Panel for the Panel Name.
- 4** Create the final entry in row 3, with *Training Sample* for the Sample Name and 4C TBNK + TruC Panel for the Panel Name.

Saving a Schedule Document

A BD Multiset Schedule document file contains all data entered at the Sign In, Set Up, Test Prefs, and Samples views, as well as preference settings. The file does not contain information from Subset Ranges, Panel Tools, Reagent Tools, and Lot IDs.

A saved Schedule document can be used for viewing previous processing, to complete processing, or to add more samples for processing. Only one Schedule document can be open at a time. You cannot print a Schedule document.

- 1** Select File > Save.
- 2** Navigate to your student folder and name the document *Multiset Schedule*.
- 3** Click Save.

TIP When you create a new Schedule document, the last information entered at the Sign In, Set Up, Test Prefs, and Samples views becomes the default for the new Schedule, regardless of whether that information was saved.

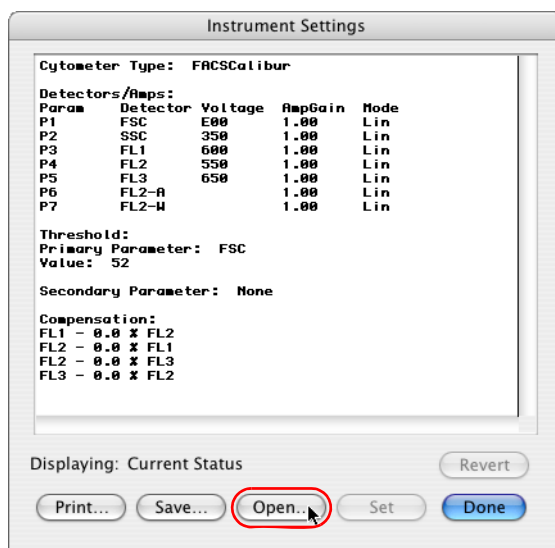
Loading Instrument Settings

Before leaving the Samples view, verify that the instrument settings from the current Lyse/No-Wash Calib file have been downloaded to the cytometer.

TIP If the BD FACSCComp software LNW assay was just run successfully, the settings from the Calib File.LNW file have already been sent to the cytometer.

- 1 Select Cytometer > Instrument Settings.

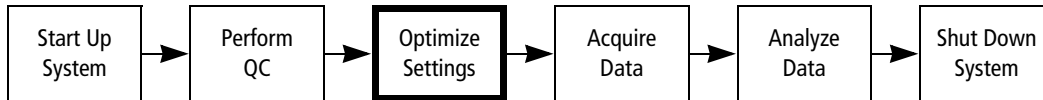
The Instrument Settings dialog is displayed.



- 2 Click Open.
- 3 Navigate to the BD Files/Instrument Settings Files folder and open Calib File.LNW.
The settings from Calib File.LNW are displayed in the Instrument Settings window.
- 4 Click Set in the Instrument Settings window to send these settings to the cytometer.
IMPORTANT You must click Set to download the instrument settings.
- 5 Click Done to close the Instrument Settings window.

Optimization

The instrument settings generated by BD FACSComp software when the LNW 4-color assay is run are appropriate for LNW samples. However, you might still need to optimize the SSC PMT voltage and the FL3 threshold setting.



In this section, you will use the Acquisition view to optimize instrument settings for your samples. The Acquisition view allows you to view a live display of sample data before and during acquisition. Data is not saved until you click Acquire.



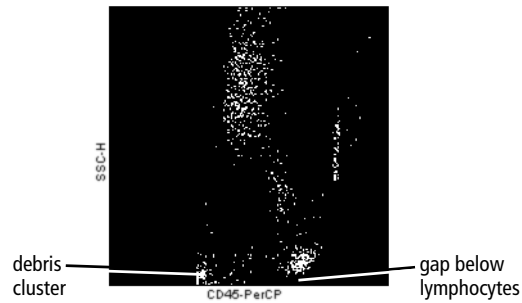
Do not modify fluorescence PMT voltages or compensation settings during optimization.

- 1 Vortex the first BD Multi-check control tube, and install it on the cytometer.
- 2 Place the cytometer in RUN mode and set the flow rate to HI.
- 3 Click Run Tests.

The application window advances to the Acquisition view.



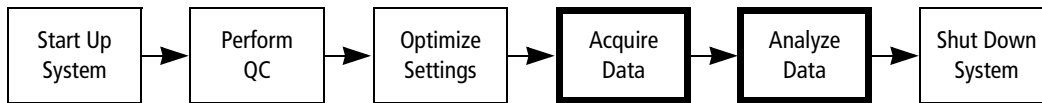
- 4 Select Cytometer > Det/Amps to display the Det/Amps window.
- 5 Select Cytometer > Threshold to display the Threshold window.
- 6 Adjust the SSC PMT voltage, if needed, so that there is a clear gap between the lymphocyte population and the bottom of the FL3 vs SSC plot, as shown in the following figure.



- 7 Adjust the FL3 threshold, if needed, so that the majority of the debris is removed. A small debris cluster must remain for the automatic gating to function properly.

Acquisition and Analysis

After optimization, acquisition and analysis of samples can begin.



- 1 Click Acquire.

Acquisition of data begins. For each tube, an initial acquisition is performed. The number of events acquired in this phase is set in Events Preferences.

The software then determines if the minimum number of events for the target population has been collected. If the minimum has not been reached, the software estimates an additional number of events to acquire to satisfy the target population requirement.

When the acquisition for a tube is complete, the data is analyzed and the application window advances automatically to the Lab Report View.

Lab Report View



As data from each tube is acquired, the analysis information and any QC messages for that tube are added to the Laboratory Report and displayed in the Lab Report view. The Laboratory Report can also be saved as a PDF file for printing and further review. Recall, you chose to automatically save the Laboratory Report in the Set Up view.

You can view the report for as long as you want, because the Until “Next” Button Pressed checkbox was selected in the Set Up view. In addition to analysis results, the Laboratory Report shows the gates and attractor regions that were used in the analysis.

The **expert lymph** gate identifies the lymphocyte population from a CD45 vs SSC plot or a CD3 vs SSC plot. Refer to the *BD MultiSet Software Reference Manual* for details about the expert lymph gate. **Attractors** then single out lymphocyte subsets from fluorescence parameter combinations. Attractor regions can automatically adapt themselves to position shifts of populations.

Manual Gating

Upon inspection, you may decide that the expert lymph gate or one or more attractors require adjustment. Refer to the *BD MultiSet Software Reference Manual* for information on how to decide when adjustment is needed. Manual adjustments can be made for the tube just analyzed. Changes are applied to the current tube only. See Manual Gating on page 220 for an exercise on manual gating.

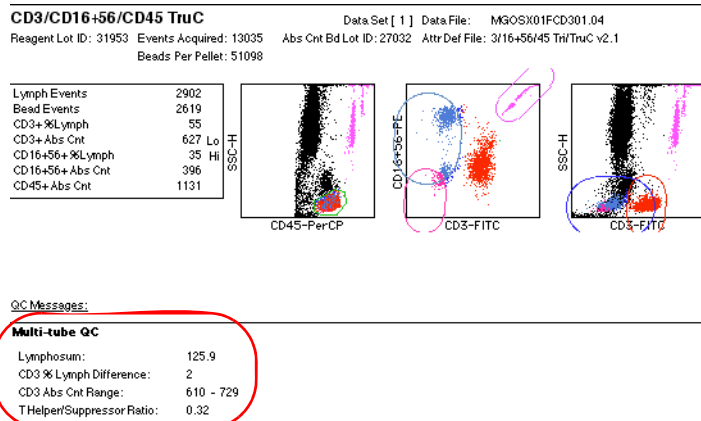
Continuing the Run

- 1 Vortex and install the second BD Multi-check control tube on the SIP. Verify that the cytometer is in RUN mode.
- 2 Click Continue.

The application window returns to the Acquisition view and data for the second control tube is acquired. When acquisition is complete, the data is analyzed and results are added to the Laboratory Report which displays at the Lab Report view.

- 3 Place a tube containing no more than 1 mL of DI water on the SIP, and place the cytometer in STNDBY mode.

Since this is the last tube in the panel, multitube QC values are displayed on the Lab Report.



TIP After all tubes in the panel have been run and before proceeding to the Physician Report view, you can rerun any of the tubes in the panel. Refer to the *BD MultiSet Software Reference Manual* for details about rerunning tubes.

- 4 Click Continue.

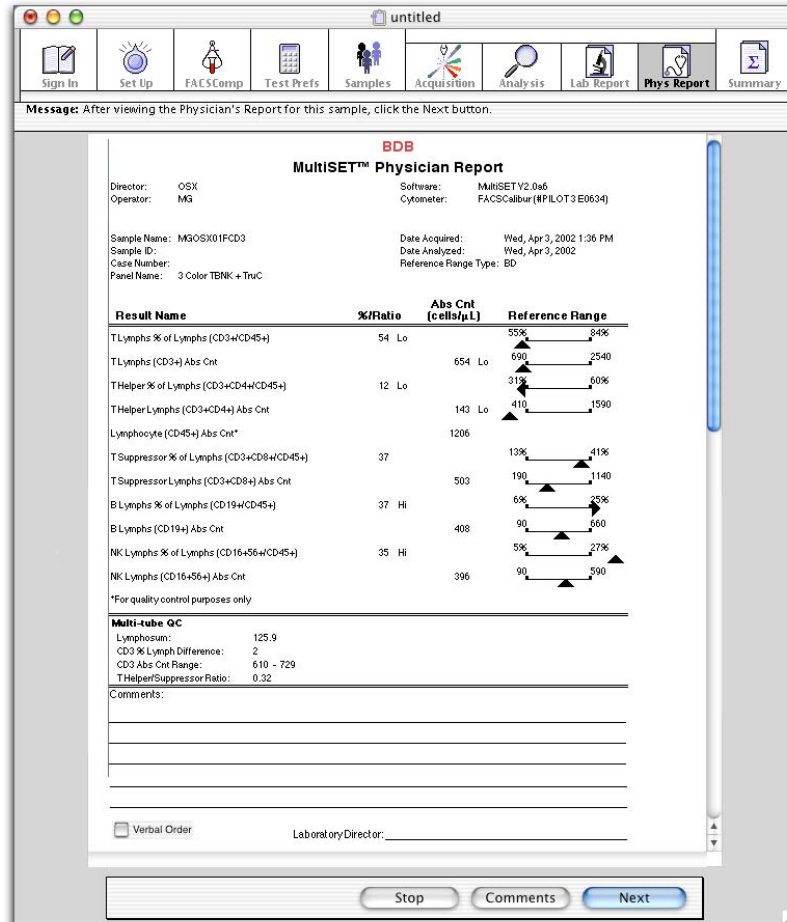
The Laboratory Report prints as specified in Printing preferences. The application window advances to the Physician Report view.

Physician Report View

The Physician Report is a summary of information for one sample, and it is displayed at the Physician Report view. The Physician Report can also be saved as a PDF file for printing and further review. In this case, you chose to automatically save the Physician Report (see Set Up View on page 200).

The Physician Report includes the results that were selected in the Test Prefs view. If more than one reagent in a panel provides a particular subset result, the average subset result is reported. The report also provides multitube results, including lymphosum, two CD3 consistency results (% CD3 and CD3 Abs Ct Range), and a T-helper/suppressor (T H/S) ratio determined from more than one tube.

The Physician Report has a Comments section where you can enter additional information. By selecting the Verbal Order checkbox, you can indicate if a test was verbally requested by a physician. For more information about using the Physician Report, refer to the *BD MultiSet Software Reference Manual*.



Running the BD Multi-Check Low Control

- 1 Install the first Multi-check Low tube on the SIP.
- 2 Verify that the flow rate is set to HI and that the cytometer is in RUN mode.
- 3 Click Next at the Physician Report view.

The Physician Report prints as specified in Printing preferences, and the application window returns to the Acquisition view.

- 4 Click Acquire.

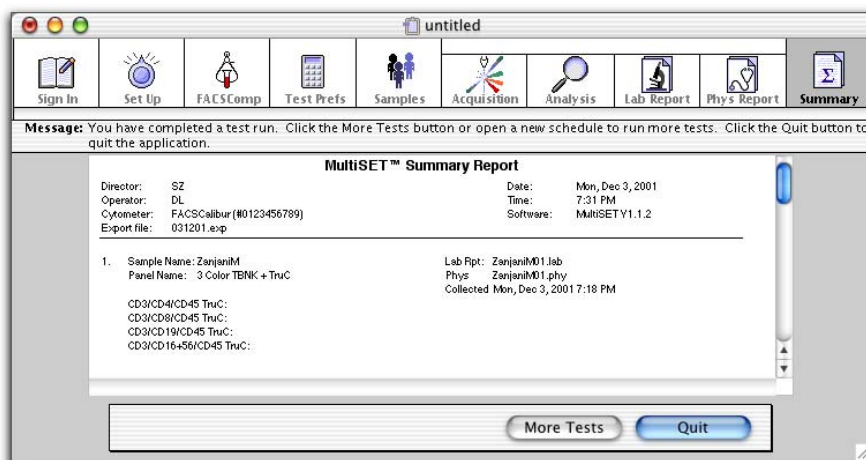
Data for the Multi-check Low tube is acquired. When acquisition is complete, the data is analyzed and results are added to the Laboratory Report, which is displayed at the Lab Report view.

- 5 Install the second Multi-check Low tube and click Continue at the Lab Report view.
- 6 After the second Multi-check Low tube has been acquired, click Continue at the Lab Report view. Click Next at the Physician Report view.

Finishing the Run

- 1 Install tubes as needed to complete the Training Sample entry.
- 2 After the second Training Sample tube has been acquired, install a tube containing no more than 1 mL of DI water on the SIP, and place the cytometer in STNDBY mode.

Since the run is complete, the application window advances from the Physician Report view to the Summary view. The Summary Report contains a list of all entries in the Schedule document. The view has no editable fields.



3 Click Quit.

If you have not saved the Schedule document or the most recent changes, a dialog appears asking if you want to save changes.

TIP Click More Tests to return to the Samples view.

Reviewing Control Results

In this section, you will review your results from the control samples.

1 Record your results.

For the CD3⁺ population, enter the average percentage and average absolute count from the two tubes.

Population	Multi-check Regular control		Multi-check Low control	
	Percentage	Absolute Count	Percentage	Absolute Count
CD3 ⁺				
CD3 ⁺ /CD4 ⁺				
CD3 ⁺ /CD8 ⁺				
CD3 ⁻ /CD16 ⁺ CD56 ⁺				
CD3 ⁻ /CD19 ⁺				

2 Compare your results with those listed on the Assay Values insert included with the BD Multi-check controls.

Using BD Multiset Tools

You can use BD Multiset software tools to enter lot IDs and to define reagents, panels, and subset ranges. The tools can be accessed from the Tools menu or from buttons on the Test Prefs view.

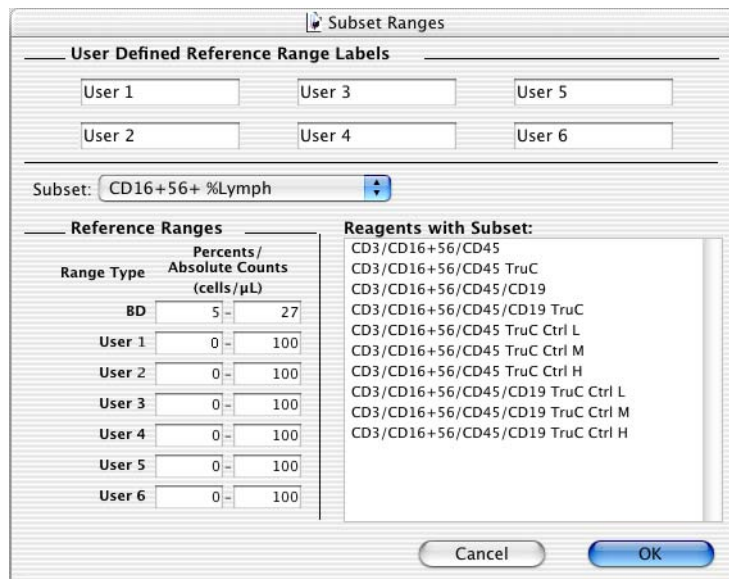
In this section, you'll practice using BD Multiset software tools to create user-defined reference ranges and panels. You'll also take a look at the Reagent Tools dialog.

BD Multiset Tools

Subset Ranges

Subset Ranges allows you to specify six user-defined reference ranges for each subset. In this exercise, you will create two user defined range labels, Adult and Pediatric, and assign ranges for the CD19+ %Lymphs subset.

- 1 Select Tools > Subset Ranges.
- 2 The Subset Ranges dialog is displayed.



- 3 Modify User Defined Reference Range Labels.
 - a Click in the upper left label field and replace User 1 with *Adult*.
 - TIP** Do NOT press Return. The Return key activates the (default) OK button, which accepts changes and closes the dialog.
 - b Click in the lower left label field and replace User 2 with *Pediatric*.

- 4 Select CD19+ %Lymphs from the Subset menu.
- 5 Type range values for the CD19+ %Lymphs Adult and Pediatric reference ranges.

NOTE These range values are for demonstration purpose only.

Reference Range	Low	High
Adult	8	24
Pediatric	5	28

User Defined Reference Range Labels

Adult User 3 User 5
 Pediatric User 4 User 6

Subset: CD19+ %Lymph

Range Type	Percents / Absolute Counts (cells/ μ L)	
BD	6	25
Adult	8	24
Pediatric	5	28
User 3	0	100
User 4	0	100
User 5	0	100
User 6	0	100

Reagents with Subset:

- CD3/CD19/CD45
- CD3/CD19/CD45 TruC
- CD3/CD16+56/CD45/CD19
- CD3/CD16+56/CD45/CD19 TruC
- CD3/CD19/CD45 TruC Ctrl L
- CD3/CD19/CD45 TruC Ctrl M
- CD3/CD19/CD45 TruC Ctrl H
- CD3/CD16+56/CD45/CD19 TruC Ctrl L
- CD3/CD16+56/CD45/CD19 TruC Ctrl M
- CD3/CD16+56/CD45/CD19 TruC Ctrl H

Cancel OK

- 6 Click OK.

Panel Tools

A panel is a reagent combination. With Panel Tools you can create new panels, modify existing panels, and copy, delete, rename, reorder, and print panels.

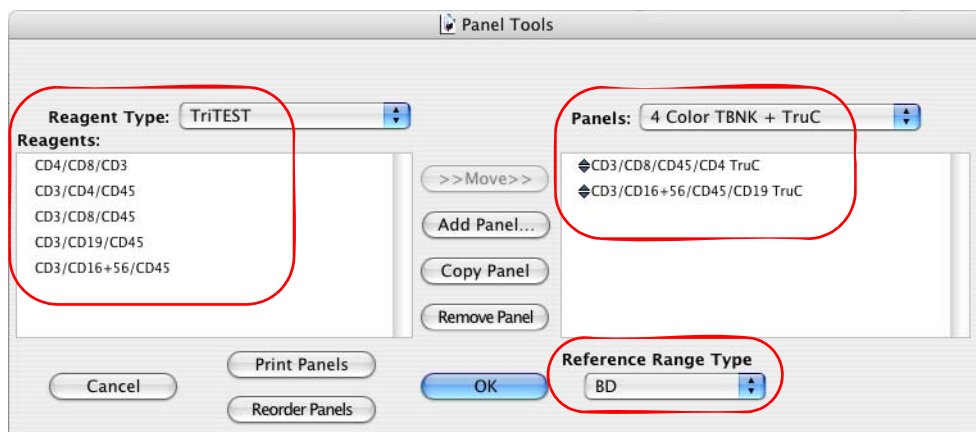
NOTE Predefined control panels are provided with BD Multiset software. You cannot create additional control panels, or modify or delete the predefined control panels. Non-control panels are provided for analyzing the BD Multiset practice data files.

In this exercise, you will create a new panel. Refer to the *BD MultiSet Software Reference Manual* for more information on using Panel Tools features.

- 1 Select Tools > Panel Tools.

The Panel Tools dialog appears.

On the left of the dialog, the Reagents list shows the reagents available in the currently selected reagent type. On the right, the reagent combination for the currently selected panel is listed. The selected reference range type for the panel is displayed below the reagent combination list.



- 2 Click Add Panel.

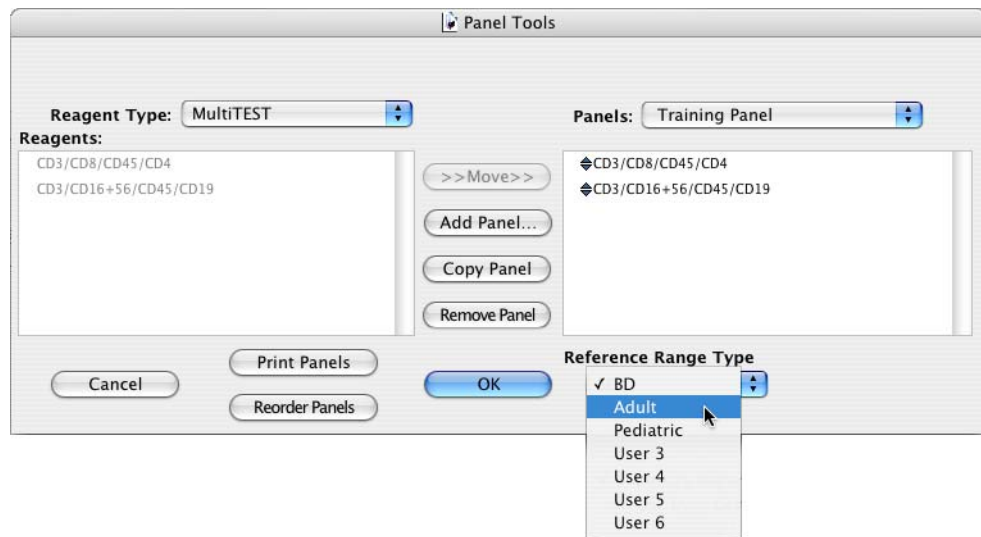
The new panel name dialog appears.

- 3 Type *Training Panel*, and click Add.



- 4 Click the Reagent Type menu control and select MultiTEST.

- 5 Add the CD3/CD8/CD45/CD4 reagent to Training Panel.
 - a Select the CD3/CD8/CD45/CD4 reagent.
 - b Click Move.
- 6 Add the CD3/CD16+56/CD45/CD19 reagent to Training Panel.
- 7 Click the Reference Range Type menu control and select Adult.



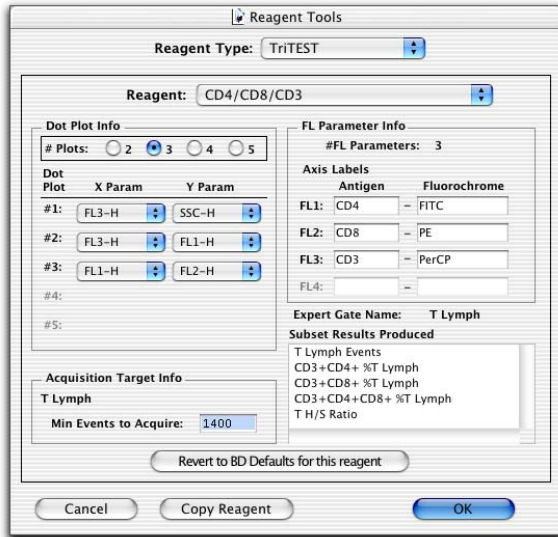
- 8 Click OK to accept changes and close Panel Tools.

Reagent Tools

With Reagent Tools, you can modify Dot Plot Info, Acquisition Target Info, and FL Parameter Info for BD-defined reagents. You can also create and customize user-defined reagents. For more information about using user-defined reagents, refer to the *BD MultiSet Software Reference Manual*.

- 1 Select Tools > Reagent Tools.

The Reagent Tools dialog appears.



- 2 Click the Reagent Type menu control and select MultiTEST.
- 3 Click the Reagent menu control and select CD3/CD8/CD45/CD4.

The Reagent Tools dialog displays the current settings for the CD3/CD8/CD45/CD4 reagent, and allows you to customize:

- The number of plots you want to view and print.
 - The X and Y parameters for each plot.
 - The axis label for each fluorescence parameter.
 - The minimum number of events to acquire for the target population.
- 4 Click Revert to BD Defaults to ensure that all settings are the original BD default values.
 - 5 Click OK.

Manual Gating

If needed, manual adjustments to the expert lymph gate and the attractors can be made for the tube just analyzed. Changes are applied to the current tube only.

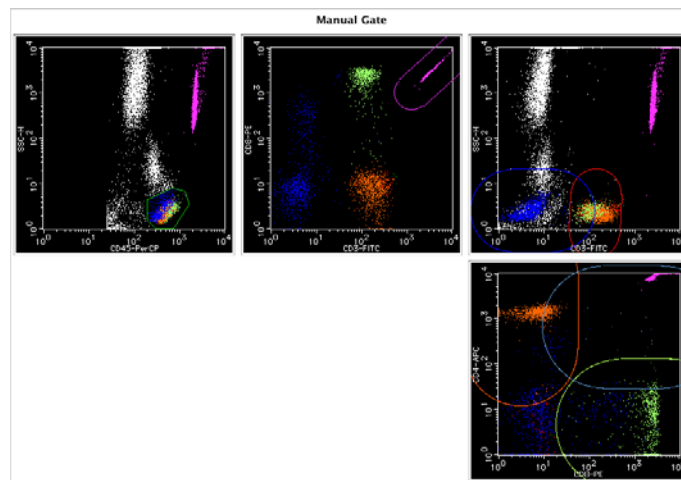
In this exercise, you will run a sample to practice using manual gating.

- 1 Select File > Open Schedule.
- 2 Navigate to your student folder and open *Multiset Schedule*.
- 3 Click the Samples icon on the BD Multiset icon bar, to display the Samples view.
- 4 Add a sample named *Manual Gating* and select 4C TBNK + TruC Panel.
- 5 Vortex one of the samples tubes, and install it on the cytometer.
- 6 Place the cytometer in RUN mode and set the flow rate to HI.
- 7 Click Run Tests.

The application window advances to the Acquisition view.

- 8 Click Acquire.
- 9 Remove the sample tube and place a tube containing no more than 1 mL of DI water on the SIP.
- 10 Place the cytometer in STNDBY mode.
- 11 Click Manual Gate.

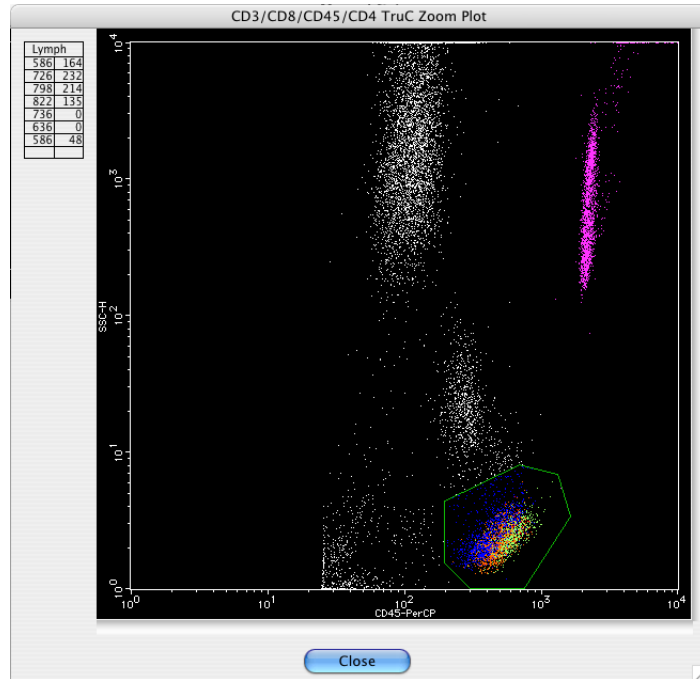
Plots are displayed that show the expert lymph gate and the attractors regions.



12 Replace the expert lymph gate with a manual gate.

- a** Double-click the CD45 vs SSC plot.

A zoomed CD45 vs SSC plot is displayed in a new window.



- b** Click inside the existing expert lymph gate to select it.

The boundary of the expert lymph gate changes from green to white.

- c** Click again to position the cursor at a starting point for the boundary of the manual gate.

- d** Continue clicking to draw the boundary of a polygon region surrounding the lymphocytes.

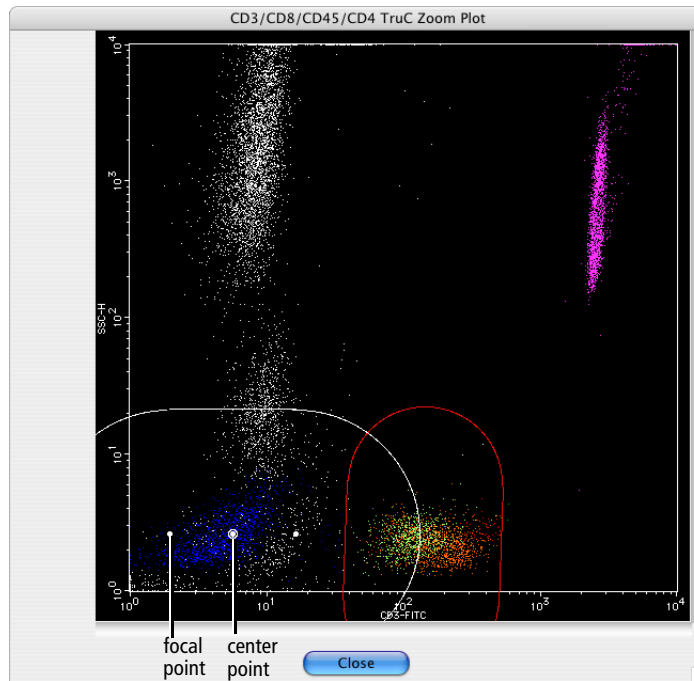
- e** Click near the starting point to complete the polygon.

The original gate disappears, and the boundary of the new gate changes from white to green.

- f** Click Close to close the zoomed plot window.

13 Adjust an attractor region.

- a Double-click the CD3 vs SSC plot to obtain a zoomed plot window.



- b Click inside an attractor to select it.

The boundary of the attractor region changes from colored to white.

- c Practice adjustments:

- To move an attractor, drag its center point.
- To change the orientation or length of an attractor, drag one of the two focal points.
- To change the size of an attractor, drag an edge.

- d Close the zoomed plot window.

NOTE Attractor changes can be saved as a user-defined reagent. Refer to the *BD MultiSet Software Reference Manual* for details about user-defined reagents.









14 Click Analyze.

The manual gates are confirmed and the data is reanalyzed. A message appears in the QC Message area of the Lab Report indicating that a manual gate has been set.

BD Multiset Software Preferences Overview

Open the MultiSET Preferences dialog by selecting MultiSET > Preferences. You can use these preferences to customize BD Multiset software features. For more information about setting individual BD Multiset software preferences, refer to the *BD MultiSet Software Reference Manual*.

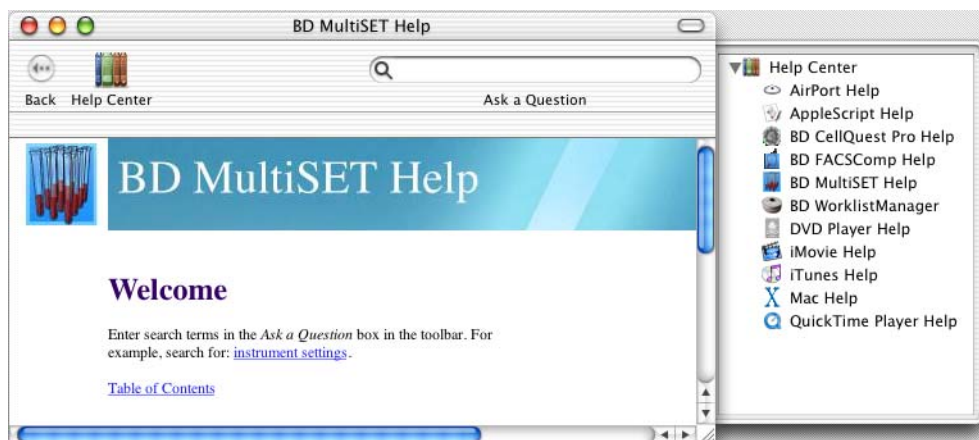
Preferences are grouped into tabbed views as shown in the following table.

Preference Group	Description
 Export	Use the Export preference to choose a delimiter for export document files and Levey-Jennings files before you transfer them to a third-party database or spreadsheet program. Delimiters are used to separate the values in a file. You can also select the values you want to export.
 Printing	Use the Printing preference to choose when to automatically print the Lab, Physician, and Summary Reports.
 Panels	With the Panels preference you can select a default panel that will appear at the Samples view for all samples in the run. Panels preferences are valid only when the data source is From Cytometer.
 Events	The Events preference defines the criteria for stopping acquisition.
 LJ Data	BD Multiset software lets you save control bead results to a file that can be exported to a third-party Levey-Jennings application. The file can contain results for either 30 or 60 controls.
 Columns	Use the Columns preference to specify up to three additional columns to the right of the Case Number column in the Samples view. The column headers and entered values are also displayed in the Lab Report and Physician Report views, are stored in data files, and can be included in an export document.
 Banner	With the Banner preference, you can display your institution's name or apply a custom banner to the top of the Lab, Physician, Summary, and Panel Reagent Report views.
 Comment	You can define and save up to 20 comments and include one of them on Physician Reports, or you can choose to delete the comments section entirely.

Using BD Multiset Software Help

When you need assistance with BD Multiset software, use the Help viewer to quickly locate the information.

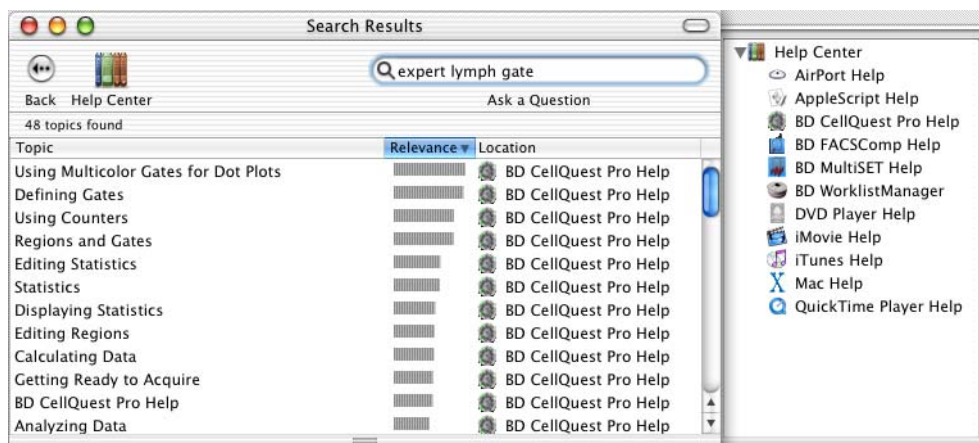
- 1 Select Help > MultiSET Help.



The fastest way to search for help is to use the Ask a Question feature. You can also search by looking through the table of contents or index.

- 2 Enter *expert lymph gate* in the Ask a Question field, and press Return.

A list of topics appears in the lower portion of the Help view. Double-click a topic to display that section of Help.



- 3 When you are finished, close the Help viewer by clicking the red button in the upper-left corner of the viewer.

BD Multiset File Naming Conventions

File Naming

When you run BD Multiset software as a standalone application, the following file-naming conventions apply.

File Type	Naming Convention	Example
Schedule document	[DDMMYY].sch	220998.sch
Lab Report	[prefix][entry number].lab OR [entry number][prefix].lab	Smith01.lab 01Smith.lab
Physician Report	[prefix][entry number].phy OR [entry number][prefix].phy	Smith01.phy 01Smith.phy
Summary Report	[DDMMYY].sum	220998.sum
FCS data	[prefix][entry number].[tube number] OR [entry number][prefix].[tube number]	Smith01.01 01Smith.01
FCS data (rerun)	[prefix][entry number].[tube number][rerun letter] OR [entry number][prefix].[tube number][rerun letter]	Smith01.01a 01Smith.01a
export document	[DDMMYY].exp	220998.exp
export document control	[DDMMYY].cxp	220998.cxp
Levey-Jennings	LJ MSET Data	LJ MSET Data

You can rerun samples and save up to five FCS data files from one tube. The rerun letter will start with an *a* and increment through the letter *e* (a–e). Only the last file saved is used for calculating statistics and reporting in the Lab and Physician Reports.

For FCS data files and Lab and Physician Reports, you can select to place the entry number before the prefix by selecting the Add Entry Number as Prefix checkbox at the Set Up view.

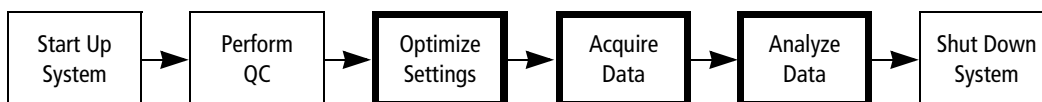
If you do not want to use the default file names (DDMMYY) for the Schedule document, export document, and Summary Report files, you can enter a new name at the Set Up view. The prefix for FCS, Lab Report, and Physician Report files is selected at the Set Up view.

BD Multiset Review

- 1 Automatic Printing Options for reports are specified at the Set Up view. T F
- 2 What lot information is entered for BD Trucount tubes?
- a Expiration date
 - b Beads/Pellet
 - c Tubes/Lot
 - d None of the above
- 3 Which of the following CANNOT be used as the entry level file name prefix?
- a Sample ID
 - b Sample Name
 - c Panel Name
 - d Case Number
- 4 What instrument adjustments are performed when optimizing samples stained with BD Tritest or BD Multitest reagents?
- _____
- _____
- 5 Why is it necessary to leave a small amount of debris when optimizing the FL3 threshold?
- _____
- _____

Using BD Multiset Software with the Loader

Acquisition of data using the Loader is a multistep process. The following figure outlines the main steps involved. This section will focus on optimization of instrument settings, data acquisition, and data analysis.



In this exercise, you will do the following.

- Prepare a BD Multiset schedule template
- Optimize instrument settings
- Specify conditions for data acquisition and storage
- Acquire data

Before You Begin

Before beginning the exercises in this section:

- Perform the procedure in System Startup on page 33.
- Run the Lyse/No-Wash (LNW) assay in BD FACSComp software.

The instructions in Performing QC and Instrument Setup on page 71 are for running the BD FACSComp software Lyse/Wash (LW) 4-color assay. The same procedure can be used to run the Lyse/No-Wash (LNW) 4-color assay by making appropriate entries at the Set Up view:

- Verify that only the Lyse/No-Wash (LNW) option is selected under Assay Selections.
- Under CaliBRITE Bead Lot IDs, verify the bead lot IDs for the FITC, PE, PerCP, APC, and Unlabeled beads.
- Under Automatic Savings Options, select the Lyse/No-Wash Summary Report checkbox.

Preparing a BD Multiset Schedule Template

When using BD Multiset software with the Loader, a BD Multiset Schedule (document) template is used to specify the following application-specific settings needed by BD WorklistManager software:

- Set Up view: Data Source and View Reports
- Test Prefs view: all settings
- Multiset Preferences: all settings

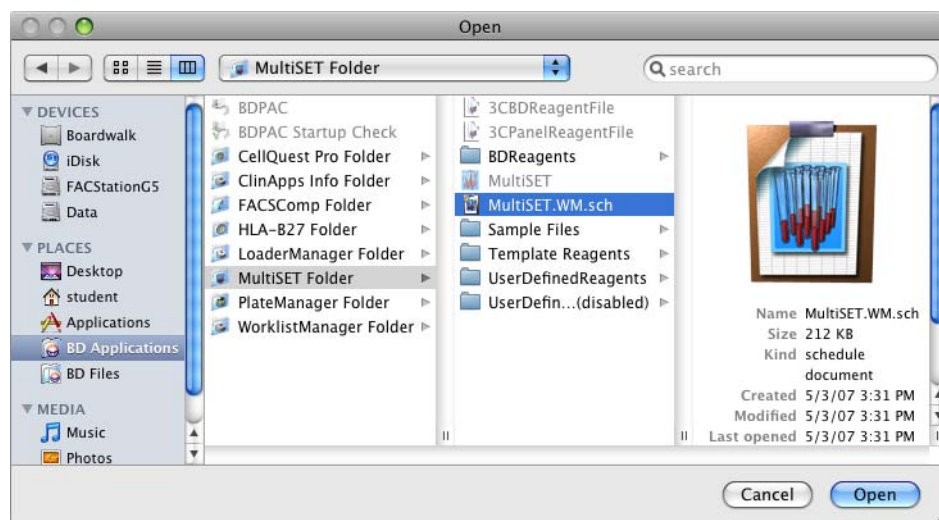
BD WorklistManager software accesses additional current settings stored by BD Multiset software:

- Lot IDs
- Subset ranges
- Panel definitions
- Reagent definitions

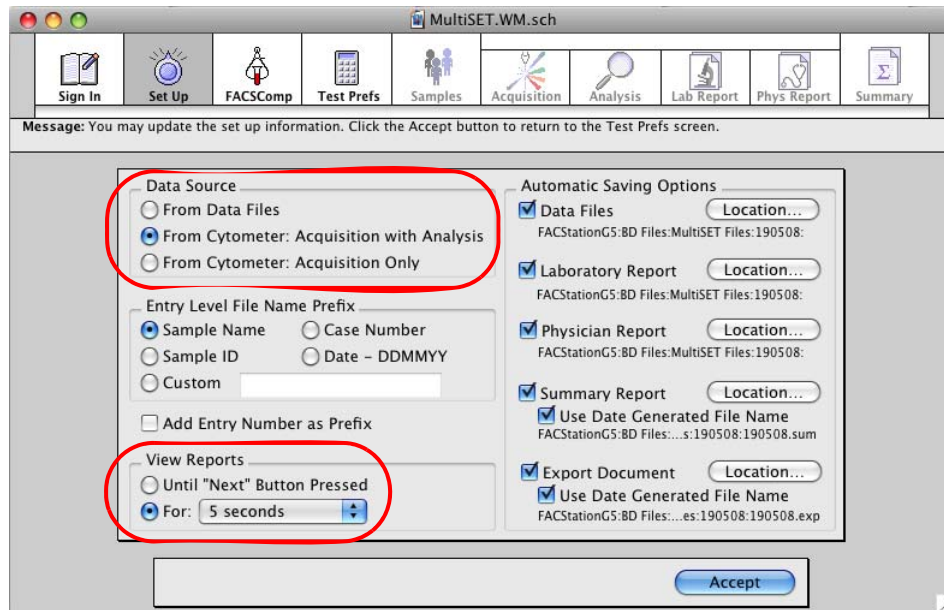
A default BD Multiset Schedule template, MultiSET.WM.sch, is installed with the software and is located at BD Applications/MultiSET Folder. In this section, you will open MultiSET.WM.sch, verify the settings used by BD WorklistManager software, and save a copy to your student folder. You will also verify that BD Trucount Lot ID information is correct.

You may need to enter (Operator) or correct (File Locations) additional information in the Schedule document to access the required views.

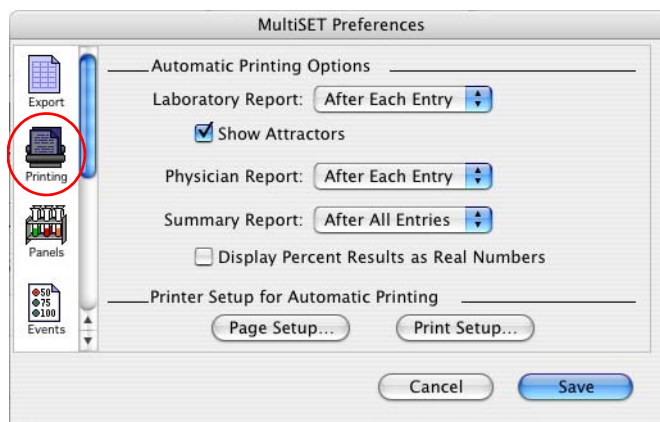
- 1 Use the Finder to open the MultiSET.WM.sch file.



- 2 Advance to the Set Up view, and verify that the Data Source and View Reports settings are as shown in the following figure.

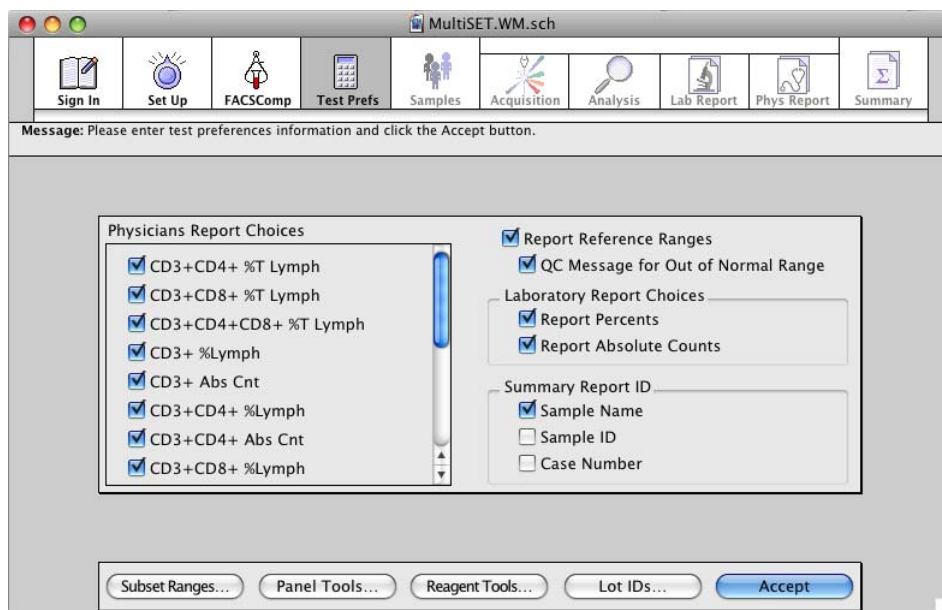


- 3 Specify automatic printing options for reports in the BD Multiset Preferences dialog.
 - a Select MultiSET > Preferences.
 - b Click the Printing icon to display the automatic printing preferences.
 - c Verify that the Automatic Printing Options are set as in the following figure.



- d Click Save to accept changes and close the dialog.
- 4 In the BD Multiset application window, advance to the FACSCComp view and click Skip FACSCComp.

- 5 At the Test Prefs view, verify that all settings are as shown in the following figure. Scroll down to verify that all Physician Report Choice checkboxes are selected.

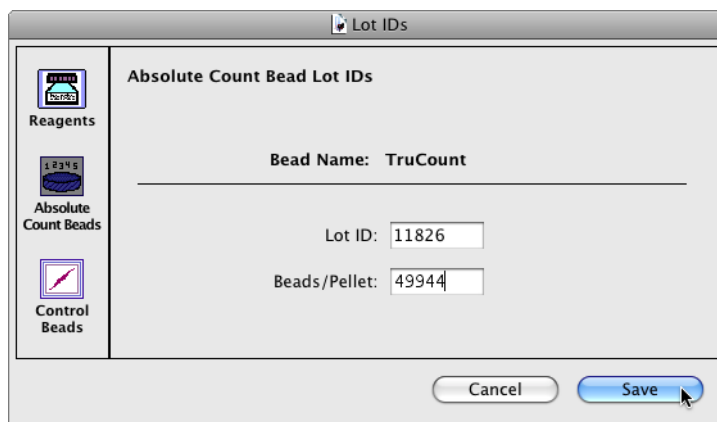


- 6 Verify the BD Trucount Lot ID information.

- a Click Lot IDs.

The Lot IDs dialog is displayed.

- b Click the Absolute Count Beads icon.



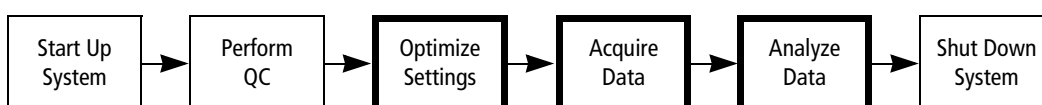
- c Enter the Lot ID and Beads/Pellet information found on the BD Trucount tube foil pouch label.
- d Click Save to close the Lot IDs dialog.

- 7 Select File > Save As and save a copy of MultiSET.WM.sch to your student folder.
- 8 Quit BD Multiset software.

TIP Only one BD Trucount lot can be used per BD WorklistManager software worklist. Lot IDs are NOT stored in the BD Multiset Schedule template. If you need to return to BD Multiset software to change the BD Trucount Lot ID information, it is not necessary to use and resave MultiSET.WM.sch.

Optimizing Settings and Acquiring and Analyzing Data

In this section, you will prepare a BD WorklistManager software schedule document and acquire data with the Loader.



Entering Information in BD WorklistManager Software

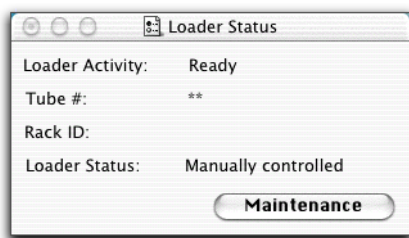
IMPORTANT BD WorklistManager software software will not start if other BD applications are already running. In addition, while BD WorklistManager software software is running, other BD applications should not be started independently.

- 1 Verify that all BD applications are closed by looking at the icons of BD applications on the Dock. Open application icons have a Mac OS dependent marking.
- 2 Start BD WorklistManager software software by clicking its icon on the Dock.



The BD WorklistManager application window opens at the Sign In view (see Figure 7-1 on page 232). BD WorklistManager automatically starts BD LoaderManager software, which checks to see if the Loader is connected.

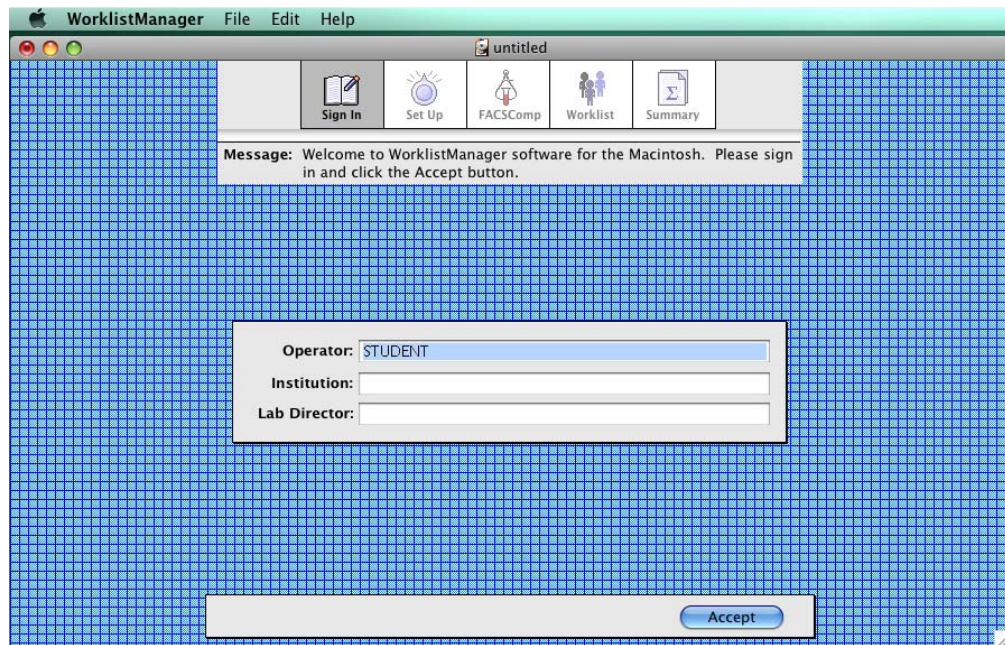
The Loader Status window appears, displaying connectivity status and also whether the Loader is controlled manually or by the computer.



TIP BD LoaderManager software can also be started independently of BD WorklistManager software. Refer to the *BD FACS Loader Reference Manual* for details.

Sign In View

Figure 7-1 BD WorklistManager software Sign In view



- 1 Type your name in the Operator field.
Only the operator field is required.
- 2 (Optional) Fill in the Institution and Lab Director fields.
- 3 Click Accept or press Return.
The application window advances to the Set Up view.

Set Up View

- 1 Make the following selections in the Set Up view. Change the locations for the Worklist Summary Report, Data Files, the BD Multiset Laboratory, Physician, and Summary Reports, and Export Files to your student folder.

The screenshot shows the 'Set Up View' dialog box with the following configurations:

- File Name Prefix:** Sample ID, Case Number, Date – DDMMYY, Custom Prefix
- Summary Report Sample Label:** Sample ID, Sample Name, Case Number
- Worklist:** Summary Report: OS X:BD Files:Workli...0503:080503.sum
- Template:** Schedule Template: OS X:BD Applicatio...er:MultiSET.WM.sch
- Automatic Saving Options:** Assay Type: MultiSET
 Data Files: OS X:BD Files:MultiSET Files:080503:
 Laboratory Report: OS X:BD Files:MultiSET Files:080503:
 Physician Report: OS X:BD Files:MultiSET Files:080503:
 Summary Report: OS X:BD Files:Multi...0503:080503.sum
 Use Date Generated File Name
 Export Files: OS X:BD Files:Multi...80503:080503.exp
 Use Date Generated File Name

- When the File Name Prefix—**Sample ID** radio button is selected, file name prefixes for FCS data files, Laboratory Report files, and Physician Report files are based on Sample ID entries in the Worklist view. You must enter a Sample ID for each worklist entry.
- A sample ID appears for each entry on the Summary Report when the Summary Report Sample Label—**Sample ID** checkbox is selected.
- When the Worklist—**Summary Report** checkbox is selected, a Worklist Summary Report file is automatically saved. The current file path is displayed. The default location is a dated folder (DDMMYY) in the BD Files/WorklistManager Files folder.
- When Automatic Saving Options—**Assay Type** is set to MultiSET, the Automatic Saving Options for BD Multiset software are displayed.

When an **Automatic Saving Options** checkbox is selected, the corresponding file is automatically saved. The current file path appears below each file type. The default location for each file type is a dated folder (DDMMYY) in the BD Files/MultiSET Files folder.

- 2 Click Assay Settings to display the Assay Settings dialog.

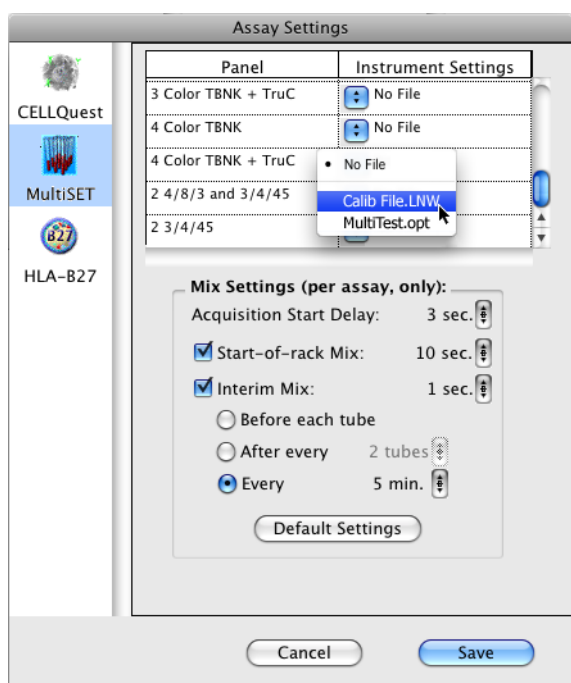
Assay Settings

In the Assay Settings dialog, you link instrument settings to panels and specify mix and printing settings.

- 1 Click the MultiSET icon.

The BD Multiset software panels are listed under the Panel column. The instrument settings saved in the BD Files/Instrument Settings Files folder are listed under the Instrument Settings column.

- 2 Link the Calib File.LNW instrument settings file with the 4C TNBK + TruC panel.
- 3 Specify Mix Settings for the 4C TNBK + TruC panel as shown in the following figure.



- 4 Click Save.

A dialog appears.



- 5 Click OK.

Saving a Worklist

Before entering sample information, save the BD WorklistManager worklist as a template that you can use to acquire data on a daily basis.

- 1 Select File > Save.
- 2 Save the worklist as *WM_MS_WorklistTemplate* in your student folder.

TIP Make the worklist template a locked stationery pad.

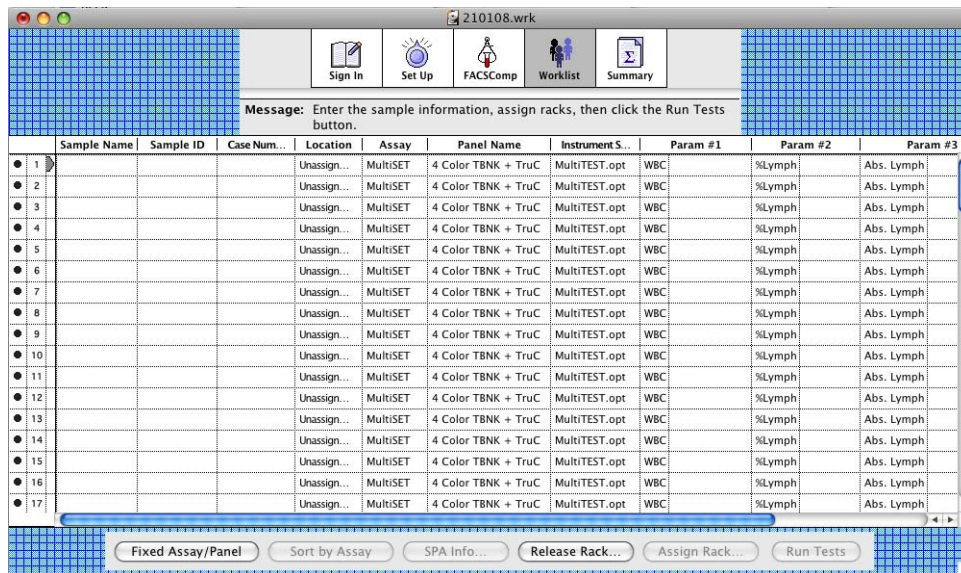
Entering Sample Information in the Worklist View

- 1 Click the Accept button in the Set Up view.

The application window advances to the FACSComp view.

- 2 Click Skip FACSComp.

The application window advances to the Worklist view. The status icons located to the left of the entry numbers indicate that no sample information has been entered.



- 3 Specify Sample ID, Assay, and Panel Name for entry 1 as shown in the following figure.

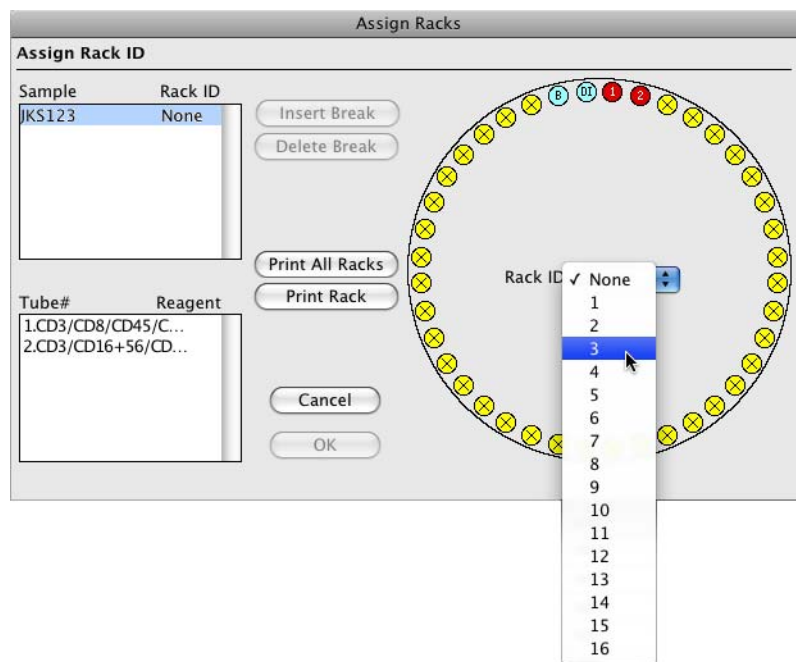
	Sample Name	Sample ID	Case Num...	Location	Assay	Panel Name	Instrument S...
1		JKS123		Unassign...	MultiSET	4 Color TBNK + TruC	MultiTEST.opt

The Instrument Settings reflects the settings linked to the selected panel. The status icon for entry 1 changes to a rack icon.

- 4 Click Assign Rack to display the Assign Racks dialog.

Assigning a Rack ID

- 1 Verify that your sample is highlighted in the Sample list, and select the Rack ID that you are using for the sample.



When a sample is highlighted in the Sample list, the dialog displays the reagent combination assigned to each tube in the panel, and indicates the position of the sample tubes in the rack sequence.

TIP All panel tubes in a given entry must reside on the same rack.

- 2 Click the Print Rack button to print the Rack Manifest, then click OK.

The Rack Manifest contains the sample information, reagent names, and tube locations as well as an overview of the rack with the entry numbers for each sample.

The location for entry 1 in the Worklist view is now displayed and the status icon for entry 1 has changed to a document icon indicating that the entry is ready to be run.



Select Save As in the following instruction to avoid overwriting your BD WorklistManager software worklist template.

- 3 Select File > Save As.
- 4 Save the worklist as *Sample Worklist* in your student folder.

Loading the Rack

- 1 Gently vortex all sample tubes.
- 2 Load the rack.
 - Load the sample tubes according to the printed Rack Manifest.
 - Load a tube containing 3 mL of a 10% bleach solution in rack position 39.
 - Load a tube containing 3 mL of DI water in rack position 40.
- 3 Place the rack on the Loader.
- 4 Close the Loader drawer completely, and place the cover on the Loader.

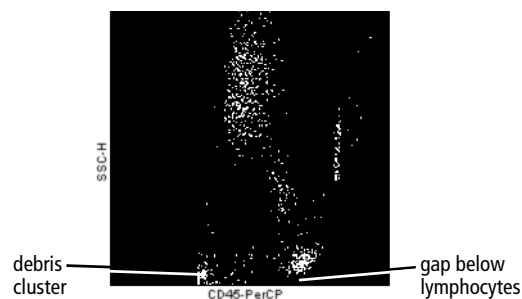
The rack initializes.

Optimizing Settings

IMPORTANT To optimize settings, you must pause the first tube during the 5-second countdown before acquisition begins. Be prepared to click Pause on the Acquisition view button panel soon after clicking Run Tests in the Worklist view.

- 1 Place the cytometer in RUN mode and set the flow rate to HI.
 - 2 Click Run Tests in the Worklist view.
- The application window advances to the Acquisition view.
- 3 Click Pause before the 5-second countdown completes.
 - 4 Select Cytometer > Det/Amps to display the Det/Amps window.
 - 5 Select Cytometer > Threshold to display the Threshold window.

- 6 Adjust the SSC PMT voltage, if needed, so that there is a clear gap between the lymphocyte population and the bottom of the FL3 vs SSC plot as shown in the figure.
- 7 Adjust the FL3 threshold, if needed, so that the majority of the debris is removed. A small debris cluster must remain for the automatic gating to function properly.



Running the Remaining Samples

- 1 Click Acquire to start acquisition for the first tube.
All tubes in the worklist entries are run, followed by a long clean.
- 2 When the cleaning cycle is complete, select File > Print to print the Summary Report.
- 3 Remove the Loader cover, pull the Loader drawer out, and remove the rack.
- 4 Install a tube containing no more than 1 mL of DI water on the SIP and place the support arm underneath the tube.
- 5 Place the cytometer in STNDBY mode.

Reviewing the Summary Report

A Summary Report is displayed in the Summary view.

The Summary Report lists all sample and reagent information, the rack location, the data files saved, and the status of the run. If an error occurred during the run, an error message appears on the Summary Report. Refer to the *BD FACS Loader User's Guide* for assistance in troubleshooting errors.

The report is automatically saved as specified at the Set Up view. You can print the Summary Report by selecting File > Print.

Sorting

After completing this chapter, you will be able to:

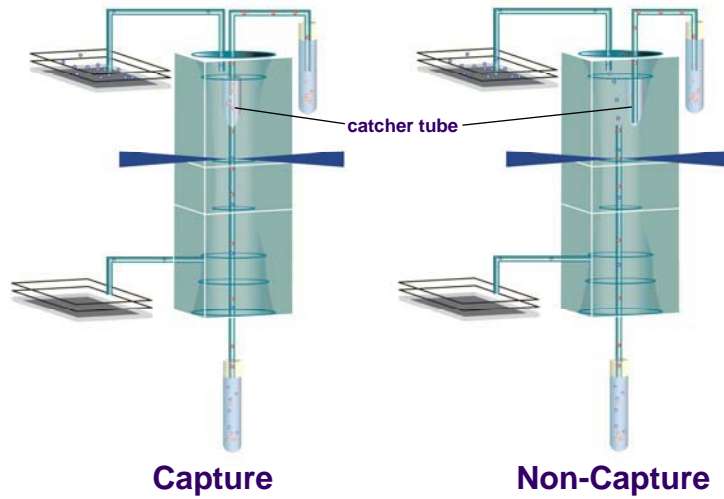
- Describe the BD FACSCalibur sorting mechanism.
- Choose an appropriate sort mode.
- Perform a sort.
- Measure sort purity.
- Maintain the sort lines.



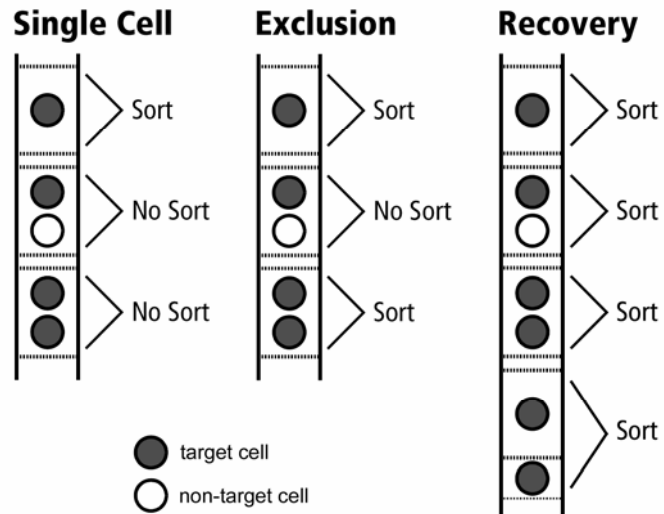
Sorting with the BD FACSCalibur



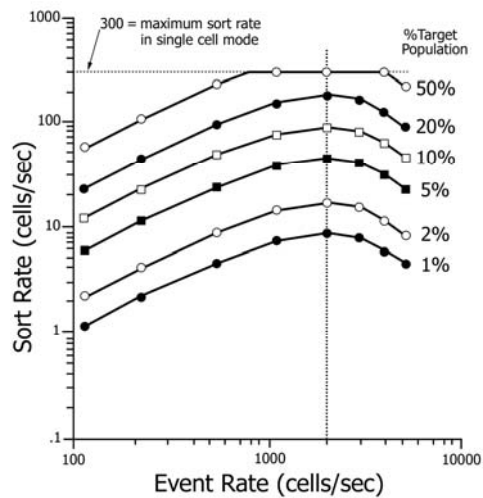
BD FACSCalibur Sorting Mechanism



Sorting Modes



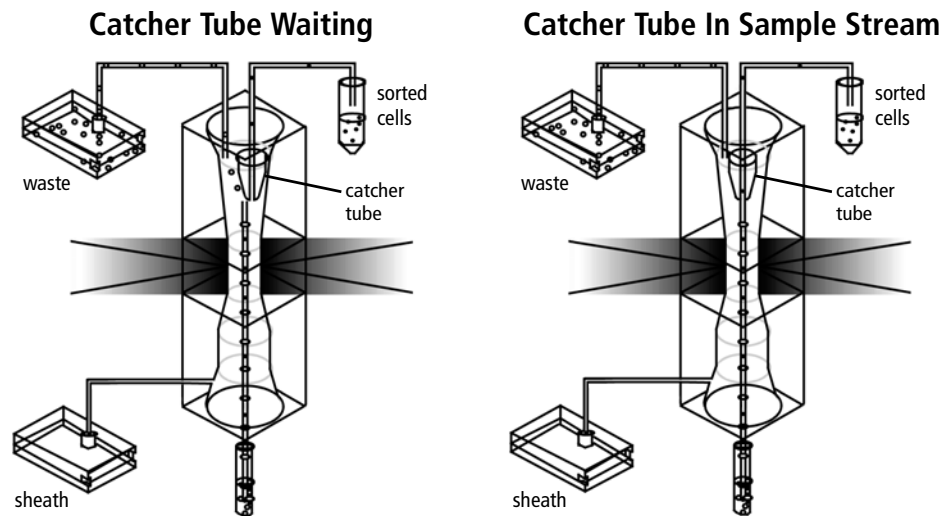
Sort Rate in Single Cell Mode



Sorting Overview

BD FACSCalibur cell sorting applications depend on real-time **sort gate** analysis to identify and track **target cells** as they pass through the flow cell. The application determines sections of the flow stream, called **sort envelopes**, that contain at least one target cell and satisfy the **sort mode** contents criteria.

The sorting mechanism moves a **catcher tube** into position in the sample stream with the precise timing needed to capture only the contents of selected sort envelopes. Captured target cells are transported to **collection station** tubes.



While waiting to capture a sort envelope, the catcher tube sits in the sheath flow and continues to collect sheath. This dilutes the sort sample. To concentrate the sorted cells, collection tubes are centrifuged after sorting.

Performance Measures

You might want to optimize one or more the following sorting performance measures:

- **Sort Rate**—target cells collected per second.
- **Sort Purity**—target cells collected per collected cells.
- **Recovery Rate**—target cells collected per sample target cells.
- **Target Count Accuracy**—target cells collected compared to the number requested.

You cannot effectively optimize all performance measures at once. For example, improving both recovery rate and sort rate is likely to lower sort purity. You can choose what to optimize by specifying a sort mode and by controlling the event rate.

Sort Modes

A sort envelope must contain at least one target population cell to be considered for collection. Additional criteria are specified by the sort mode.

Sort Mode	Contents Criteria	When to Use
<p>Single Cell</p>	<p>The sort envelope contains a single target cell and no additional cells.</p>	<ul style="list-style-type: none"> To obtain high sort purity To optimize the accuracy of the sorted target cell count
<p>Exclusion</p>	<p>The sort envelope contains a target cell and no non-target cells.</p>	<ul style="list-style-type: none"> To obtain high sort purity To obtain a better recovery rate than single cell mode
<p>Recovery</p>	<p>The sort envelope contains a target cell and may also contain additional cells. Also, if a target cell is located immediately following the selected sort envelope, the catcher tube stays in the flow stream long enough to capture it as well.</p>	<ul style="list-style-type: none"> To obtain a high sort recovery rate

Event Rate

The acquisition event rate has a significant effect on sorting performance measures. When sorting, the BD FACSCalibur flow rate should always be set to LO. However, you can still achieve a desired event rate by controlling the sample concentration.

Reducing Event Coincidence

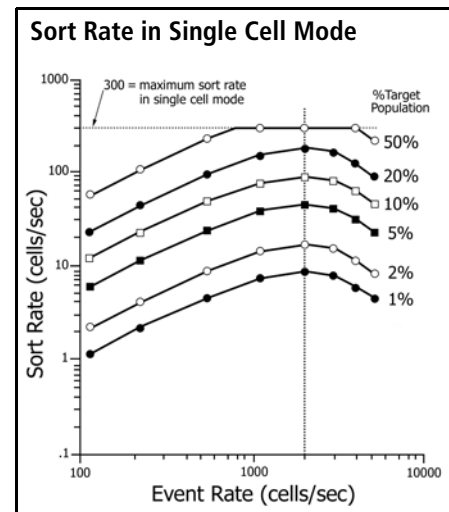
At higher event rates, it is more likely that a sort envelope with a target cell will also contain another cell. This is called **coincidence**. By diluting the sample, you can lower the event rate and reduce coincidence. When coincidence is reduced, there is almost always improvement in the sort rate and recovery rate for single cell mode, and the sort purity for recovery mode.

When the %target population is low, reducing high coincidence levels will improve the sort rate and recovery rate for exclusion mode. Coincidence levels have negligible effect on recovery rate in recovery mode, and on sort purity in single cell and exclusion sort modes.

Effect on Sort Rate

At lower event rates, there is little coincidence, and so increasing the event rate will improve the sort rate in all sort modes. However, in single cell and exclusion modes, increased coincidence at higher event rates will eventually cancel out this improvement, and the sort rate will drop again.

Sort rate performance is easiest to characterize in single cell mode. The figure shows the relationship between sort rate, event rate, and the percentage of target population in the sample. Sort rate is optimized at an event rate of about 2000 cells per second. At LO flow rate, 2000 cells per second is achieved at a cell concentration of 10^7 cells per mL.



Catcher Tube Movement

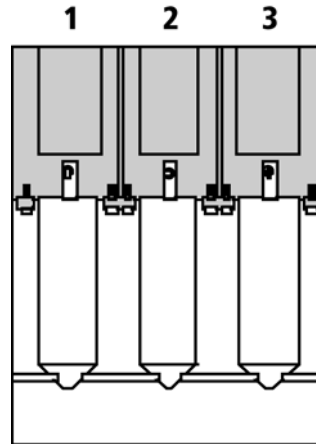
To prevent overheating, the catcher tube is not allowed to capture more than 150 sort envelopes in any half second. Therefore, the sort rate for single cell mode has a maximum value of 300 cells per second. In exclusion and recovery modes, sort rate can exceed 300 cells per second because multiple target cells can be collected from each sort envelope.

In recovery mode, as the event rate increases, sort rate continues to improve, but sort purity declines. When the %target population is high, the recovery rate will eventually decline because the catcher tube movement will reach its limit, and target cells will pass through in envelopes that cannot be collected.

Collection Station

At the collection station, sorted cells are deposited into 50-mL **collection tubes**. Up to three collection tubes can be installed in the three **collection ports**. The sorting system detects how many collection tubes are installed and fills tubes starting at the left (tube 1). With the flow rate set to LO, it takes about 9 minutes to fill a collection tube with 40 to 45 mL of fluid.

If all installed collection tubes fill before any of the end sorting criteria has been met, sorting continues but the sorted sample is sent to waste. To continue sorting after the collection tubes are filled, pause the acquisition, replace one or more collection tubes with new tubes, then restart or resume.



Preparing Collection Tubes



Recovery and viability of sorted cells will be reduced if the collection tubes are not coated.

Coat collection tubes with a bovine serum albumin (BSA) solution to help maintain cell integrity and increase cell yield during centrifugation.

The BSA should be filtered through a 0.45- μ m filter before use. Coat the collection tubes with 4% BSA in 1X PBS.

- 1 Fill one to three 50-mL conical tubes with a 4% BSA (in 1X PBS + 0.1% NaN₃).
Store remaining BSA at 2° to 8°C.
- 2 Place the tubes on ice or in the refrigerator for at least 1 hour.
- 3 Pour the BSA solution from the tubes into a bulk container or into other conical tubes.
- 4 The BSA solution may be recycled for 1 month.

Sorting Viable Cells



Using commercial sheath fluid for sorting cells can affect cell viability.

When sorting viable cells, use sterile, filtered (0.22 μ m), phosphate-buffered saline (PBS, pH 7.2–7.4) as sheath fluid.

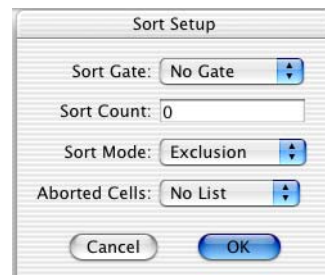
Sorting With BD CellQuest Pro Software

This section describes BD CellQuest Pro software sorting features.

Sort Setup

Select **Acquire > Sort Setup** to display the Sort Setup dialog, where you can specify the following options:

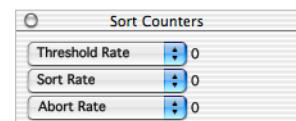
- **Sort Gate**—the sort gate defines the target population. Click the menu control to select the sort gate from the current list of gates. If No Gate is selected, acquisition is performed without sorting.
- **Sort Count**—the number of target cells to be sorted. Specify zero for continuous sorting.
- **Sort Mode**—Click the menu control to select the sort mode. See Sort Modes on page 243 for a description of sort modes and how to decide which one to use.
- **Aborted Cells**—Click the menu control to select List or No List. When List is specified, the data from aborted cells is acquired. Aborted cells are cells that cannot be reliably measured for sorting. The most common cause of an aborted cell is a cell that follows too closely in time behind another cell.



Sort Counters

Select **Cytometer > Sort Counters** to display the Sort Counters window. There are eight different values that can be displayed (all rates are given in events per second):

- **Threshold Rate** or **Threshold Total**—the event rate or the total number of events triggering the threshold detector, including aborted events.
- **Auxiliary Rate** or **Auxiliary Total**—the event rate or the total number of events that the cytometer is processing, including aborted events.
- **Sort Rate** or **Sort Total**—the event rate or the total number of events that are sorted target cells.
- **Abort Rate** or **Abort Total**—the event rate or the total number of events that are aborted events.



Select from threshold and auxiliary values in the top counter, auxiliary and sort values in the middle counter, and sort and abort values in the bottom counter.

Ending a Sort

The following options are available for specifying the end of a sort:

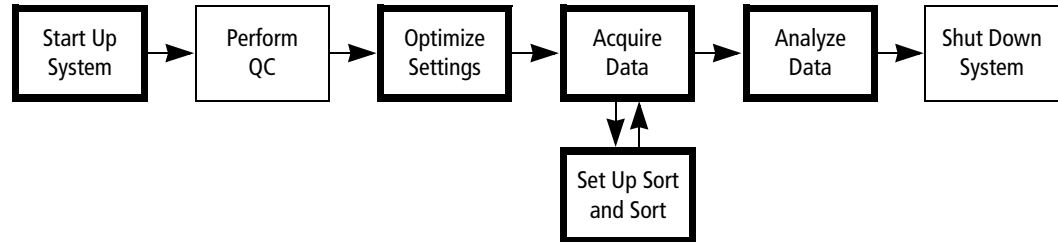
- Clicking Pause and then Abort (Acquisition Control window) will end a sort.
- When NOT acquiring data (Set Up checkbox selected) and a Sort Count > 0 is specified in the Sort Setup dialog, sorting ends when the count is reached.
- When acquiring data, the sort can be ended based on time by specifying a large Event Count so that the specified Time will expire before the Event Count is reached.

The following table describes additional options when acquiring data (with the Set Up checkbox cleared).

Sort Setup selection	Acquisition & Storage selection	Outcome
Sort Count = 0	Event Count or Time specified in Collection Criteria field	Sort and acquisition end when Event Count or Time is reached, whichever comes first.
Sort Count > 0	Event Count or Time specified in Collection Criteria field	Sort and acquisition end when Sort Count, Event Count, or Time is reached, whichever comes first.

Sorting Practice

Sorting cells on a flow cytometer is a multistep process. The following figure outlines the main steps involved. This module includes instructions for system start up, optimizing instrument settings, sorting, and acquiring and analyzing pre- and post-sort data.



IMPORTANT BD Calibrite beads are used with this exercise. A similar procedure can be used with other sample types including peripheral blood samples, bone marrow, cell lines, and mouse cells. When using cells, replace the sheath with sterile PBS, and coat the collection tubes with BSA.

Collection Station Ports

In this exercise, only the first collection port will be used. Refer to the *BD FACSCalibur Flow Cytometer Reference Manual* for instructions for using multiple ports.

Materials

The following BD Calibrite bead tubes will be provided by your instructor for these exercises:

- Unlabeled beads
- Mixed beads (FITC beads, PE beads, and Unlabeled beads)

Starting Up the System

Perform system startup as described in System Startup on page 33.

IMPORTANT The sort samples provided are beads, so no change to the sheath is needed. When cells are used, replace the sheath with sterile PBS.

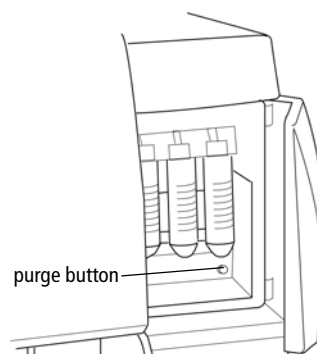
Purging the Sort Lines

Purge the sort lines to check that they are unblocked before sorting.

- 1 Install a tube of DI water on the SIP.

TIP When sorting viable cells, use a tube of sterile PBS water to check the lines.

- 2 Place the cytometer in RUN mode.
- 3 Install a 50-mL tube in the first (leftmost) collection port.
- 4 Press the sort-line purge button.



After the sort-line purge button is pressed, the valve remains open for approximately 30 seconds. Verify that liquid drips from the collection port into the 50-mL tube.

- 5 Continue to press the sort-line purge button every 30 seconds until 5 to 10 mL of fluid has been collected.
- TIP** When using multiple ports, repeat steps 3 to 5 with each additional port.
- 6 Place the cytometer in STNDBY mode.

Performing QC

Follow the procedure in Performing QC and Instrument Setup on page 71.

Optimizing Settings

In this section, you will optimize instrument settings for your target cells. If needed, review Optimizing Cytometer Settings on page 103 for detailed instructions on optimization tasks.

- 1 Start BD CellQuest Pro software.
- 2 Select Acquire > Connect to Cytometer.
- 3 Open the 4-Color Beads Experiment document that you created and saved during the BD CellQuest Pro Workflow exercise.
- 4 Load the 4-Color Beads Settings instrument settings that you saved after optimization in the BD CellQuest Pro Workflow exercise. If the 4-Color Beads Settings instrument settings file is unavailable, use the Calib file settings.

NOTE The sorting experiment uses just two colors with the FL1 and FL2 detectors. Therefore, only the FL1 and FL2 detectors voltages are adjusted, and only the FL2 – %FL1 and FL1 – %FL2 compensation settings are optimized.

- 5 Install the Unlabeled beads tube on the SIP.
- 6 Set the flow rate to MED to conserve sample, and place the cytometer in RUN mode.
- 7 Optimize the following settings:
 - a Adjust the FSC amp gain and SSC detector voltage.
 - b Adjust the FSC threshold setting.
 - c Gate (G1=R1) on the population of interest (singlet beads).
 - d Adjust the FL1 and FL2 detector voltages.
- 8 Install the Mixed beads tube on the SIP and optimize the FL2 – %FL1 and FL1 – %FL2 compensation settings.

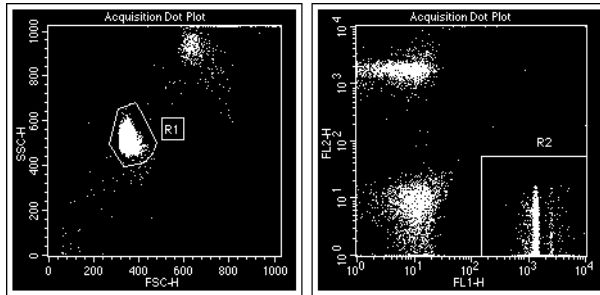
The FL2 – %FL1 and FL1 – %FL2 compensation settings can be adjusted from the Mixed beads tube, because it does not contain a double-positive population.

- 9 Save a new instrument settings file named *2-Color Sort Settings*.

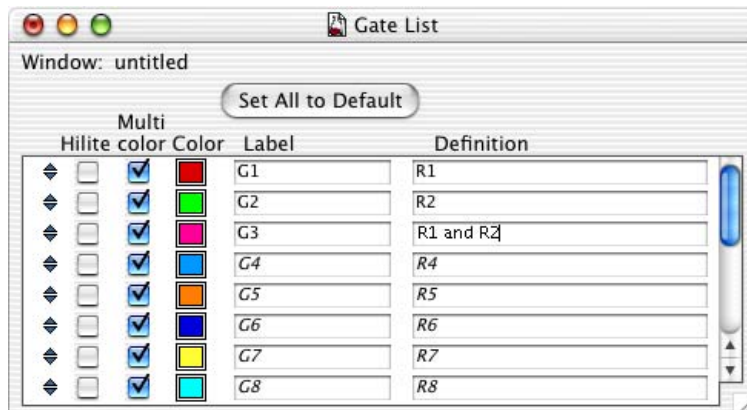
Creating a Sort Gate

Create a logical gate for FITC⁺ singlet beads.

- 1 With the Mixed beads tube still on the SIP, draw a rectangular region (R2) encompassing the FITC⁺ cell population in the FL1 vs FL2 plot.



- 2 Click Pause, followed by Abort.
- 3 Select Gates > Gate List to display the Gate List window.
- 4 Change the definition for G3 to **R1 AND R2**, and press Return.



- 5 Close the Gate List window.

Collecting a Pre-Sort Data File

You will collect a data file from your sample before sorting. This file will be used later to help determine the purity of the sorted sample.

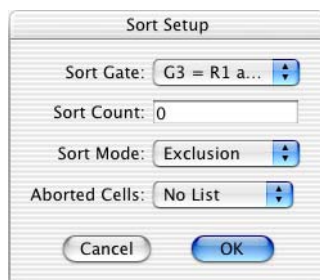
- 1 In the Acquisition Browser, specify settings to save a 10,000 event data file named *Pre-Sort* in your student folder.
- 2 Clear the Setup checkbox in the Acquisition Control window.
- 3 With the mixed beads tube still on the SIP, set the flow rate to HI and acquire the data file.
- 4 Place a tube of DI water on the SIP.
- 5 Place the cytometer in STNDBY mode.

Preparing the Sort Setup and Sort Counters

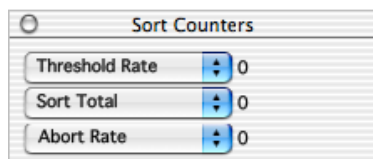
- 1 Select Acquire > Sort Setup.

The Sort Setup dialog appears.

- 2 Make the following selections in the Sort Setup dialog.



- 3 Click OK.
- 4 Select Cytometer > Sort Counters to display the Sort Counters window.
- 5 Select Threshold Rate, Sort Total, and Abort Rate in the Sort Counters dialog.



Sorting the Target Cells

- 1 Select the Setup checkbox in the Acquisition Control window.

When the Setup box is selected, cells are sorted continuously without saving data to a file.

- 2 Install a 50-mL conical tube in the first (leftmost) collection port.

IMPORTANT Since cells are not used in this exercise, a non-coated collection tube can be used. When cells are used, coat the collection tubes with BSA.



If you Pause and then Resume or Restart during a sort, sorting collection will start again at time zero in the first (leftmost) collection port, and the tube may overflow.

If you need to Pause, replace full and partially full collection tubes before clicking Resume or Restart.

- 3 Place the cytometer in RUN mode and set the flow rate to LO.
- 4 Install the Mixed beads tube onto the SIP.
- 5 Click Acquire in the Acquisition Control window.
- 6 When the collection tube is nearly full, end the sort by clicking Pause and then Abort.
- 7 Remove the Mixed beads tube and place a tube of DI water on the SIP.
- 8 Place the cytometer in STNDBY mode.

Concentrating the Sample

The instructor will perform this procedure while you continue with Cleaning the Sort Lines on page 254.

- 1 Remove the collection tube from the collection station and cap it.
- 2 Spin the tube at 300 x *g* for 5 minutes.
- 3 Aspirate the supernatant without disturbing the pellet.
- 4 Vortex the pellet and resuspend it with 0.5 mL of PBS.
- 5 Transfer the suspension to a 12 x 75-mm BD Falcon™ test tube.

Cleaning the Sort Lines

After sorting is completed, clean the sort lines using the syringe provided. This is done to prevent saline deposits from accumulating in the line.

IMPORTANT When sorting biohazardous material, perform the cleaning procedure described with a 10% bleach solution, followed by twice the volume of DI water.

Preparing the Syringe

- 1 Disconnect the tubing from the syringe by twisting the luer fitting counterclockwise.
- 2 Fill the syringe with DI water.

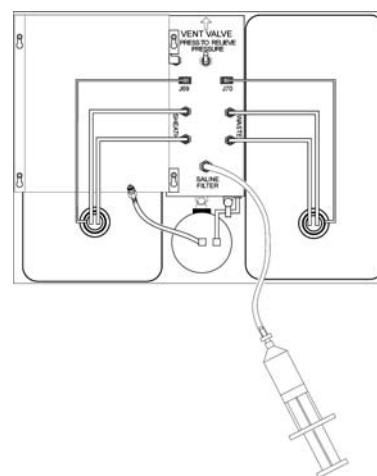
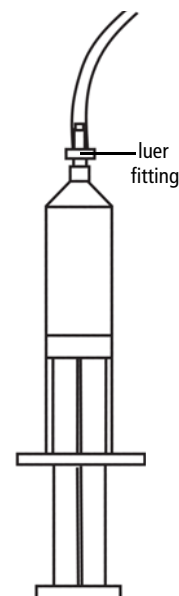
Place the syringe nozzle in a container filled with DI water and slowly pull out the plunger until the syringe is full.

- 3 Bleed any air out of the syringe by holding it luer-end up and gently pushing in the plunger.
- 4 Reconnect the tubing to the syringe by turning the fitting clockwise.
- 5 If necessary, bleed any air out of the tubing by pressing the tip of the quick-disconnect against the side of a beaker while gently pushing on the syringe plunger.
- 6 Disconnect the upper tubing of the sheath filter from the SALINE FILTER port by squeezing the metal clip on the quick-disconnect and pulling the connector from the fitting.
- 7 Connect the tubing from the syringe to the SALINE FILTER port.

Push firmly until you hear a click.

Performing the Clean

- 1 Place a tube of DI water on the SIP.
- 2 Set the flow rate to HI and place the cytometer in RUN mode.
- 3 Install a 50-mL tube in the first (leftmost) collection port.
- 4 Press the sort-line purge button.



- 5 Slowly and firmly apply pressure to the syringe plunger. Continue to depress the plunger until approximately 10 mL of fluid has been dispensed from the syringe and delivered to the collection tube.

TIP After the sort-line purge button is pressed, the valve remains open for approximately 30 seconds. Continue pressing the purge button, as needed, and depressing the plunger until approximately 10 mL of fluid have been collected.

TIP When using multiple ports, repeat steps 3 to 5 with each additional port.

Collecting a Post-Sort Data File

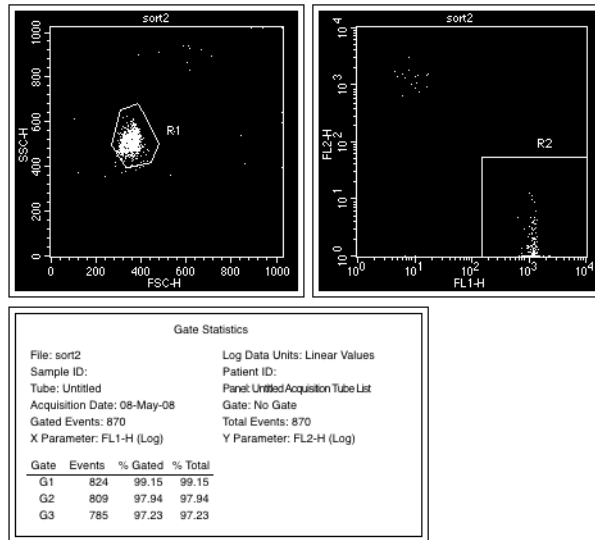
- 1 Remove the syringe and reconnect the sheath filter tubing to the SALINE FILTER port.
- 2 Select Acquire > Sort Setup.
- 3 Click the Sort Gate menu control and select No Gate.
- 4 Click OK.
- 5 Place the cytometer in STNDBY mode.
- 6 Place a tube containing no more than 1 mL of DI water on the SIP.
- 7 Set the flow rate to HI and place the cytometer in RUN mode.
- 8 Before collecting the post-sort data file, run DI water for 3 minutes. This will eliminate carryover from the pre-sort sample tube.
- 9 In the Acquisition Browser, specify settings to save a 1000 event data file named *Post-Sort* in your student folder.
- 10 Place the sorted sample tube on the SIP, and acquire the data file.
- 11 Place the cytometer in STNDBY mode.
- 12 Place a tube containing no more than 1 mL of DI water on the SIP.

Verifying Sort Purity

Verify that the sort purity is greater than 95%.

- 1 If needed, change the FSC vs SSC and FL1 vs FL2 plots to Analysis plots.
- 2 Verify that both plots display ungated post-sort data.
- 3 Display Gate Statistics for either plot.

The %Total for G3 is the sort purity.



- 4 Display the pre-sort data in both plots and compare the %Total for G3.

Sorting Tips

This section lists useful tips you should consider to obtain the best sorting performance from your BD FACSCalibur system.

Testing Sorting Performance

To ensure optimal performance of the instrument, periodically check the sort purity performance. Use the procedure in *Sorting Practice* on page 248 to test sorting performance. A sort purity of greater than 95% indicates that the instrument is performing well and that good sample handling techniques are being used.

Sheath Fluid

- Verify that the sheath tank cap is properly tightened before sorting. A poorly tightened sheath tank cap could alter the flow rate, which could compromise the timing of the catcher tube movements.
- After tightening the sheath tank cap, listen for a hissing noise. Loosen and retighten the cap if this occurs. If the hissing continues, it indicates there is a small hole in either the cap or the tank and one or both need to be replaced.
- Verify that the sheath tank is sufficiently full before beginning a sort to avoid having to refill during a sort.

Cell Recovery and Viability

- Store tubes of sorted cells on ice if they cannot be centrifuged immediately after collection.
- If viability is a concern, centrifuge cells immediately after collection and place concentrated cells on ice in a protein-supplemented medium.
- Viability may be improved if 5 mL of chilled medium with 20% serum is placed in each BSA-coated collection tube prior to sorting.
- Ensure that the last sorted cells are recovered from the sort lines.

Remove the sort tube, install a tube of PBS on the SIP, and continue sorting for 20 seconds.

- Recovered cells will have a large amount of BSA present. If your application (example, Western blot analysis) requires low levels of extraneous protein, resuspend the cells in a small volume (about 1 mL) of PBS after centrifuging, transfer to a clean tube, add PBS to wash, and centrifuge again.

Aseptic Sorting

If you need to perform an aseptic sort, refer to the *BD FACSCalibur Flow Cytometer Reference Manual* section on Aseptic Sorting for detailed instructions.

Low Target Cell Count

When starting with a low sample target cell count, both purity and recovery can be optimized by using an event rate of 1000 events per second or lower in single cell mode.

Threshold

- If the threshold level is too low, the instrument will detect debris, and the coincidence of debris with your target cell could prevent the cell from being sorted.
- If the threshold level is too high, events that do not trigger the system cannot be excluded by the sort logic. For example, if red cells and platelets are in your sample and must be rejected from the sorted fraction, the scatter threshold level must be low enough to allow them to be detected.

Long Sorts

- Vortex your sample occasionally during long sorts to avoid sample settling.



If you Pause and then Resume or Restart during a sort, sorting collection will begin again starting in the first (leftmost) collection port, and the collection tube may overflow.

If you need to Pause, replace full and partially full collection tubes before clicking Resume or Restart.

Sample settling is most often noticed as a decrease in the event rate during the sort. If the sample is removed from the sample injection port for vortexing, stop acquisition and sorting by clicking Pause in the Acquisition Control window or in the Browser. To avoid sorting cells while the sample tube is pressurizing, wait a few seconds after replacing the tube before clicking Restart or Resume. If you wish to continue a sort that has been paused without restarting the counters, click Resume rather than Restart.

- Reset the Collection Station to tube 1 after tube 3 is full.

After all three tubes have been filled, install three new tubes in the collection station. Select Pause, then Restart or Resume. The counters are not reset if you select Resume, but are reset to 0 if you select Restart.

Eliminating Clogs

Check your sort lines if the sorted sample does not reach the collection station.

Small salt crystals may form in the sort line or in the collection station port lines. They can usually be loosened by gently massaging the tubing. If not, use the clog removal method described in *BD FACSCalibur Flow Cytometer Reference Manual*, or the alternate method specified here.

Testing the Sort Line

Check the main sort line.

- 1 Install a tube of DI water on the SIP.
- 2 Place the cytometer in RUN mode and set the flow rate to HI.
- 3 Open the top of the instrument and disconnect the sort line that leads down into the valve block on the collection station.

IMPORTANT Do not disconnect the tubing from the catcher tube. It is very hard to reconnect and may result in a service call.

If sheath fluid comes out of this line when it is disconnected, there is no clog in this line. Reconnect the line and proceed to the second test.

If sheath fluid does not come out, this line is clogged. Examine the line for visible particles (probably salt crystals). Gentle pressure on the outside of the line at the point of the clog sometimes dislodges it.

Testing the Collection Station Port Lines

Check the individual collection station port lines leading from the valve block to the collection tubes.

- 1 Disconnect the line to port 1 at the valve end.
- 2 Attach an 18-gauge luer stub to a 10-mL syringe filled with DI water.
- 3 Fit the needle stub into the end of the tubing.
- 4 Apply pressure on the syringe barrel, forcing water into the tubing and out port 1.
- 5 Repeat steps 1 to 4 on the remaining two port lines.

Reconnect all the tubing and purge the lines as described in *Purging the Sort Lines* on page 249.

Sorting Review

1 Name the three sort modes.

2 If you want to optimize both sort purity AND recovery rate, which sort mode would you use?

3 If you wanted to ensure that you collected the maximum possible percentage of a target cell population, what sort mode would you use?

4 Why should you precoat collection tubes with BSA?

5 What can you do to increase the event rate?

- a Change to a higher flow rate.
- b Concentrate your sample.
- c Add sterile PBS to your sample.
- d None of the above.

6 Name three ways to end a sort.

7 When sorting viable cells, use _____ for sheath fluid.

BD FACS Loader Option

After completing this chapter you will be able to:

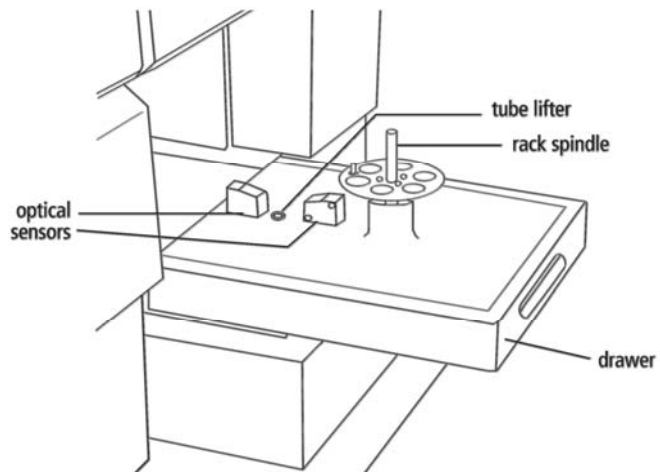
- Identify the major components of the Loader hardware.
- Operate the BD FACS Loader using BD WorklistManager and BD LoaderManager software.
- Perform Loader troubleshooting and diagnostics.
- Perform proper cleaning procedures for the Loader.



BD FACS Loader

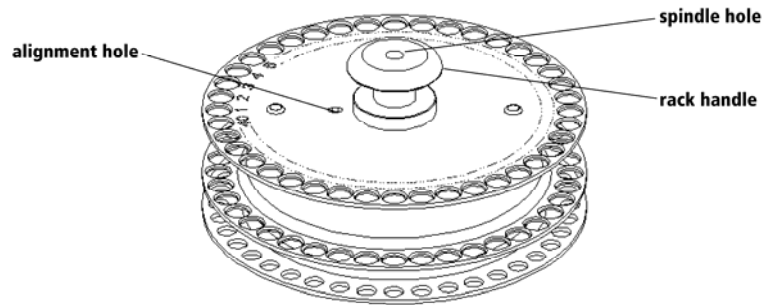


BD FACS Loader Components

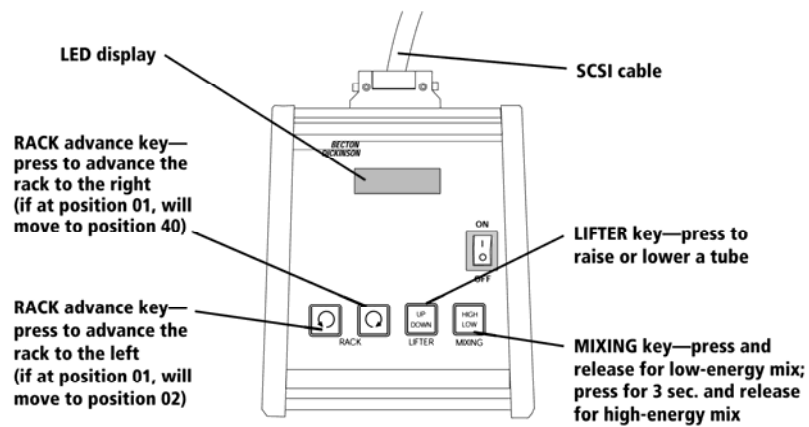




BD FACS Loader Components

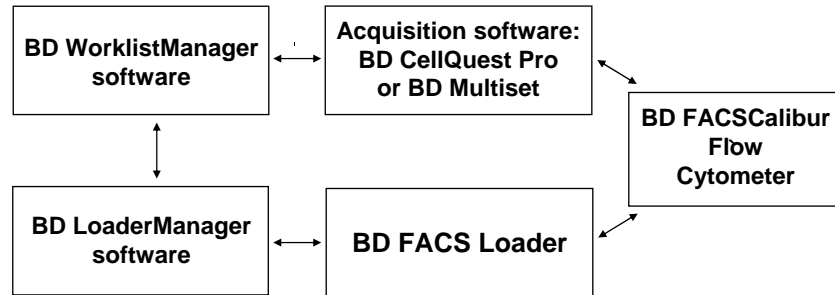


BD FACS Loader Components





Cytometer + Loader + Software



Prepare an Acquisition Document

- Specify stopping criteria in Acquisition & Storage window.
- Select or create a panel.
- Load Tubes from Panel in Acquisition Browser.
- Save document in BD Applications/WorklistManager Folder/CellQuest Experiment Folder.

6



Optimize Instrument Settings

Save instrument settings in
BD Files/Instrument Settings File

7



Link Panel with Instrument Settings

- Start WorklistManager.
- In the Set Up View, click Assay Settings.
- Link each BD CellQuest panel with Instrument Settings.
- Specify Mix Setting.

8

Starting Up the Loader and Using It in Manual Mode

In this exercise you will start up the Loader and operate it using the remote keypad control.

Starting Up the Loader

- 1 Perform the procedure in System Startup on page 33.
- 2 Verify that the Loader rack and cover are not on the Loader, and then turn on the Loader power from the Loader keypad control.
- 3 Remove the DI water tube from the Sample Injection Port (SIP) by holding the bottom of the test tube and swinging the Droplet Containment Module (DCM) arm to the left.

Installing the Rack and Loader Cover

- 1 Place a rack on the Loader with tubes in positions 1 and 2—each tube containing about 1 mL of DI water.

To install a rack, position the hole in the rack handle over the center spindle of the Loader drawer and rotate the rack until the alignment guide pin fits into the small alignment hole at the top of the rack.

- 2 Push the Loader drawer completely in.
- 3 Place the Loader cover on the Loader drawer.

The Loader cycles and the Loader keypad displays *Tube 01*.



- 4 Place the cytometer in RUN mode.

Using the Loader Keypad Control

- 1 On the Loader keypad, press the MIXING key for approximately 3 seconds, then release to activate the high-energy mix.
- 2 After the mix completes, press the LIFTER key to raise the tube in rack position 1 onto the SIP.

When the tube has pressurized, the RUN button indicator light turns green. The lifter remains in the up position.

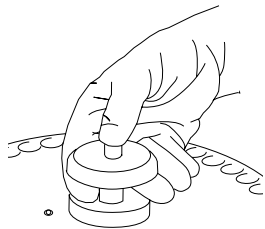
TIP If the tube does not pressurize, the run button remains orange, a message flashes on the keypad, an alarm beeps, and the lifter returns to the down position. To stop the alarm, verify that the lifter is completely down and then remove and replace the Loader cover. If pressurization problems persist, refer to the troubleshooting section in the *BD FACS Loader User's Guide*.

- 3** After the RUN button turns green, press the LIFTER key to lower the tube.
- 4** Press the rack advance key () to index the rack to position 2.
The Loader keypad displays *Tube 02*.
- 5** Press the LIFTER key to raise the tube in rack position 2 onto the SIP.
- 6** After the RUN button turns green, press the LIFTER key to lower the tube.
- 7** Use the RACK back key () to index the rack to position 1.

Removing the Loader Cover and Rack

- 1** Remove the Loader cover.
- 2** Remove the Loader rack.

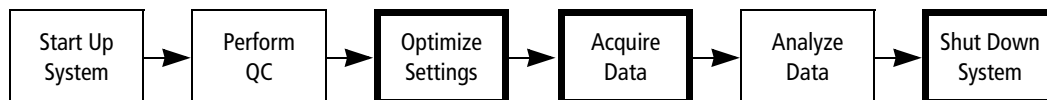
To remove a rack from the Loader, slide the Loader cover forward and pull the Loader drawer out. Place your thumb on the center spindle that protrudes through the top of the rack handle. Press down with your thumb while gently pulling up on the handle with two fingers, as illustrated in the following figure.



- 3** Place a tube containing no more than 1 mL of DI water on the SIP, with the support arm underneath.
- 4** Place the cytometer in STNDBY mode.

Using BD CellQuest Pro Software with the Loader

Acquisition of data using the Loader is a multistep process. The following figure outlines the main steps involved. This section will focus on optimization of instrument settings, data acquisition, and shutdown.



In this exercise, you will do the following.

- Prepare a BD CellQuest Pro Experiment document
- Optimize instrument settings
- Specify conditions for data acquisition and storage
- Acquire data

Before You Begin

Before beginning the exercises in this section, perform the procedures in:

- System Startup on page 33
- Performing QC and Instrument Setup on page 71

Preparing a BD CellQuest Pro Experiment Document

When using BD CellQuest Pro software with the Loader, a BD CellQuest Pro Experiment document is used to specify information needed by BD WorklistManager software.

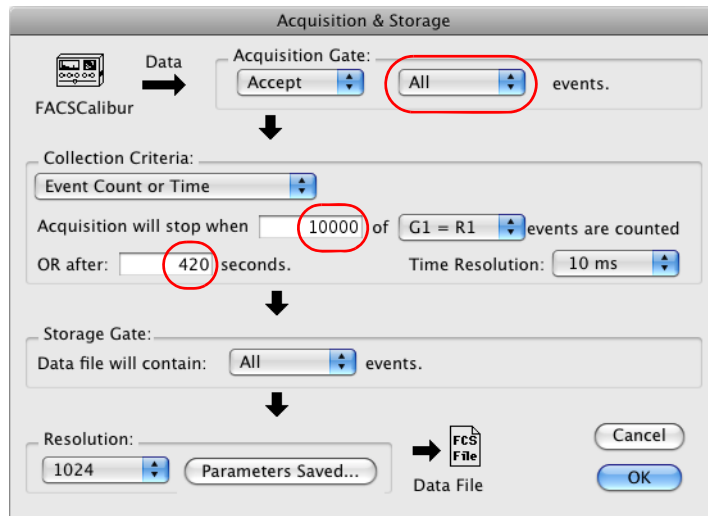
In this section, you will modify your 4-color Beads Experiment document for use with BD WorklistManager software.

- 1** Using the Finder, navigate to your 4-color Beads Experiment document and open it in BD CellQuest Pro software.
- 2** Format all dot plots to be Acq -> Analysis plots.
 - a** Select all the plots in the document.
 - b** Select Windows > Show Inspector.
 - c** In the Inspector, click the Plot Type menu control and select Acq -> Analysis.

- 3 Select Windows > Show Browser.
- 4 Verify that the 4-color Bead panel that you specified in Creating a Reagent Panel on page 117 is loaded in the Browser's acquisition tube list.
- 5 Select Acquire > Acquisition & Storage.

The Acquisition & Storage dialog appears.

- 6 Specify settings as shown in the following figure.



With these settings, acquisition will end when 10,000 events in G1 are counted, or after 420 seconds, whichever comes first.

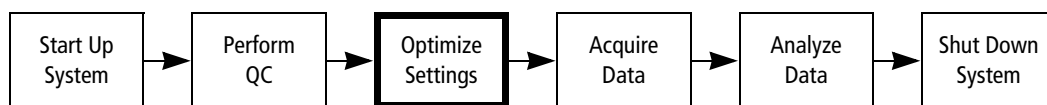
NOTE For walkaway operation, if you select Event Count only, or enter a time limit of more than 10 minutes, BD WorklistManager software will time out if the specified number of events is not collected before 10 minutes. Data is not saved when a time out occurs, and you might not have enough sample left to run the assay.

- 7 Save the Experiment document for use with the Loader.
 - a Select File > Save Document As.
 - b Navigate to the BD Applications/WorklistManager Folder/CellQuest Experiments Folder.
 - c Name the document *Loader 4-color Beads*.
 - d Click Save.

BD CellQuest Pro Experiment documents must be saved in the CellQuest Experiments Folder if they are to be used for acquisition and analysis with the Loader.

Optimizing Instrument Settings

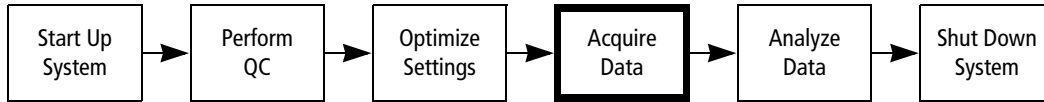
In this exercise, you will optimize instrument settings in BD CellQuest Pro software.



- 1** Verify that the Loader 4-color Beads Experiment document is still open.
- 2** Select Acquire > Connect to Cytometer (⌘-B).
- 3** Open the Detectors/Amps, Threshold, Compensation, and Status windows.
- 4** Use the Loader in manual mode (see Starting Up the Loader and Using It in Manual Mode on page 266) with the unlabeled, FITC, PE, PerCP, and APC control tubes to perform the standard optimization sequence (Optimizing Cytometer Settings on page 103):
 - Adjust the FSC amp gain and the SSC detector voltage.
 - Adjust the FSC threshold setting.
 - Gate on the population of interest.
 - Adjust FL1, FL2, FL3, and FL4 detector voltages.
 - Adjust fluorescence compensation.
- 5** Save the optimized instrument settings.
 - a** Select Cytometer > Instrument Settings.
 - b** Click Save in the Instrument Settings window.
 - c** Navigate to the BD Files/Instrument Settings Files folder and name the file *4-color Loader Settings*.
 - d** Click Save.
 - e** Click Done to close the Instrument Settings window.
- 6** Quit BD CellQuest Pro software.

Acquiring Data

In this exercise, you will enter information in BD WorklistManager software and acquire data.



Entering Information in BD WorklistManager Software

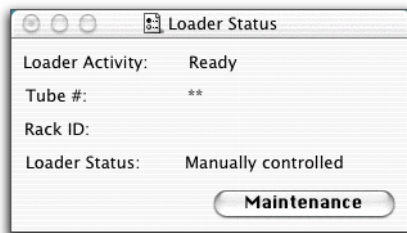
IMPORTANT BD WorklistManager software will not start if other BD applications are already running. In addition, while BD WorklistManager software is running, other BD applications should not be started independently.

- 1 Verify that all BD applications are closed by looking at the icons of BD applications on the Dock. Open application icons have a Mac OS dependent marking.
- 2 Start BD WorklistManager software by clicking its icon on the Dock.



The BD WorklistManager application window opens at the Sign In view (see Figure 9-1 on page 272). BD WorklistManager software automatically starts BD LoaderManager software, which checks to see if the Loader is connected.

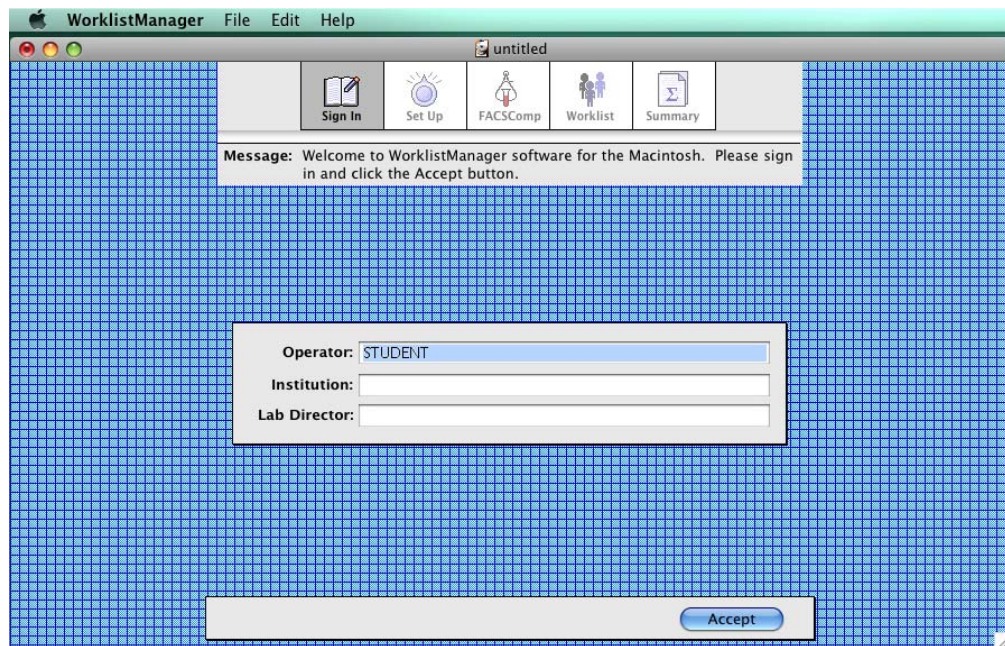
The Loader Status window appears, displaying connectivity status and also whether the Loader is controlled manually or by the computer.



TIP BD LoaderManager software can also be started independently of BD WorklistManager software. Refer to the *BD FACS Loader Reference Manual* for details.

Sign In View

Figure 9-1 BD WorklistManager software Sign In view



- 1 Type your name in the Operator field.
Only the operator field is required.
- 2 (Optional) Fill in the Institution and Lab Director fields.
- 3 Click Accept or press Return.
The application window advances to the Set Up view.

Set Up View

- 1 Make the following selections in the Set Up view. Change the Locations for the Worklist Summary Report, Data Files, and Export Files to your student folder.

The screenshot shows the 'Assay Settings' dialog box with the following configurations:

- File Name Prefix:** Sample Name, Case Number, Sample ID, Date - DDMMYY, Custom Prefix
- Summary Report Sample Label:** Sample Name, Sample ID, Case Number
- Worklist:** Summary Report: Location... Mac OS X:BD Files:W...0403:150403.sum
- Template:** Schedule Template: Location...
- Automatic Saving Options:** Assay Type: CELLQuest
 Data Files: Location... Mac OS X:BD Appli...o Folder:Data files:
 Laboratory Report: Location...
 Physician Report: Location...
 Summary Report: Location...
 Use Date Generated File Name
 Export Files: Location... Mac OS X:BD Appli...Folder:Export files:
 Use Date Generated File Name

Buttons at the bottom: Assay Settings..., Accept

- When the File Name Prefix—**Sample ID** radio button is selected, file name prefixes for FCS data files are based on Sample ID entries in the Worklist view. You must enter a Sample ID for each worklist entry.
- A sample ID appears for each entry on the Summary Report when the Summary Report Sample Label—**Sample ID** checkbox is selected.
- When the Worklist—**Summary Report** checkbox is selected, a Worklist Summary Report file is automatically saved. The current file path is displayed. The default location is a dated folder (DDMMYY), in the BD Files/WorklistManager Files folder.
- When Automatic Saving Options—**Assay Type** is set to CELLQuest, the Automatic Saving Options for BD CellQuest Pro assays are displayed.

When an **Automatic Saving Options** checkbox is selected, the corresponding file is automatically saved. The current file path appears below each file type. The default location for each file type is a dated folder (DDMMYY) in the BD Files/CellQuest Pro Folder.

- 2 Click Assay Settings to display the Assay Settings dialog.

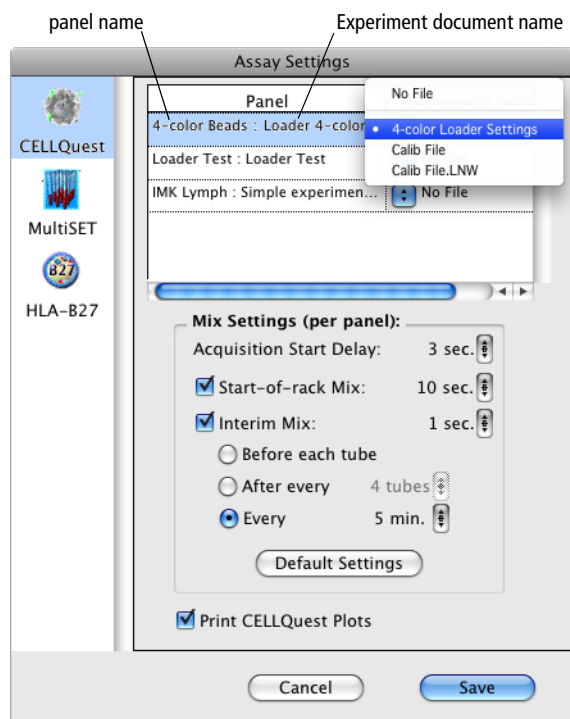
Assay Settings

In the Assay Settings dialog, you link instrument settings to panels and specify mix and printing settings.

- 1 Click the CELLQuest icon.

Only panels in BD CellQuest Pro Experiment documents saved in BD Applications/WorklistManager Folder/CellQuest Experiment Folder are listed under the Panel column. The instrument settings saved in BD Files/Instrument Settings Files are listed under the Instrument Settings column.

- 2 Link the 4-color Loader Settings instrument settings file with the 4-color Beads panel in the Loader 4-color Beads Experiment document.
- 3 Specify Mix Settings for the 4-color Beads panel as shown in the following figure.



- 4 Verify that the Print CELLQuest Plots checkbox is selected.

When the Print CellQuest Plots checkbox is selected, a copy of the BD CellQuest Pro document will print after each tube in a panel is acquired.

NOTE Settings specified in the BD WorklistManager Page Setup and Print dialogs apply only to the Summary Report and Rack Manifest printouts.

- 5 Click Save.

A dialog appears.



- 6 Click OK.

Saving a Worklist

Before entering sample information, save the BD WorklistManager worklist as a template that you can use to acquire data on a daily basis.

- 1 Select File > Save As.
- 2 Save the worklist as *WM_CQ_WorklistTemplate* in your student folder.

TIP Make the worklist template a stationery pad.

Entering Sample Information in the Worklist View

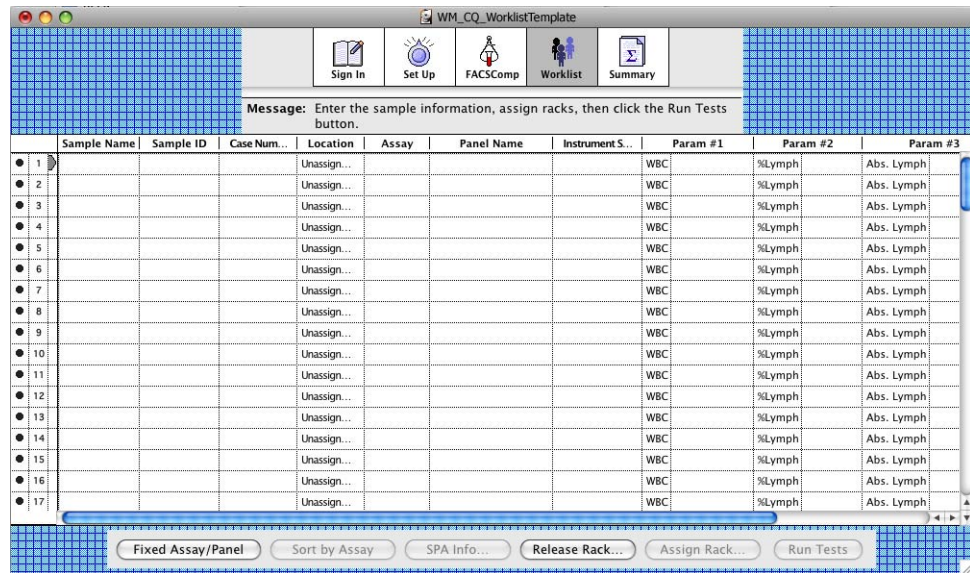
In this section you will make entries for three samples.

- 1 Click the Accept button in the Set Up view.

The application window advances to the FACSCComp view.

- 2 Click Skip FACSCComp.

The application window advances to the Worklist view. The status icons located to the left of the entry numbers indicate that no sample information has been entered.



- 3 Specify Sample ID, Assay, and Panel Name for three entries as shown in the following figure.

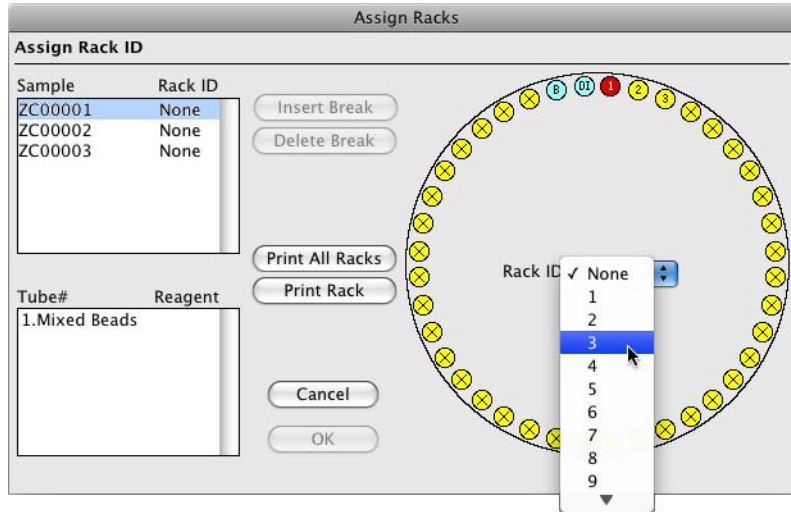
	Sample Name	Sample ID	Case Number	Location	Assay	Panel Name	Instrument Settings
1		ZC00001		Unassigned	CELLQuest	4-color Beads : Loader 4-Color Beads	4-color Loader Settings
2		ZC00002		Unassigned	CELLQuest	4-color Beads : Loader 4-Color Beads	4-color Loader Settings
3		ZC00003		Unassigned	CELLQuest	4-color Beads : Loader 4-Color Beads	4-color Loader Settings

Instrument Settings indicates the settings linked to the selected panel. The status icons for entries 1 through 3 change to rack icons.

- 4 Click Assign Rack to display the Assign Racks dialog.

Assigning a Rack ID

- 1 Verify that your sample is highlighted in the Sample list, and select the Rack ID that you are using for the sample.



When a sample is highlighted in the Sample List, the dialog displays the tube number and the tube name (label) assigned in the panel, and indicates the position of the sample tube in the rack.

NOTE All panel tubes in a given entry must reside on one rack.

- 2 Click the Print Rack button to print the Rack Manifest, and then click OK.

The Rack Manifest contains the sample information, reagent names, and tube locations as well as an overview of the rack with the entry numbers for each sample.

The Locations for entries 1 through 3 are now displayed in the Worklist view, and their status icons have changed to document icons indicating that the entries are ready to be run.



Select Save As in the following instruction to avoid overwriting your BD WorklistManager software worklist template.

- 3 Select File > Save As.
- 4 Save the worklist as *Sample Worklist* in your student folder.

Running Samples

- 1** Gently vortex all sample tubes.
- 2** Load the rack.
 - Load the sample tubes according to the printed Rack Manifest.
 - Load a tube containing 3 mL of a 10% bleach solution in rack position 39.
 - Load a tube containing 3 mL of DI water in rack position 40.
- 3** Place the rack on the Loader.
- 4** Close the Loader drawer completely, and place the cover on the Loader.

The rack initializes.
- 5** Place the cytometer in RUN mode and set the flow rate to HI.
- 6** Click the Run Tests button in the Worklist view.

The samples are run and the cleaning cycle is performed.
- 7** Remove the Loader cover, pull the Loader drawer out, and remove the rack.
- 8** Place a tube containing no more than 1 mL of DI water on the SIP and place the support arm underneath the tube.
- 9** Place the cytometer in STNDBY mode.

Reviewing the Summary Report

A Summary Report is displayed in the Summary view.

The Summary Report lists all sample and reagent information, the rack location, the data files saved, and the status of the run. If an error occurred during the run, an error message appears on the Summary Report. Refer to the *BD FACS Loader User's Guide* for assistance in troubleshooting errors.

The report is automatically saved as specified at the Set Up view. You can print the Summary Report by selecting File > Print.

- 1** When you have finished reviewing the report, quit BD WorklistManager software.

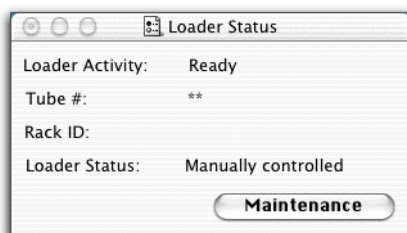
Cleaning

This section describes how to perform a Long Clean using BD LoaderManager software, and how to shut down the Loader. A Long Clean runs bleach and DI water through the cytometer for approximately 12 minutes, and is performed automatically at the end of each Worklist run. Run a Long Clean manually if you stop a Worklist before it is complete, or for additional, optional cleaning.

- 1 Quit BD WorklistManager software, if it is open.
- 2 Start BD LoaderManager software by clicking its icon on the Dock.

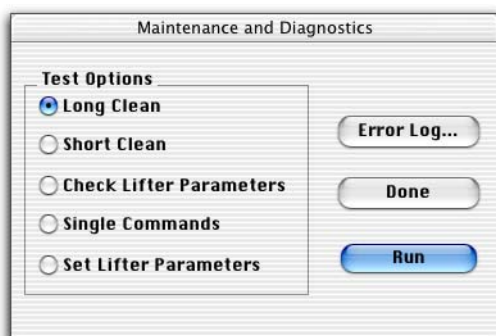


The Loader Status window appears.



- 3 Click the Maintenance button.

The Maintenance and Diagnostics dialog appears.



- 4 Verify that the Long Clean option is selected and click Run.

A message appears, prompting you to install bleach and rinse tubes.

- 5** Load a rack with cleaning tubes.
 - Load a tube containing 3 mL of a 10% bleach solution in rack position 39.
 - Load a tube containing 3 mL of DI water in rack position 40.
- 6** Place the rack on the Loader.
- 7** Close the Loader drawer completely, and place the cover on the Loader.

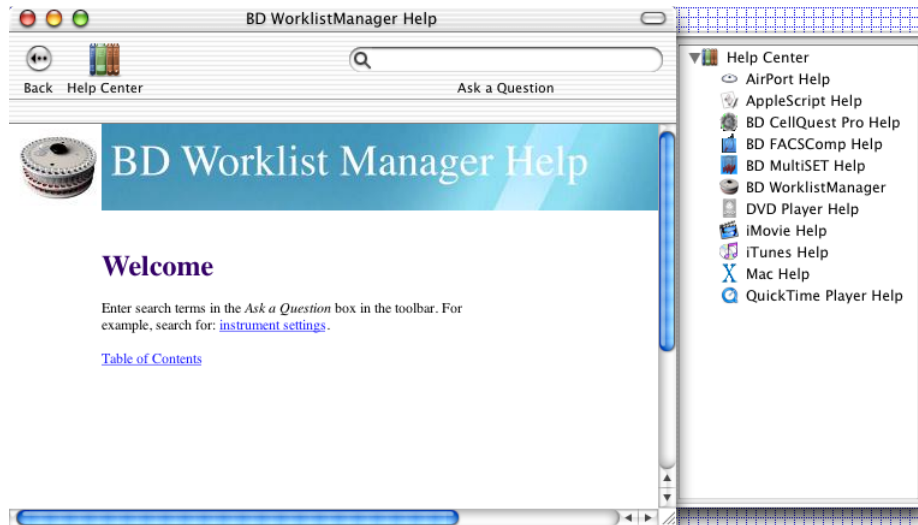
The rack initializes.
- 8** Click Run.

A cleaning status dialog appears.
- 9** When the cleaning cycle is complete, remove the Loader cover and rack.
- 10** Install a tube containing no more than 1 mL of DI water on the SIP.
- 11** Place the cytometer in STNDBY mode.
- 12** Quit BD LoaderManager software.
- 13** Close the Loader drawer. Leave the rack out and the cover off.

Using BD WorklistManager Software Help

When you need assistance with BD WorklistManager software, use the Help viewer to quickly locate the information.

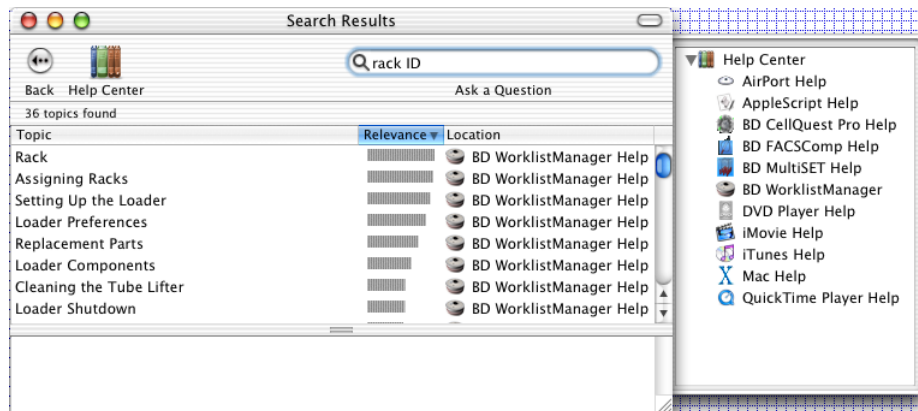
- 1 Select Help > BD WorklistManager Help.



The fastest way to search for help is to use the Ask a Question feature. You can also search by looking through the table of contents or index.

- 2 Enter *rack ID* in the Ask a Question field, and press Return.

A list of topics appears in the lower portion of the Help view. Double-click a topic to display that section of Help.



- 3 When you are finished, close the Help viewer by clicking the red button in the upper-left corner of the viewer.

Loader Review

- 1 Where must BD CellQuest Pro Experiment documents be saved to be used with BD WorklistManager software?

- 2 Where must optimized instrument settings be saved to be used with BD WorklistManager software?

- 3 Why should Event Count or Time be specified as the Acquisition Criteria in a BD CellQuest Pro Experiment document to be used with BD WorklistManager software?

- 4 The Rack Manifest includes:

- a Sample information and tube locations for a rack.
- b A summary of the analysis results for all tubes in a rack.
- c Certification of rack manufacturing integrity.
- d All of the above.
- e None of the above.

Lab Exercise: Troubleshooting

In this exercise, the instructor will demonstrate a Loader run. Several conditions will have been set to simulate problems you might encounter during a Loader run.

- 1 Observe error messages that appear, determine causes, and propose solutions. Record notes in the following table.

Observation or Error Message	Causes	Solutions

2 Describe the proper way to interrupt a Loader run.

3 Describe the procedure for cleaning the Loader lifter.

4 Where is the Loader error log file located?

Refer to the *BD FACS Loader User's Guide* for additional information about rerunning samples and interrupting the Loader during a run.

BD High Throughput Sampler Option

After completing this module, you will be able to use the BD High Throughput Sampler (HTS) to:

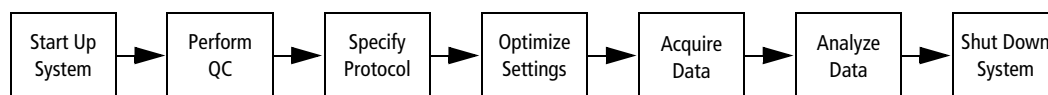
- Prepare BD CellQuest Pro Experiment documents for acquisition and analysis with BD PlateManager software.
- Set up a plate protocol in BD PlateManager software.
- Acquire and analyze samples using BD PlateManager software.
- Export an analysis results file.
- Perform daily cleaning with BD PlateManager software.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

Introduction

This chapter provides step-by-step instructions for performing a basic run using the BD High Throughput Sampler (HTS).

Acquisition and analysis of data using the HTS is a multistep process.



Materials

BD Calibrite beads will be used in the exercises in this chapter to demonstrate the data acquisition process. These same steps can be used for the immunophenotyping of many cell types including peripheral blood samples, bone marrow, cell lines, and mouse cells.

BD FACS Sheath Solution with Surfactant

To prevent bubble formation in the flow cell, use only BD FACS sheath solution with surfactant when acquiring samples with the HTS. Note that this sheath solution is intended for research use only, and should be used only with the HTS option. It should not be used for sorting or in vitro diagnostic (IVD) applications.

Preparing BD CellQuest Pro Experiment Documents

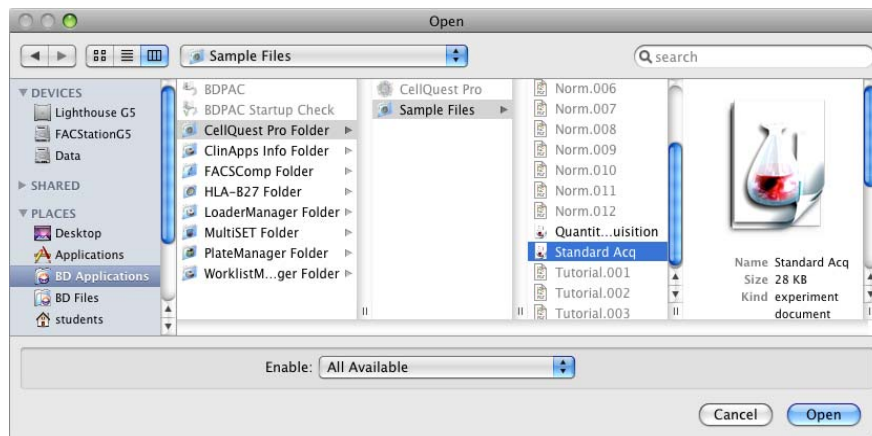
BD PlateManager software works in tandem with BD CellQuest Pro software for data acquisition and analysis. Before acquiring samples with the HTS, you will prepare BD CellQuest Pro acquisition and analysis documents to be used with BD PlateManager. You will not need to prepare new Experiment documents for every run. The documents you create can be reused with multiple BD PlateManager protocols.

TIP It is possible to use the same Experiment document for both acquisition and analysis. Make all plots acquisition-to-analysis and ensure that the necessary statistics windows are visible in the document.

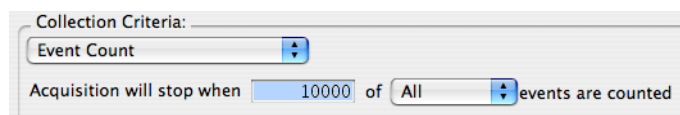
Preparing an Acquisition Document

In this section, you will modify the Standard Acq Experiment document for use with BD PlateManager software. The Standard Acq document is an Experiment document containing four plots and statistics views. The document is a stationery pad.

- 1 Using the Finder, navigate to the Standard Acq document and double-click to open it in BD CellQuest Pro software.



- 2 Select Acquire > Acquisition & Storage.
The Acquisition & Storage dialog appears.
- 3 Click the Collection Criteria menu control and select Event Count.
- 4 Type 10,000 for the event count



5 Click OK to close the Acquisition & Storage dialog.

TIP To ensure that the event count can be reached, you will need to specify a sample volume (in BD PlateManager software) and use a sample concentration that are sufficient.

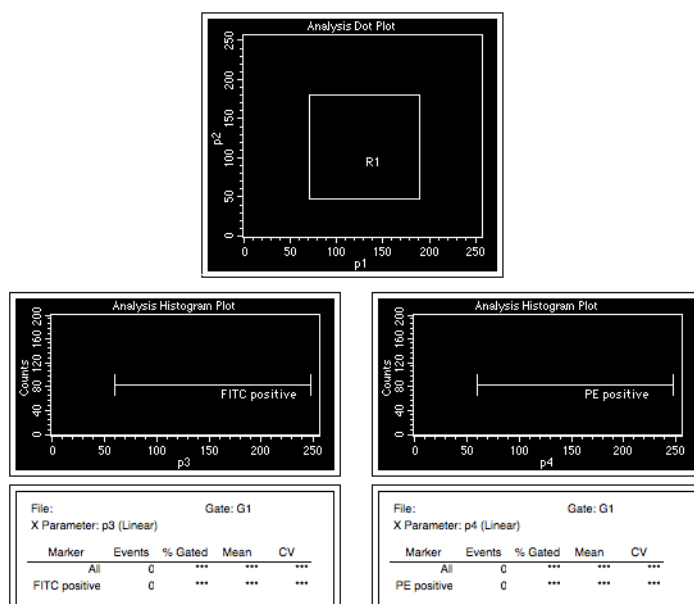
6 Select File > Save Document As.

7 Name the document *PM_CQ_Acquisition* and save it in your student folder.

8 Select File > Close Document.

Preparing an Analysis Document

In this section, you will create an analysis document for use with BD PlateManager software. When complete, your analysis document should look like the one in the following figure.



1 Select File > New Document.

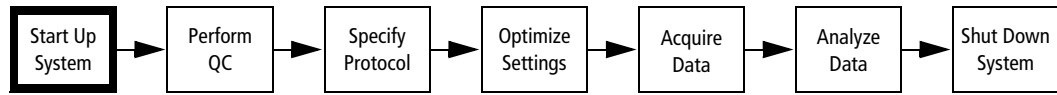
A blank Experiment document opens.

2 Create the following Analysis plots:

- p1 vs p2 dot plot
- p3 histogram
- p4 histogram

- 3** Create a rectangular region (R1) in the center of the p1 vs p2 dot plot.
Make the region large enough to encompass the singlet bead population.
- 4** Create a histogram marker (M1) on the p3 histogram
- 5** Rename this marker *FITC-positive*.
 - a** Select the M1 marker.
 - b** Select Gates > Marker List.
 - c** Change the M1 label to *FITC-positive*.
- 6** Create a histogram marker (M1) in the p4 histogram. Rename this marker *PE-positive*.
- 7** For each histogram:
 - a** Use the Inspector to gate on G1=R1.
 - b** Select Stats > Histogram Stats to display histogram statistics.
- 8** Edit the histogram statistics views:
 - a** Command (⌘)+click to select both histogram statistics views.
 - b** Select Stats > Edit Histogram Stats to obtain the Edit Histogram Statistics dialog.
 - c** Make selections in the Edit Histogram Statistics dialog so that your statistics views show the same information as in the figure on page 288.
- 9** Select File > Save Document As.
- 10** Name the document *PM_CQ_Analysis* and save it in your student folder.
- 11** Select CellQuest Pro > Quit CellQuest Pro.

System Startup



BD FACS Sheath Solution with Surfactant

To prevent bubble formation in the flow cell, use only BD FACS sheath solution with surfactant when acquiring samples with the HTS.

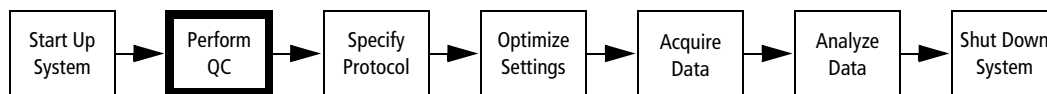
Startup Procedure

For BD FACSTFlow Supply System users:

- 1 Check the sheath and waste cubitainers to ensure that the sheath is not empty and the waste is not full.
- 2 Turn on the power to the BD FACSTFlow Supply System.

If you are not using the BD FACSTFlow Supply System, perform the startup procedure in System Startup on page 33. You will be loading tubes manually in the next section, so leave the HTS unit powered off for now.

Instrument QC



You will use BD FACSComp software to perform instrument QC and to generate an instrument settings file. The cytometer should be in tube-based acquisition mode in order to use BD FACSComp software. If the cytometer is already in tube-acquisition mode, skip ahead to Performing QC on page 292.

Setting Up for Tube-Based Acquisition

To switch to tube-based acquisition mode:

- Remove the HTS sample coupler.
- Remove the sample injection tube (SIT) protector and install the DCM sleeve.
- Place the acquisition control switch in tube-based acquisition mode.



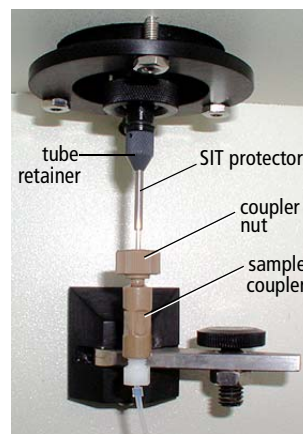
Any instrument surface or material that comes in contact with biological specimens can transmit potentially fatal disease. Use universal precautions when handling instrument hardware. Wear suitable protective clothing and gloves.


- 1 Place the cytometer in STNDBY mode.
- 2 Remove the BD High Throughput Sampler safety cover.

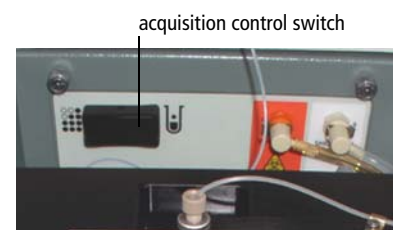
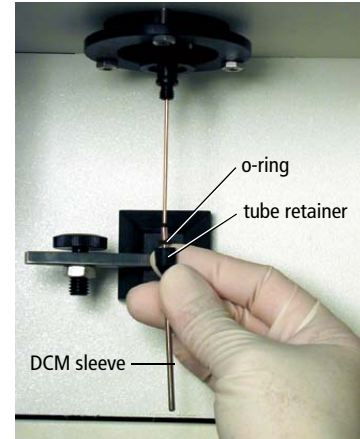


The SIT can be bent and damaged when removing the sample coupler and SIT protector. Be sure to slide these parts **STRAIGHT DOWN** when removing them from the SIT in steps 3 and 4.

- 3 Detach the sample coupler from the cytometer sample injection tube (SIT).
 - a Loosen the coupler nut.
 - b Once the coupler moves freely, gently pull straight down on the coupler to remove it from the SIT.
- 4 Remove the SIT protector. Unscrew the tube retainer and slide the SIT protector straight down. To avoid bending the SIT, do not slide the SIT protector at an angle.



- 5 Reinstall the standard DCM sleeve.
 - a Transfer the o-ring and tube retainer from the SIT protector to the DCM sleeve.
 - b Slide the sleeve straight up over the SIT. To ensure that the sleeve seats properly, hold the sleeve up while screwing the tube retainer onto the SIP.
- 6 Install a tube of DI water on the SIP. Place the tube support arm under the tube.
- 7 Switch the acquisition control switch to tube-based acquisition mode. The acquisition control switch is located on the BD FACSCalibur cytometer directly behind the HTS unit. Press the side of the switch next to the  icon to select tube-based acquisition mode.



Performing QC

- 1 Run the 4-color Lyse/Wash assay in BD FACSComp software as described in Performing QC and Instrument Setup on page 71. Store the prepared BD Calibrite bead tubes (labeled A and B) in the refrigerator for use later in this chapter.

At the completion of the run, an instrument settings file named Calib File is automatically saved in the BD Files > Instrument Settings Files folder.

- 2 After the instrument QC run is complete, install a tube containing no more than 1 mL of deionized (DI) water on the sample injection port (SIP) and place the cytometer in STNDBY mode.

Preparing an Instrument Settings File

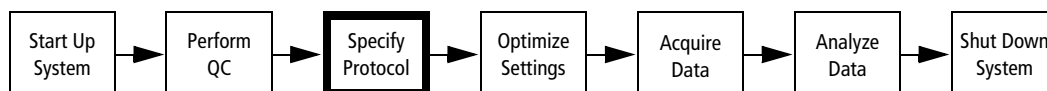
In this section, you will duplicate and rename the Calib File instrument settings file so it can be modified by BD PlateManager software.

- 1 Use the Finder to navigate to BD Files folder > Instrument Settings Files folder and select Calib File.
- 2 Select File > Duplicate.

A new file, Calib File copy, appears in the file listing.
- 3 Change the name of the Calib File copy file to *PM_CQ_Settings*.

Specifying a Protocol

In this section, you will use BD PlateManager software to specify an experiment protocol including the plate layout, well type (setup, standard, or sample), data file prefixes, sample volume, mixing volume, acquisition document, and instrument settings file.



Specifying a Plate Layout

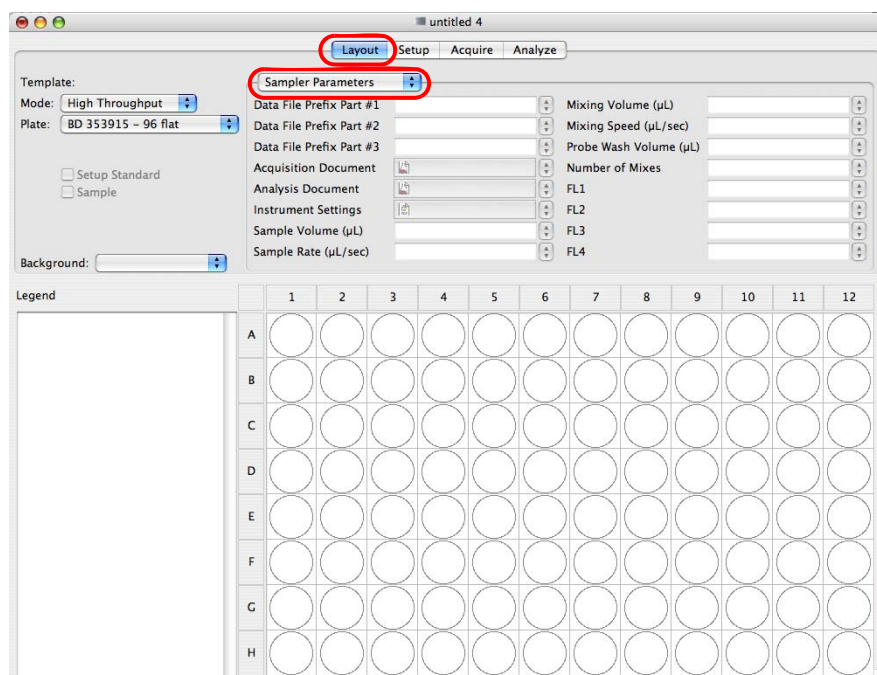
- 1 Start BD PlateManager software by clicking its icon on the Dock.



The main application window appears with the Layout view selected.

BD PlateManager software is organized into four workflow views: Layout, Setup, Acquire, and Analyze. You can navigate to a view by clicking its view button near the top of the window.

The keyword view selector can be used to change the display of the Layout view. For this exercise, keep its value set to Sampler Parameters.



2 Click the Mode menu control and select Standard.

- In Standard mode, the BD High Throughput Sampler can transfer 2–200 µL per well.
- In High Throughput mode, the BD High Throughput Sampler can transfer 2–10 µL per well.

TIP Setup standard wells are always acquired in the standard fluidic mode to allow for sufficient acquisition time. If you select the high throughput mode for the plate, acquisition in the Setup view will default to standard mode.

For more information about throughput modes, refer to the *BD High Throughput Sampler User's Guide*.

3 Click the Plate menu control and select *BD 353910 – 96 U*.

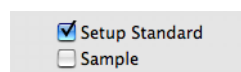
Items in the Plate menu are part numbers with descriptions that correspond to BD Discovery Labware multiwell plates.

IMPORTANT The HTS unit is compatible only with standard depth 96- and 384-well microtiter plates. Refer to the *BD High Throughput Sampler User's Guide* for a list of compatible plates.

Specifying Settings for the Setup Standard Wells

TIP When specifying parameters for specific wells, ensure that the wells remain selected.

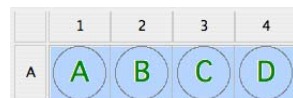
- 1 Select wells A1 through A4 by dragging the mouse over the well icons in the plate layout section.
- 2 Select the Setup Standard checkbox.



Wells A1 through A4 will be used to optimize settings.

TIP Designate several wells as setup standard wells to ensure that you do not run out of sample during setup.

The setup standard wells are labeled with a letter indicating their order of acquisition.



Since data files will not be saved for the setup standard wells, leave the Data File Prefix fields blank.

3 Specify the Acquisition Document for the setup standard wells.

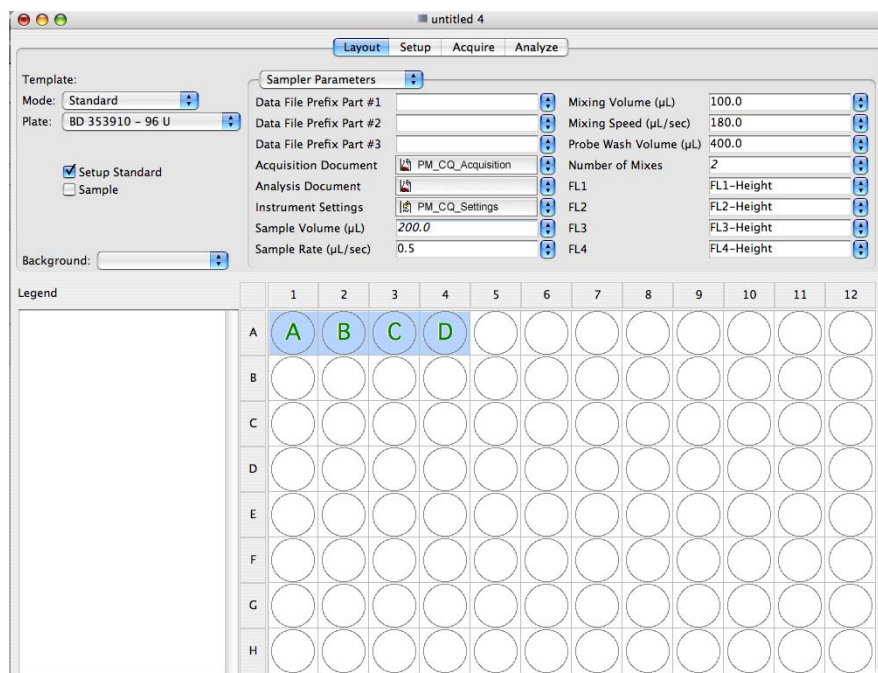
- a Click the icon in the Acquisition Document field.
- b Navigate to your student folder and open PM_CQ_Acquisition.

Since data files will not be saved for the setup standard wells, leave the Analysis Document field blank.

4 Specify the Instrument Settings file for the setup standard wells.

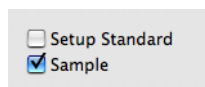
- a Click the icon in the Instrument Settings field.
- b Navigate to BD Files > Instrument Settings Files and open PM_CQ_Settings.

5 Verify that the default values are specified for Sample Volume, Sample Rate, Mixing Volume, Mixing Speed, Probe Wash Volume, Number of Mixes, FL1, FL2, FL3, and FL4, as shown in the following figure.



Specifying Settings for the Sample Wells

- 1 Select wells B1 through B4 and C1 through C4 by dragging the mouse over the well icons in the plate layout section.
- 2 Select the Sample checkbox to designate the selected wells as samples.



Setup Standard
 Sample

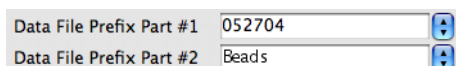
The sample wells are numbered indicating their order of acquisition.

	1	2	3	4
A	A	B	C	D
B	1	2	3	4
C	5	6	7	8

- 3 Specify Data File Prefixes for the sample wells.

You can specify up to three different prefixes. In this exercise, date and sample name are used as prefixes.

- a Type the current date in yymmdd format in the Data File Prefix Part #1 field.
- b Type *Beads* in the Data File Prefix Part #2 field.



Data File Prefix Part #1 052704
Data File Prefix Part #2 Beads

BD PlateManager software adds a file name suffix to indicate the well location. For example, a data file generated from well B1 in this exercise would be named 052704BeadsB01.a. The .a extension indicates the first acquisition of the well. If the same well is reacquired, it is named 052704BeadsB01.b.

- 4 Specify the acquisition document for the sample wells.
 - Click the Acquisition Document menu control and select PM_CQ_Acquisition.



Data File Prefix Part #3
Acquisition Document PM_CQ_Acquisition

- 5 Specify the Analysis Document for the sample wells.

- a Click the icon in the Analysis Document field.
- b Navigate to your student folder and open PM_CQ_Analysis.

- 6** Specify the Instrument Settings file for the sample wells.
- Click the Instrument Settings menu control and select PM_CQ_Settings.
- 7** Specify settings for the Sample Volume, Sample Rate, and the Mixing Volume.

- Type 100.0 in the Sample Volume (μL) field.

The sample volume range in the Standard fluidic mode is 2–200 μL.

- Click the Sample Rate (μL) menu control and select 1.0.

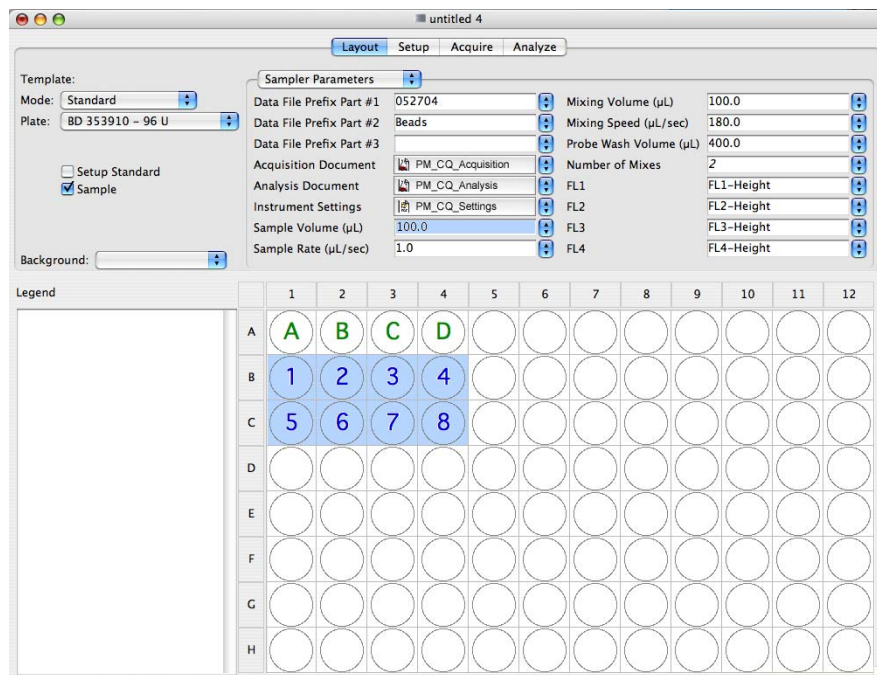
The sample rate range is 0.5–3.0 μL/second.

- Type 100.0 in the Mixing Volume (μL) field.

The mixing volume specifies the volume of sample that will be aspirated and dispensed back into the well during a mix. This volume should be set at 50–60% of the total well volume.

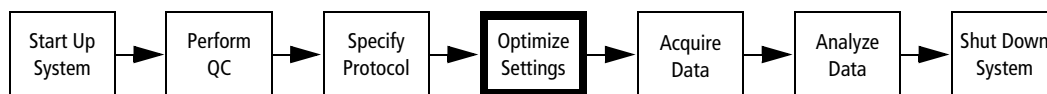
- 8** Verify that the default values are specified for Mixing Speed, Probe Wash Volume, Number of Mixes, FL1, FL2, FL3, and FL4, as shown in the following figure.

Refer to the *BD High Throughput Sampler User's Guide* for more information about these parameters.



Optimizing Instrument Settings

In this exercise, you will prepare the multiwell plate, set up the instrument for plate-based acquisition, and optimize the instrument settings for beads.



Preparing the Multiwell Plate

TIP As an aid in preparing the multiwell plate, you can print out the plate layout by selecting the Layout view, and then selecting File > Print.

- 1 Retrieve your BD Calibrite bead tubes and gently vortex both tubes.
- 2 Add bead suspension to wells as specified in the following table. If you run out of mixed bead, prepare another 1 mL of the mixed bead suspension.

Wells	Amount	From Tube
A1	200 μ L	A (unlabeled beads + APC beads)
A2-A4	200 μ L	B (mixed beads)
B1-B4	200 μ L	B (mixed beads)
C1-C4	200 μ L	B (mixed beads)

Setting Up for Plate-Based Acquisition

To switch to plate-based acquisition mode:

- Remove the DCM sleeve and install the SIT protector.
- Install the HTS sample coupler.
- Place the acquisition control switch in plate-based acquisition mode.



Any instrument surface or material that comes in contact with biological specimens can transmit potentially fatal disease. Use universal precautions when handling instrument hardware. Wear suitable protective clothing and gloves.

- 1 Place the cytometer in STNDBY mode.
- 2 Remove the BD High Throughput Sampler safety cover.

3 Remove the tube of DI water from the SIP.

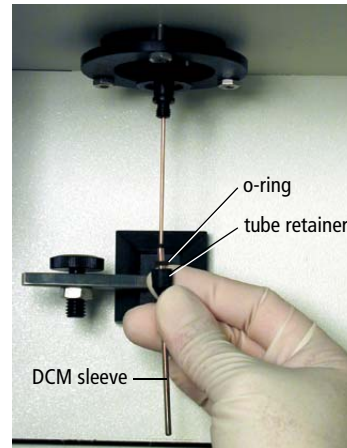


The SIT can be bent and damaged when removing the DCM sleeve. Be sure to slide the DCM sleeve STRAIGHT DOWN when removing it from the SIT.

4 Remove the DCM sleeve.

- Unscrew the tube retainer that holds the DCM sleeve onto the sample injection port (SIP), and carefully remove the sleeve.

TIP Store the DCM sleeve near the flow cytometer so that you can reinstall it when you need to switch back to tube-based acquisition mode.

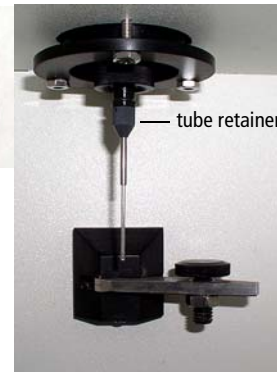


5 Install the SIT protector.

The SIT protector is a modified sleeve that prevents the sample injection tube from bending during installation of the sample coupler.



- a Transfer the o-ring and tube retainer from the SIT protector to the DCM sleeve.
- b Slide the protector over the SIT, and push up on the tube retainer until you can screw it onto the SIP. Tighten the tube retainer.



6 Attach the sample coupler to the cytometer SIT.

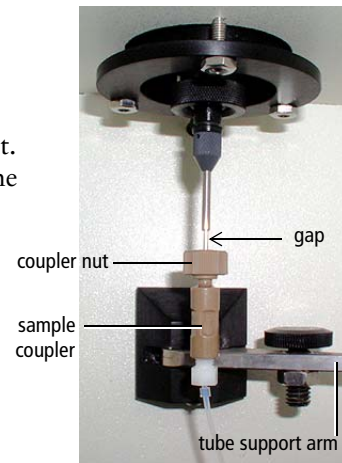



Make sure the coupler nut is loose before you install it. The coupler should slide easily onto the SIP. Forcing the coupler when the nut is too tight could bend the SIT.

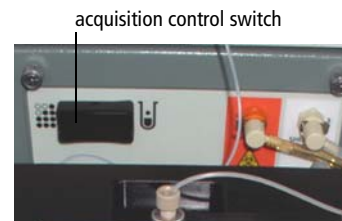
7 Slide the sample coupler onto the SIP until it reaches a hard stop. Make sure the sample coupler tubing is not kinked or twisted. Hold the coupler with one hand while you tighten the nut with the other hand.

There should be a gap between the tightening nut and the bottom of the SIT protector. If you don't see a gap, unscrew the tube retainer, push the SIT protector all the way up, and retighten the tube retainer.

Make sure the coupler is securely connected to the SIT.



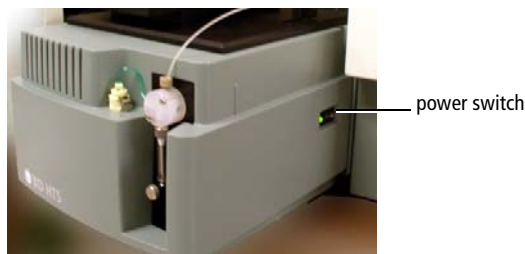
8 Switch the acquisition control switch to plate-based acquisition mode ().



Running the Standard Setup Wells

- 1 Turn on the HTS power switch.

The power switch is on the right side of the HTS unit.



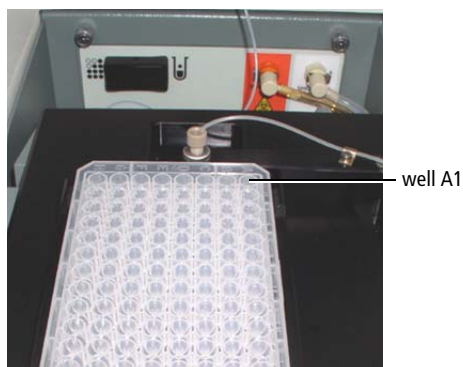
- 2 Remove the safety cover from the HTS unit.



To prevent damage to the probe, always remove the multiwell plate cover before you put a plate on the HTS plate holder.

- 3 Place your plate on the plate holder.

Verify that the multiwell plate type matches the type selected in the plate field of the BD PlateManager software. Orient the plate with well A1 on the back-right corner of the stage.



- 4 Replace the HTS safety cover.

- 5 Place the cytometer in RUN mode.

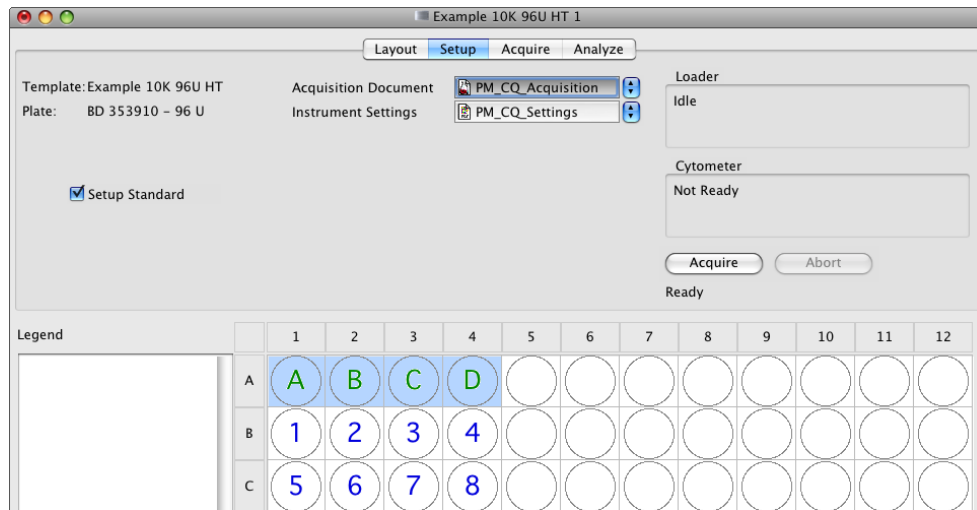
The flow rate selection on the cytometer is ignored when the cytometer is in plate-based acquisition mode. The protocol settings determine the flow rate.



When the HTS unit is set up in a plate acquisition mode, and the RUN button is pressed, the button will remain green, whether or not the system is pressurized. Once BD CellQuest Pro software is started, BD PlateManager software displays the instrument status in the status field in the Setup and the Acquire views.

- 6 Display the Setup view in the BD PlateManager window.
- 7 Select the setup standard wells (A1–A4).

The acquisition document and instrument settings you selected for the setup standard wells are displayed in the upper pane of the Setup view.



- 8 Prime the BD High Throughput Sampler system twice to eliminate any bubbles.
 - a Select Sampler > Prime.
The Sampler menu is inactive during the prime.
 - b When the menu item is available, prime again.
- 9 Click the Acquire button (NOT the Acquire view selector).

The Save PlateManager dialog for saving the BD PlateManager protocol appears.

A saved protocol file contains all the selections made thus far. A protocol file can be opened at a later date to display the experiment setup and reanalyze data, if needed.

When a protocol is saved, BD PlateManager software creates a folder with the same name as the protocol. The following files are saved in the new folder: FCS data files, the protocol file, and copies of the acquisition document, analysis document, and instrument settings file.

- 10** Name the protocol *HTS_Beads* and save it in your student folder.

BD CellQuest Pro software is started and the *PM_CQ_Acquisition* document opens. The following windows are displayed: Acquisition Control, Counters, Detector/Amps, Threshold, Status, and Compensation.

BD CellQuest Pro software acquires the first setup standard well (A1).

- 11** Adjust the FSC and SSC settings to place the singlet beads on scale.

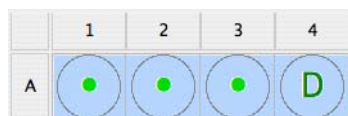
For this optimization, the singlet beads should be placed so that they will be encompassed by the region R1 in the *PM_CQ_Analysis* document

- 12** Adjust the gate to encompass the singlet beads and the threshold, if needed.

- 13** When you have finished making adjustments, you can wait for the acquisition of well A1 to complete, or you can manually advance to the next setup well. To manually advance:

- a Click the BD PlateManager icon on the Dock to make it the current application.
- b Click Pause—wait for the system to pause.
- c To resume acquisition for the next well, click Acquire.

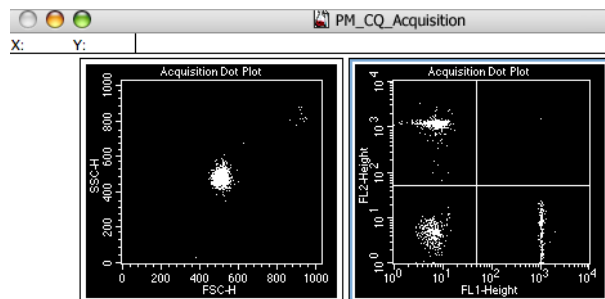
After each well is acquired, a green dot appears in the well to indicate that the well has been sampled.



TIP To reacquire a well, select an acquired well with a green dot and then select the Setup Standard checkbox. The well is assigned a letter indicating its acquisition order.

IMPORTANT Do not click Abort unless there is a problem with the loader during acquisition. If you click Abort during well acquisition, the aspirated sample will go straight to waste.

- 14** Use wells A2 to A4 to adjust compensation for the single-positive bead populations.



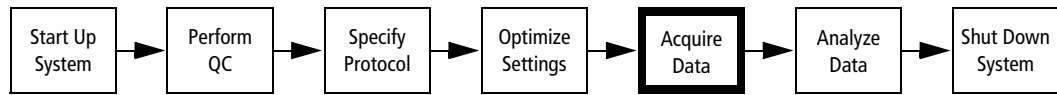
Saving Instrument Settings

After instrument settings are optimized, the changes should be saved to use during acquisition of sample wells.

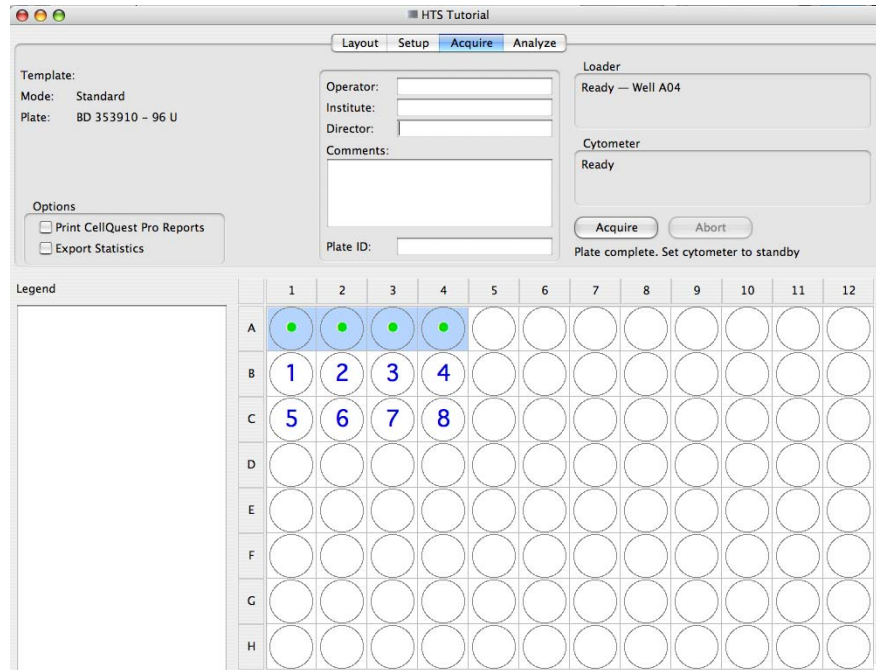
- 1** In BD CellQuest Pro software, click Pause and Abort in the Acquisition Control dialog.
- 2** Select Cytometer > Instrument Settings.
- 3** The Instrument Settings dialog appears.
- 4** Click Save.
- 5** Navigate to the HTS_Beads protocol folder in your student folder and overwrite the PM_CQ_Settings file with the adjusted instrument settings.
- 6** If needed, complete acquisition by clicking Pause in BD PlateManager software.

Acquiring Data

In this section, you will acquire data for the sample wells.



- 1 Display the Acquire view in the BD PlateManager window.



- 2 Type your name in the Operator field.
The default value is the user name of the account logged into the computer.
- 3 (Optional) Type entries in the Institute and the Director fields.
Information in these fields defaults to the most recent entries.
- 4 (Optional) Enter an identifier in the Plate ID field.
In this field, you can enter a unique tracking ID for your plate.
- 5 Verify that the Print CellQuest Pro report and Export Statistics checkboxes are cleared.

TIP If the Print CellQuest Pro report function is selected in the Acquire view, a BD CellQuest Pro document will be printed for each acquired well. This means if you




acquire a full plate, at least 96 pages will be printed. When using Print CellQuest Pro report or the Export Statistics during acquisition, ensure that the plots in the acquisition document are Acq -> Analysis plots.

- 6 Verify that the multiwell plate is properly installed in the HTS unit and the safety cover is on.
- 7 Click Acquire.

BD CellQuest Pro software launches and the acquisition document is displayed. Acquisition for the first sample well begins.

TIP The Acquisition Control is disabled in BD CellQuest Pro software. If you need to pause the run, click the BD PlateManager icon on the Dock to make it the current application, and click Pause in BD PlateManager software. When paused, acquisition of the current well completes. Click Continue to resume the run at the next well, or click Abort to stop the run.

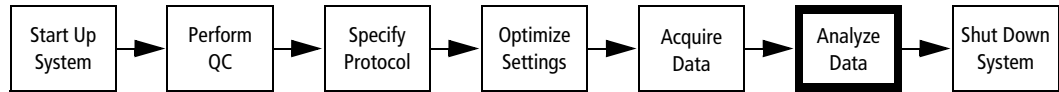
To monitor the acquisition status of the wells, click the BD PlateManager software icon in the dock to make it the current application. During acquisition, wells are labeled according to their status:

Well	Status
	injecting
	acquired
	failed

- 8 When acquisition of all the wells is complete, remove the plate from the HTS, and place the cytometer in STNDBY mode.

Analyzing Data

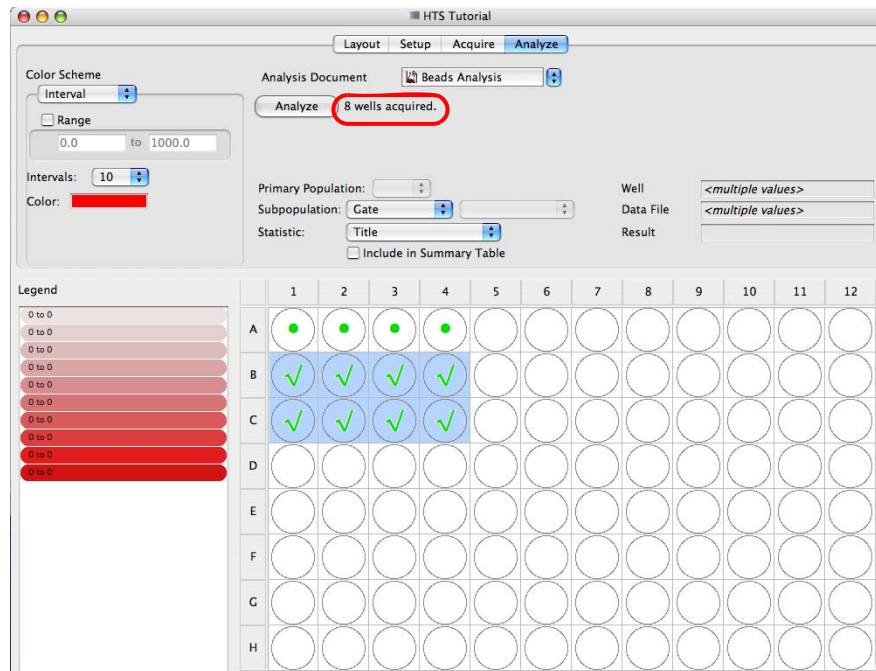
In this section, you will perform a batch analysis of the data files and view the results in a color-coded display. You will also generate a summary table that contains the results for the acquired wells. The summary table can be exported and viewed in third party spreadsheet applications such as Microsoft Excel.



Performing Batch Analysis of Data Files

- 1 Display the Analyze view in the BD PlateManager window.

All the acquired sample wells display green check marks. Below the analysis document field, the software displays the number of wells that have been acquired.



- 2 Select File > Save.

The updated protocol is saved in your student folder. The saved protocol includes links to the data files for each well. As long as the data files remain in the protocol folder, the protocol file can be reopened at a later date and the data reanalyzed.

- 3 Select the acquired sample wells.

- 4 Verify that the correct analysis document is specified in the Analysis Document field.
- 5 Click Analyze to begin the batch analysis.

The analysis document opens in BD CellQuest Pro software. All selected sample well data files are analyzed.

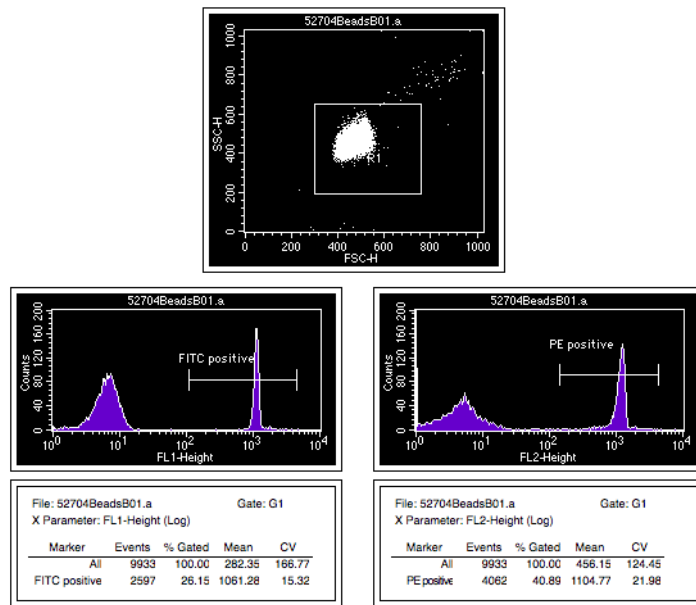
Checking Analysis Results

- 1 Double-click the B1 well in the plate layout of the Analysis view in BD PlateManager software.

BD CellQuest Pro software displays the data file for this well in the analysis document.

- 2 Modify the gate region and the histogram markers, if needed.

TIP When analyzing multiple data files containing similar populations, Snap-To regions can be used to automate population analysis from file to file. See Using Snap-To Regions on page 150.



TIP You can also change the statistic views or display new plots.

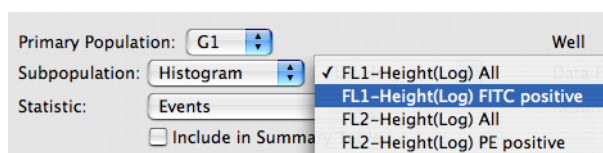
- 3 Select File > Save Document to save the modified analysis document.
- 4 Repeat steps 1 to 3 with several acquired sample wells.
- 5 When you are satisfied with your revised analysis document, repeat the batch analysis.

Displaying Color-Coded Results

With BD PlateManager software, you can display analysis results in a color-coded plate view. You select the statistics to display and specify the color scheme. A legend to the left of the plate view displays a key for the color-coded interval values.

In this section, you will specify to display the fluorescence means for the populations defined by the FITC- and PE-positive markers in the analysis document.

- 1 Select all acquired sample wells in the plate layout.
- 2 Click the Primary Population menu control and select G1.
- 3 In the Subpopulation fields, select Histogram and FL1-Height(Log) FITC positive.

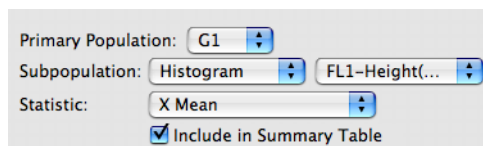


- 4 In the Statistic field, select X Mean.

The mean fluorescence intensity for the FITC-positive population is displayed in a color-coded plate view. By default, the results are subdivided into 10 intervals.

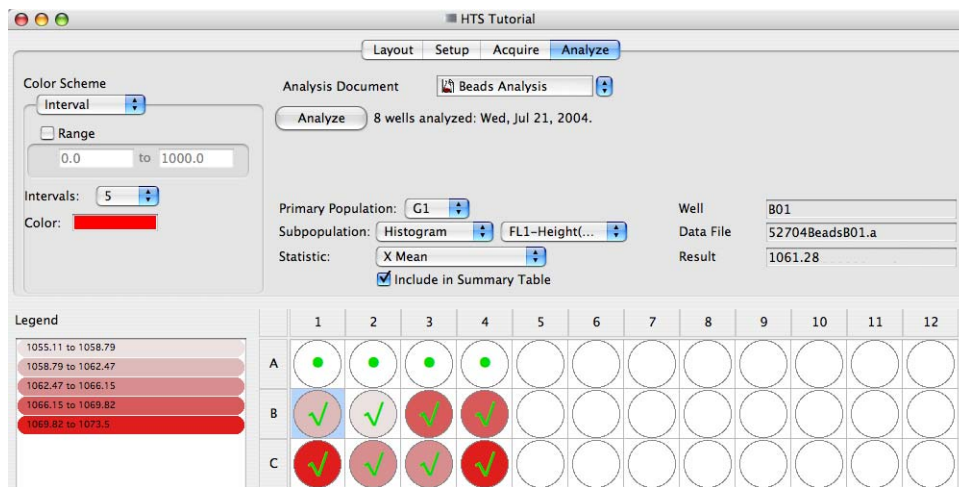
- 5 Select the Include in Summary Table checkbox.

The results for the FITC-positive marker are added to the Export Summary Table.



- 6 In the Intervals field, select 5.

The result range is redistributed into 5 intervals and the well results are updated.



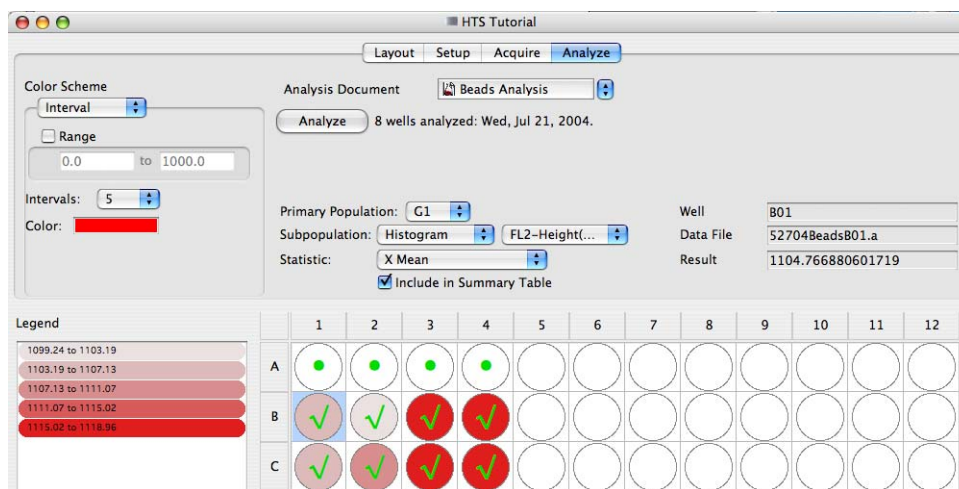
- 7 Click well B1.

Information for well B1 is displayed in the Well, Data File, and Results fields.

Well	B01
Data File	52704BeadsB01.a
Result	1061.28

- 8 Check results for several other wells.
- 9 In the right Subpopulation field, select FL2-Height(Log) PE positive.

The mean fluorescence intensity for the PE-positive population is now displayed.



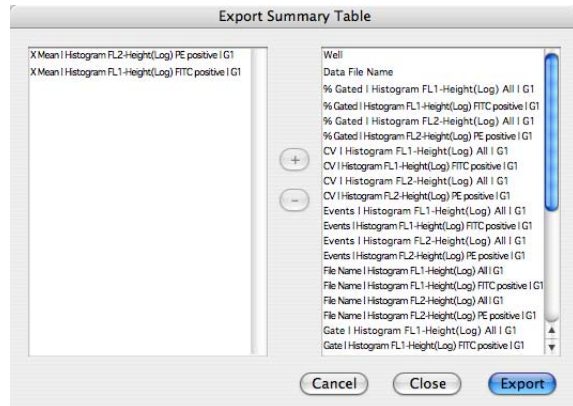
- 10 Select File > Print to print the current plate view and legend.

Exporting a Summary Table

In this section, you will generate a summary table containing results for all the acquired wells and view this file in Microsoft Excel software.

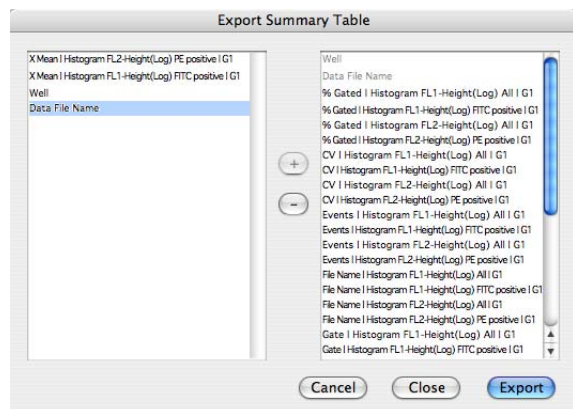
- 1 Select File > Export Summary Table.

The export summary table dialog appears with the X mean for the FITC- and PE-positive markers already selected.



The right column in the dialog contains all the fields available for export. The left column displays the fields selected for export.

- 2 Select additional file and statistics information to be exported to the summary table.
 - a In the right column, select Well and then click the plus sign (+).
 - b In the right column, select Data File Name and then click the plus sign (+).



- 3 Click Export to generate the Export Summary Table.
- 4 Name the file *Export_Table* and save it in the HTS_Beads protocol folder.

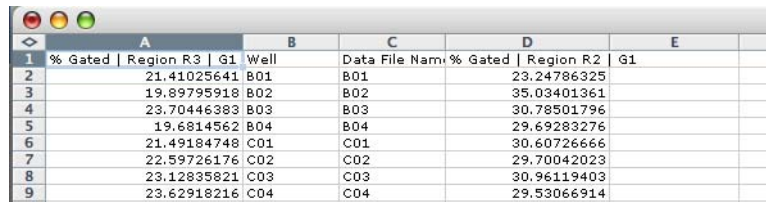
- 5 Select File > Save.

The updated protocol document is saved in your student folder.

- 6 Select PlateManager > Quit PlateManager.

- 7 Navigate to the exported file and double-click the file icon to open the file.

The summary file opens in Microsoft Excel software.



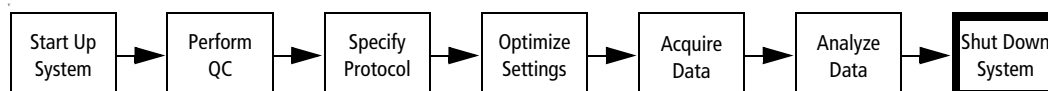
	A	B	C	D	E
1	% Gated Region R3 G1	Well	Data File Name	% Gated Region R2	G1
2	21.41025641	B01	B01	23.24786325	
3	19.89795918	B02	B02	35.03401361	
4	23.70446383	B03	B03	30.78501796	
5	19.6814562	B04	B04	29.69283276	
6	21.49184748	C01	C01	30.60726666	
7	22.59726176	C02	C02	29.70042023	
8	23.12835821	C03	C03	30.96119403	
9	23.62918216	C04	C04	29.53066914	

- 8 Select Excel > Quit Excel to close the spreadsheet.

Shutting Down

At the end of each day, run the daily cleaning procedure to clean and properly maintain the system. The daily cleaning procedure should also be performed after running the following:

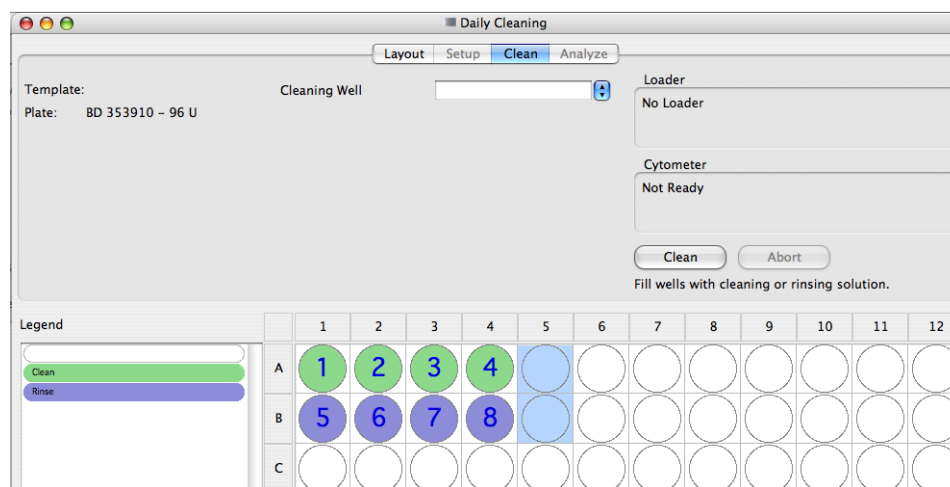
- Viscous samples
- Dyes such as propidium iodide, acridine orange, and thiazole orange
- Potentially biohazardous samples



Daily Cleaning

During the daily clean, the instrument dispenses cleaning solution from predefined wells and performs a sequence of mixing, aspiration, and rinsing. A daily cleaning protocol installed with the software guides you through the procedure. Allow 15 minutes to complete the cleaning procedure.

- 1 If needed, set up the cytometer for plate-based acquisition (see Setting Up for Plate-Based Acquisition on page 298) and start BD PlateManager software.
- 2 Select Sampler > Daily Cleaning to display the daily cleaning protocol.



Wells A1 to A4 are designated for cleaning, and wells B1 to B4 are designated for rinsing.

TIP If needed, you can change the location of the cleaning and rinsing wells. Refer to the *BD High Throughput Sampler User's Guide* for details.

- 3 Verify that the specified plate type matches the plate type that you will use for cleaning.

TIP To change the plate type, display the Layout view and select the desired plate type in the Plate field. Return to the Clean view.

IMPORTANT Sampler Parameters in the Daily Clean protocol have been optimized for the instrument cleaning procedure. Changing these parameters may decrease the effectiveness of cleaning.

- 4 Fill wells A1 to A4 of a 96-well microtiter plate with a 10% bleach solution.
- 5 Fill wells B1 to B4 with DI water.
- 6 Remove the HTS safety cover and place the plate on the plate holder.

Verify that the plate is oriented with well A1 on the back-right corner of the plate holder.

- 7 Replace the safety cover.
- 8 Place the cytometer in Run mode, and click Clean.

BD PlateManager software launches BD CellQuest Pro software to check the cytometer status. The instrument performs a homing sequence, and cleaning begins. When the cleaning cycle is complete, a completion message appears on the Clean view.



- 9 Remove and discard the microtiter plate, or save it and use the unused wells.
- 10 Select Sampler > Prime.

Priming the BD High Throughput Sampler system at the end of the day prevents crystals from forming in the syringes.

The Sampler menu becomes inactive during the prime. After the priming is completed, the Sampler menu becomes active.

- 11 Verify that the priming is completed (the Sampler menu is active) and place the cytometer in STNDBY mode.
- 12 BD FACsFlow Supply System users should turn off the power to the BD FACsFlow Supply System.
- 13 Turn off power to the HTS unit and the cytometer.

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Data Management

After completing this chapter, you will be able to:

- Identify elements of the BD FACStation file structure
- Organize your documents and data files
- Lock files and create stationery pads
- Archive and back up important documents and data files
- Use and maintain external storage devices

Data Management

337452 Rev. A

File Types

Data files



Norm.001

Export stats files



untitled stats



090403.exp

Report files



Alice Wonderland01.job



Alice Wonderland01.pdf

Document files



untitled



010403.mxl

Instrument Settings files



InstrSettings

2



File Organization

Data can be organized by:

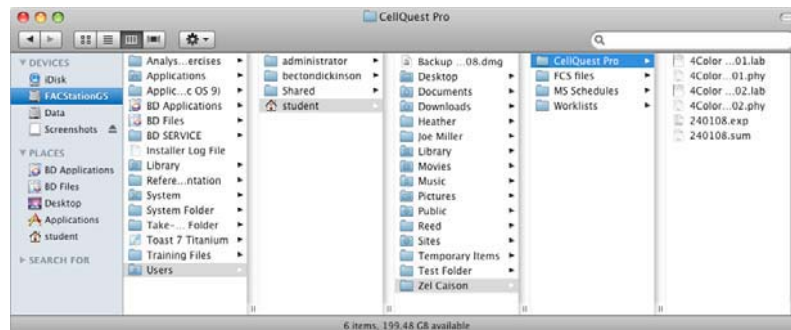
- Topic
- Date
- BD Files
- Users—OSX Home Directory

3



Data Management

Files Organized by OSX Home Directory



4



Protecting Data: Archiving

- Long-term storage of data
- Example method:

At the end of each day, copy all of the Experiment documents and FCS data files created that day to an external storage device OR burn the files to CD.

5



Protecting Data: Backup

- Safeguard against loss or damage.
- Automated method example:
Time Machine (Mac OS X v10.5 Leopard).
- Manual method example:

At the end of each week, create one or more disk images of all the data on your hard drive, save the images to an external storage device, then delete the previous week's backup.

6

BD FACStation File Structure

In this section, you will learn the types of files of the data management system and how to organize them.

Types of BD FACStation Files

Following is a list of types of files and documents along with examples of icons you might see as you use the BD FACStation™ data management system.

Data Files



A data file consists of unprocessed data. It contains all measured parameters for each particle (event) in a sample, as well as information describing the sample. Data files created by BD FACStation software are in a Flow Cytometry Standard (FCS) 2.0 format. Your BD FACStation software applications can analyze FCS 2.0 files.

Data files contain instrument settings used at the time data was collected. You can retrieve instrument settings from FCS files and use them to collect data, if needed.

NOTE If you have FCS 1.0 files, you can convert to FCS 2.0 using BD FACSCConvert software. Refer to the *BD FACSCConvert Software User's Guide* for details.

Export Stats Files



An export statistics file is a text file used to transfer statistical results to other applications such as spreadsheet programs or database programs. At the left are examples of two export statistics file icons, one from BD CellQuest Pro software and one from BD Multiset software.



Report Files



Alice Wonderland01.lab



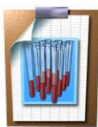
Alice Wonderland01.phy

A report file contains the results of a single test or group of tests. Typically, report files for a single test are PDF files. At the left are examples of two report file icons (Laboratory and Physician Reports) from BD Multiset software.

Document Files



untitled



010403.sch

Documents contain information created while using a software application. The type of information stored in documents can include plot formats, page layout, statistical markers, and acquisition setup options.

Once saved, documents can be opened and used again at a later time. This saves the user the time it takes to set up software options each time the program is used. The figures on the left show an example of a BD CellQuest Pro software Experiment document icon and a BD Multiset software schedule document icon.

Instrument Settings Files



InstrSettings

An instrument settings file contains the information necessary to set up the cytometer electronics for an application. Saved instrument settings can be recalled and sent to the cytometer. All BD data acquisition software programs can save and open instrument settings files.

Organizing your Files

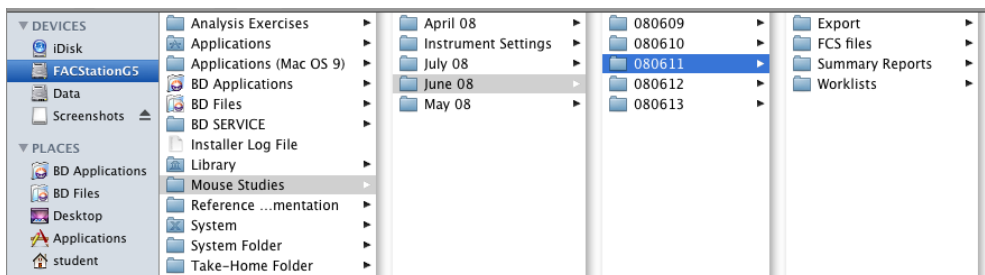
You can choose from several methods to organize your files, including:

- **topic**—research studies or lab applications
- **date**—month and date created
- **BD files**—by application, default for BD software
- **user**—Home directory

Filing by Topic

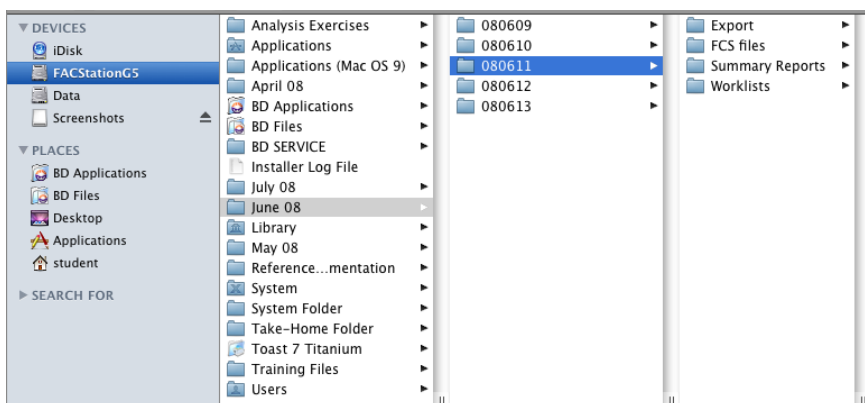
When filing by topic, folders are organized first by study or application, and then by month and date. It is easy to locate a particular study by the unique name given. The month folder can easily be archived by dragging it to a storage device icon.

TIP The example demonstrates the use of the yymmdd format, which is useful for sorting folders by date.



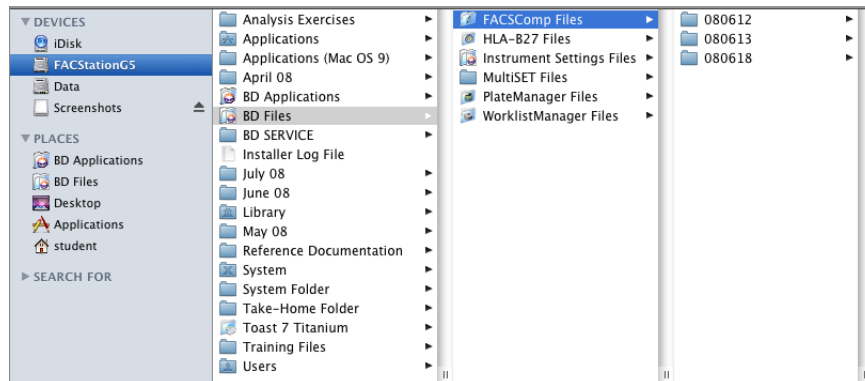
Filing by Date

When filing by date, folders are organized first by first by month, and then by dates within the month. With this system, it is easy to locate a particular experiment by date.



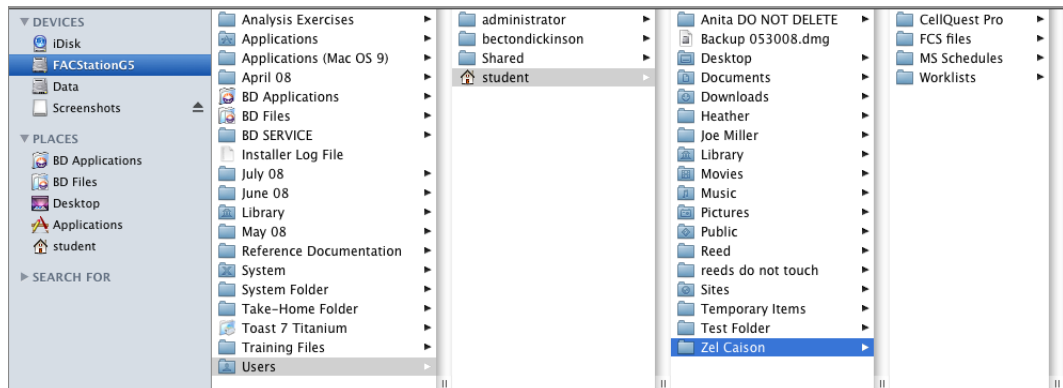
Filing by BD Files

When filing by BD Files, folders are organized first by software application (example: FACSCComp Files), and then by dated folders.



Filing by User

When filing by user, folders are organized first by user account in the Documents folder of the Home directory folder created by the operating system for each user. The documents folder can be further organized by topic, application, or date.



Storage Partitions

Computers provided by BD contain three partitions, FACStation, Data, and FSE Restore. The FACStation partition contains all the files and applications that came with your computer. The Data partition is initially empty. If you organize your data by topics or date, you can save your data on either partition. When you save data using the default BD locations or the Home directory, data is saved to the FACStation partition.

Locking and Using Documents as Templates

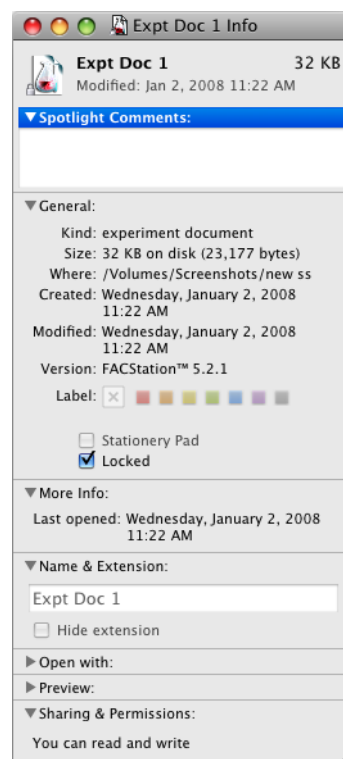
Locking or making a document a stationery pad protects the original document from being modified or deleted. Use this feature if a document is used repeatedly for routine work, but you do not want the original to be modified. You can open and read a locked document but cannot make changes unless you first use the Save As function to save a copy of the original.

When you open a stationery pad, a copy of the original document is opened. You can make changes to the new document, rename, and save it, without changing the stationery pad. Use stationery pads as a master for creating similar documents.

To lock a document or save a document as a stationery pad, do the following.

- 1 Click the icon of the document you want to lock or make a into a stationery pad.
- 2 Select File > Get Info.
- 3 Select the Stationery Pad checkbox to make the document a stationery pad, or select the Locked checkbox to lock the document.
- 4 Close the Info dialog.

The following figure shows standard, locked, and stationery pad BD CellQuest Pro Experiment document icons. A locked document has a padlock to the left of the document icon. A stationery pad icon has a curled lower right corner instead of a curled upper right corner.



Standard Document



Expt Doc 1

Locked Document



Expt Doc 1

Stationery Pad



Expt Doc 1

Protecting Files

For optimal software performance, perform the following steps on a weekly basis, or as needed depending on the volume of data collected. Properly archiving data allows you to quickly retrieve saved experiments and ensures that data is not lost. The following table summarizes the archiving process.

When	Activity	Purpose
Daily	Archive	Creates a copy of documents and data files to be preserved indefinitely. Archived files can be accessed as needed.
Weekly	Backup	Creates a copy of documents and data files as a safeguard against loss or damage. Backup is used to restore documents and data files to the computer.
Monthly	Delete older archived files	Removes non-current documents and data files to free memory for future use.

Creating a Backup Disk Image (Administrators Only)

The following procedure can be used to create a disk image of a data folder.

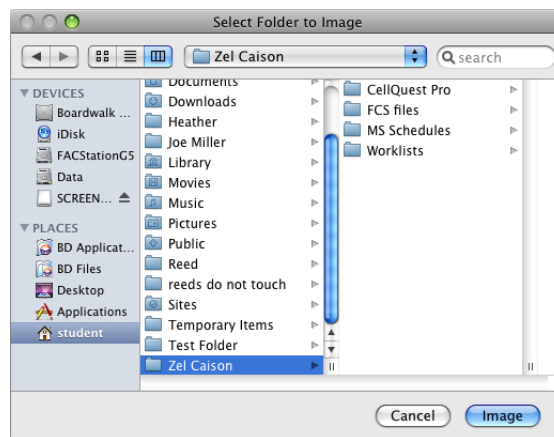
IMPORTANT The instructions in this procedure are for MAC OS 10.5.2. Consult your Disk Utility Help documentation for instructions for creating a disk image on an earlier MAC OS.

- 1 Start the Disk Utility application (Applications/Utilities/Disk Utility).

The Disk Utility workspace appears.

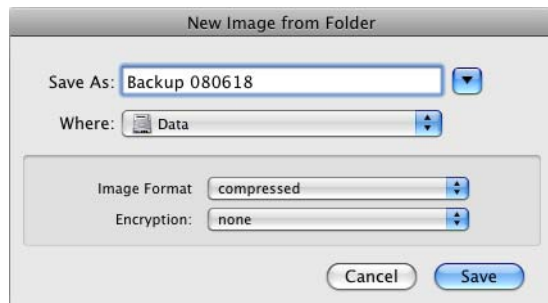
- 2 Select File > New > Disk Image from Folder.

The Select Folder to Image dialog appears.



- 3 Navigate to the data folder, and click Image.

The Select Folder to Image dialog closes and the New Image from Folder dialog appears.



- 4 In the New Image from Folder dialog:
 - a In the Save As field, type a name for the disk image.
 - a Specify a destination for the disk image.
 - b Verify that the image format is set to Compressed.
 - c Verify that Encryption is set to None.
 - d Click Save.

The New Image from Folder dialog closes and a Progress window is displayed while the image is created.

- 5 When the Progress window closes, open a Finder window, navigate to the disk image, and copy it to an external storage device.

Restoring files from a Backup Image

To restore the files contained in a disk image, open the image and copy its files to the desired location(s).

Optional External Storage Devices

External storage devices can provide additional file storage space. Some offer removable media with storage capacity, media longevity, and access speed comparable to that of your computer hard disk.

External Storage Device Tips

Before using your storage device and its media, consult the storage device and media documentation.

- When an operable storage device is properly connected to your computer, an icon is displayed on your desktop. You can access the device memory the same way you access your computer hard drive.
- Some storage devices must be connected and powered on before the computer is powered on.
- Some media must be formatted before they can be used.
- If the power fails while a disk is in the drive, do not attempt to remove the disk until power is restored.



Improperly connecting or disconnecting some storage devices may result in data loss.



Turning the computer off before removing media from some storage devices may result in data loss.



Communication problems resulting in data loss may be encountered when saving directly from an application to external data storage devices.

To avoid data loss, first save the data to your hard drive, and then copy it to your external data storage.

Data Management Review

- 1 You've created a template file that you want to protect from being accidentally overwritten or deleted. What can you do to protect the file?

- 2 A stationery pad can be deleted.

T F

- 3 You want to be able to easily archive all the new files on your computer each day. What method should you use to organize your files?

- 4 You want to be able to easily prevent the other users of your computer from accessing the documents and data files that you create. What method should you use to organize your files?

- 5 How do you make a document a stationery pad?

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Appendix A

Worksheets

Day 1 Review

If you need help answering the following questions, see the appropriate module, as indicated in parentheses.

- 1 What cellular property most influences forward scatter (FSC)? (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)

- 2 What cellular property most influences side scatter (SSC)? (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)

- 3 Why is it necessary to turn on the computer after the cytometer? (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)

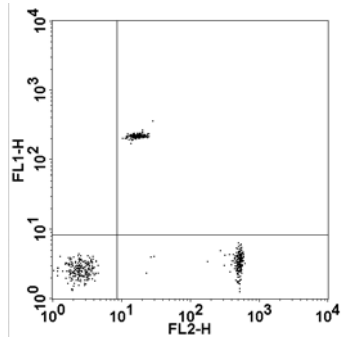
- 4 Describe the daily shutdown and monthly maintenance procedures recommended for proper maintenance of the cytometer. (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)

5 Why is it important to add APC beads to tube A if you are running a 4-color BD FACSCComp assay? (Chapter 3, BD FACSCComp Software)

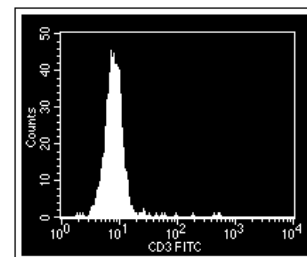
6 List the four functions that BD FACSCComp software performs in the order they are performed. (Chapter 3, BD FACSCComp Software)

7 What wavelengths pass through a 650 LP filter? (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)

8 Which compensation network should be adjusted? (Chapter 2, Instrument Controls in BD CellQuest Pro Software)



9 What instrument adjustment would you make to move the population in the histogram plot to the left? (Chapter 2, Instrument Controls in BD CellQuest Pro Software)

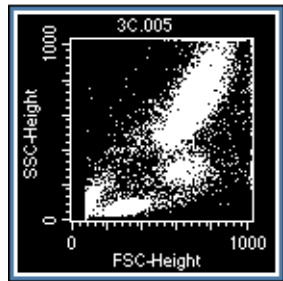


- 10 BD FACSCComp software sets up the cytometer for all applications. (Chapter 3, BD FACSCComp Software)
- T F
- 11 To display very large cells on an FSC plot, you will most likely use the E01 FSC detector setting. (Discussed in class.)
- T F
- 12 An event is processed only when the signal is above the threshold setting. (Chapter 2, Instrument Controls in BD CellQuest Pro Software)
- T F
- 13 For best detection of dim signals, log amplification is preferred. (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)
- T F
- 14 It is best to leave a tube filled with 4 mL of DI water on the SIP. (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)
- T F
- 15 If the support arm is not placed under the sample tube, your sample will be directly aspirated into the waste tank. (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)
- T F
- 16 Why is compensation performed? (Chapter 2, Instrument Controls in BD CellQuest Pro Software)
-
- 17 Why can't BD FACSCComp software set FL4 PMT voltage using unlabeled beads? (Chapter 3, BD FACSCComp Software)
-
-
-

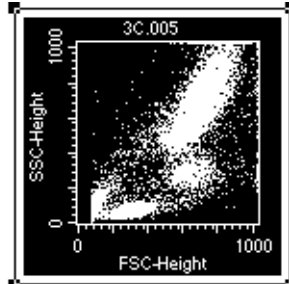
Day 2 Review

See Chapter 4, BD CellQuest Pro Workflow for help answering the following questions.

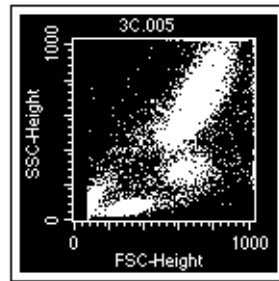
- 1 For each view, select the correct description.



View A



View B



View C

_____ Deselected

_____ Selected

_____ Active

- 2 Name three things you can do with a plot when it is selected.

- 3 List the order in which the following settings should be optimized.

_____ FSC threshold level

_____ Fluorescence detectors

_____ Compensation settings

_____ FSC amplifier and SSC detector

_____ Gate population of interest

- 4 How do you ensure that no data will be stored while optimizing the cytometer?

5 When optimizing the instrument, why is it necessary to set a gate encompassing the population of interest before adjusting the detector voltages?

6 Data files are saved as part of the Experiment document.

T F

7 On an instrument that has been optimized, changing the detector voltages will necessitate a readjustment of compensation.

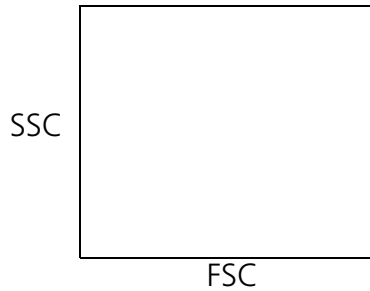
T F

8 To display acquired data in a plot, you should select File > Open Document.

T F

Final Review

- 1 Cell A is 10 microns and is not very complex. Cell B is 30 microns and is complex. Draw a circle to represent cell A and a square to represent cell B in the following plot. (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)



- 2 Why is it necessary to place a tube of DI water on the SIP rather than leaving no tube on the SIP at the end of the day? (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)

- 3 In what folder are the FACSComp Calib File and Calib File.LNW stored? (Chapter 3, BD FACSComp Software)

- 4 While reviewing the BD FACSComp software Summary Report, you notice that FL1 Sensitivity Test failed. Name two resources that are available for you to use to troubleshoot this problem. (Chapter 3, BD FACSComp Software)

- 5 What are you adjusting when you increase or decrease the voltage on a PMT? (Chapter 2, Instrument Controls in BD CellQuest Pro Software)

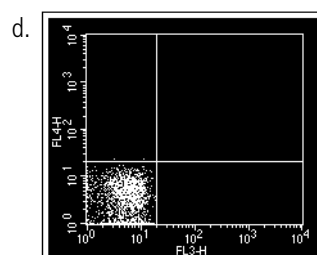
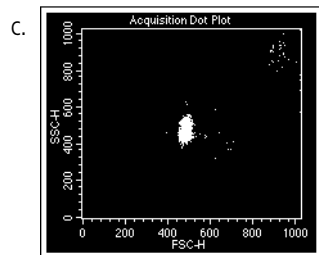
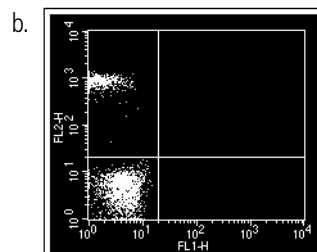
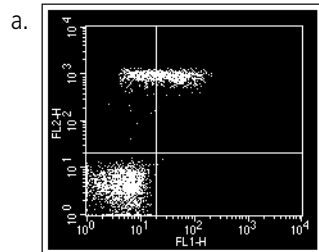
- a Detector
- b Threshold
- c Test Pulse

6 If you are running samples stained with FITC and APC reagents, would you need to adjust compensation? If yes, which compensation networks do you need to adjust? (Chapter 2, Instrument Controls in BD CellQuest Pro Software)

7 You created a template file that you want to protect from being accidentally overwritten or deleted. What can you do to protect the file? (Chapter 11, Data Management)

8 Optimization is a multistep process. List the five basic steps in the order they should be performed. (Chapter 4, BD CellQuest Pro Workflow)

9 You place a tube containing unlabeled and PE-labeled beads on the cytometer. All compensation networks are set to 0.0%. Which of the following plots displays data that you would NOT expect to see? (Chapter 2, Instrument Controls in BD CellQuest Pro Software)



- 10 Which tubes can be used for optimizing compensation? (Chapter 4, BD CellQuest Pro Workflow)
- a A tube containing only one positive population.
 - b A tube containing a double-positive population.
 - c A tube containing two mutually exclusive positive populations.
 - d a and b
 - e a and c
 - f All of the above
- 11 Which software program will monitor the daily performance of your instrument and provide instrument settings for human lymphocyte immunophenotyping?
-
- 12 Which tube can you use to optimize FSC and SSC adjustments? (Chapter 4, BD CellQuest Pro Workflow)
- a CD3/CD8/CD45/CD4
 - b Unstained control
 - c CD3/CD16+CD56/CD45/CD19
 - d Any of the above
- 13 Which type of plot displays only a single parameter? (Chapter 4, BD CellQuest Pro Workflow)
- a Histogram
 - b Dot plot
 - c Contour plot
 - d Density plot
- 14 BD CellQuest Pro software gets the cytometer type and configuration from the BDPAC (Power PC) application. Where is this file located? (Discussed in class.)
-

15 How would you change an FSC vs SSC plot to a FL1 vs FL2 plot in BD CellQuest Pro software? (Chapter 4, BD CellQuest Pro Workflow)

- a use the Edit menu
- b use the File menu
- c click the parameter labels on the plot
- d use the Stats menu

16 For 1-color acquisition, you need to set compensation. (Chapter 2, Instrument Controls in BD CellQuest Pro Software)

T F

17 If the cytometer is having problems communicating with the computer, which would you check? (*Demonstrated in class.*)

- a BDPAC
- b GPIO cable connection
- c a and b

18 When the cytometer is in STNDBY mode, the sample injection tube will drip onto the benchtop when the support arm is to the side of the SIP. (Chapter 1, BD FACSCalibur Cytometer Overview and Operation)

T F

19 To define a logical gate, what menu item would you use? (Chapter 6, BD CellQuest Pro Software Analysis Tools)

20 When acquiring data, why is it not a good idea to accept only the events in a region encompassing a specific population for data storage? (*Discussed in class.*)

BD Multiset Software Questions

Answer the following questions only if you completed the BD Multiset software (with BD CellQuest Pro) exercise.

- 1 What is the purpose of adjusting FL3 threshold?

- 2 Why was the FL3 threshold adjusted, rather than the FSC threshold?

- 3 When using BD Trucount tubes, you must enter information in the WBC and Lymph fields to calculate absolute counts.

T F

Sorting Questions

Answer the following questions only if you completed the *Sorting* exercise.

- 1 When would you use the Recovery sort mode?

- 2 When would you use the Single Cell sort mode?

- 3 To maximize the sort rate in single cell mode, the event rate should be about:

- a 1000 events per second
- b 2000 events per second
- c 5000 events per second
- d 10,000 events per second

- 4 Which flow rate should be used during sorting?
- a LO
 - b LO or MED, depending on the sort mode.
 - c MED
 - d HI
- 5 The maximum number of catcher tube movements is:
- a 150 movements per 0.5 seconds
 - b dependent on the event rate
 - c 10,000 movements per collection tube
 - d 100,000 movements per sort
- 6 Which of the following would you NOT do before sorting:
- a Set the flow rate to LO.
 - b Define a Sort Gate.
 - c Select a Sort Mode.
 - d Measure the sort purity.
- 7 Collection tubes used for collecting cells should be coated with:
- a Sterile PBS
 - b 10% bleach
 - c 4% BSA
- 8 You should first run bleach and then DI water through the sort lines after sorting biohazardous material.

T F

BD FACS Loader Questions

Answer the following questions only if you completed Chapter 9, BD FACS Loader Option.

- 1 To use an instrument settings file in BD WorklistManager software, it must be in the BD Files/Instrument Settings File folder.

T F

2 In BD WorklistManager software, if the Assay Type is CellQuest, the BD CellQuest Pro Experiment document to be used for acquisition should be saved in what folder?

3 Why is it beneficial to use *Event Count or Time* as the stop acquisition criterion in a BD CellQuest Pro Experiment document used with BD WorklistManager software?

4 When making a panel in a BD CellQuest Pro Experiment document to be used in BD WorklistManager software, why is it a good idea to change the tube names from the default names (Tube #)?

5 You can run BD LoaderManager software maintenance and diagnostic procedures while in BD WorklistManager software.

T F

6 BD FACSCComp software can be launched from within BD WorklistManager software.

T F

7 When running more than one rack, bleach and water must be in positions 39 and 40 on each rack.

T F

8 You can add samples to a rack that's already been assigned if first you release the rack.

T F

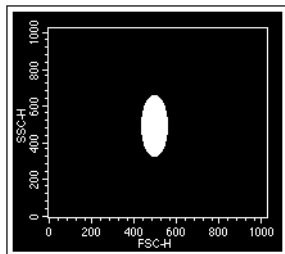
9 Tubes from the same panel can be split between two racks.

T F

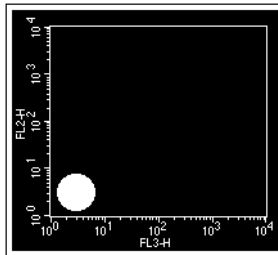
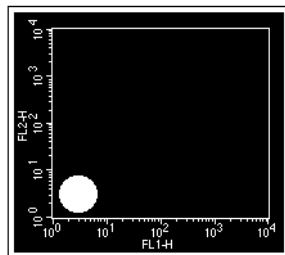
Troubleshooting Exercise

For this exercise, do the following:

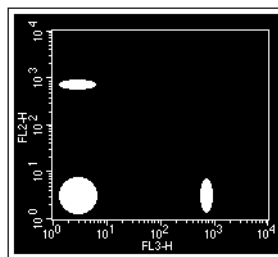
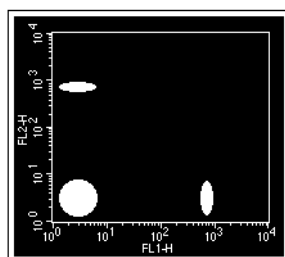
- Do not use Calib settings or copy them from the printout.
 - In the table provided on page 343, list all the problems and solutions you encounter throughout the exercise.
- 1 Adjust the appropriate instrument settings to place the unlabeled BD Calibrite beads in the center of the FCS vs SSC plot.



- 2 Adjust the appropriate instrument settings to place the unlabeled BD Calibrite beads in the lower left corner of the FL1 vs FL2 plot and the FL3 vs FL2 plot.



- 3 Using the mixed beads, adjust the appropriate compensation networks to correct for over- or undercompensation.



Problems	Solutions

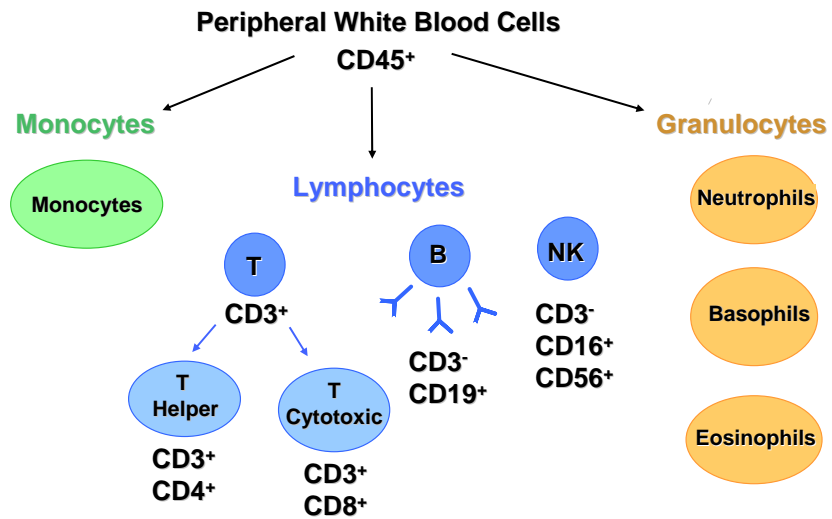
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Appendix B

Lab Practice



Lysed Whole Blood Components



2



Lab Practice Options

- Lyse/Wash (using BD Cellquest Pro software)
 - > with manual tube loading
 - > with automatic tube loading on the Loader
- Lyse/No-Wash (using BD Multiset software)
 - > with manual tube loading
 - > with automatic tube loading on the Loader

3



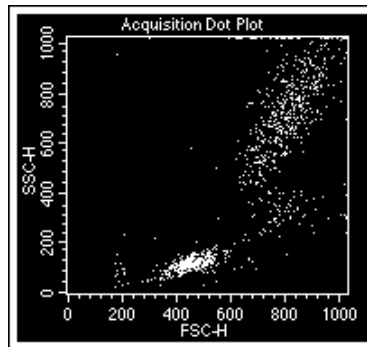
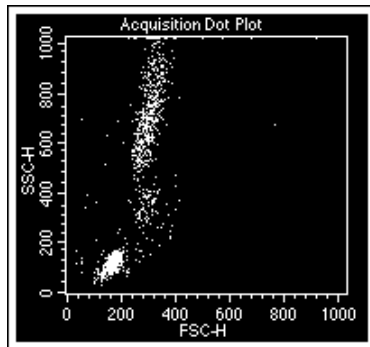
Lyse/Wash Samples

- **Optimization**
 - Unstained control
 - **FITC control (CD3 FITC)**
 - **PE control (CD8 PE)**
 - **PerCP control (CD45 PerCP)**
 - **APC control (CD4 APC)**
- **Acquisition**
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC

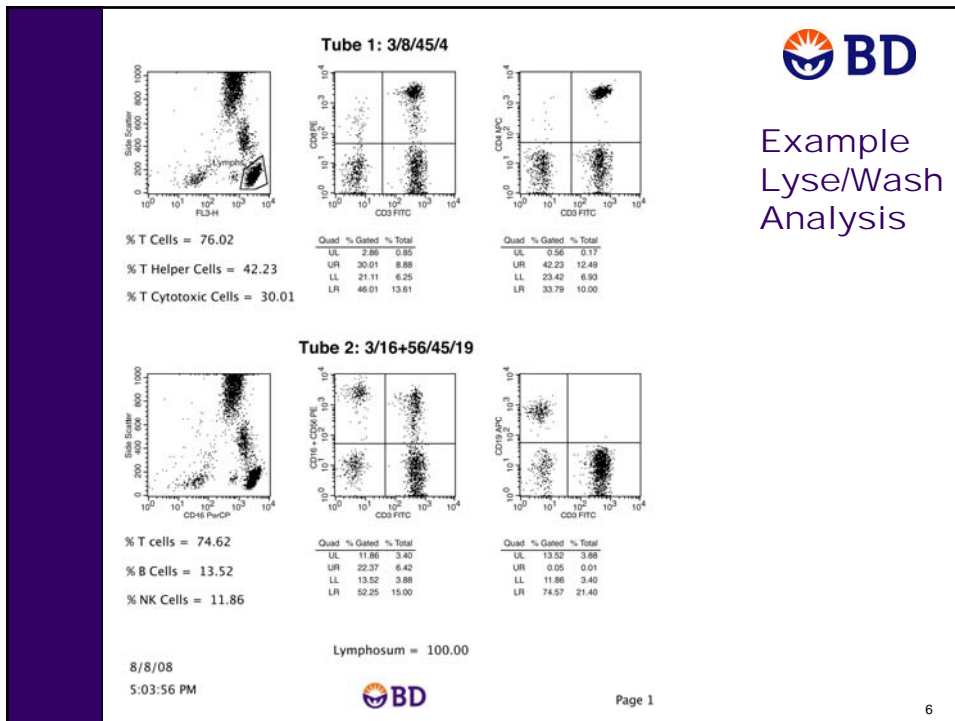
4



Lyse/Wash Scatter Optimization



5



Lyse/No-Wash Samples

All samples are prepared in BD Trucount tubes.

- **BD Multi-check control**
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC
- **BD Multi-check CD4 Low control**
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC
- **Test sample**
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16+56 PE/CD45 PerCP/CD19 APC

7

Lab Practice—Lyse/Wash Manual Tube Loading

In this exercise, you will use the BD FACSCalibur cytometer to collect and analyze 4-color TBNK data from a normal peripheral blood sample.

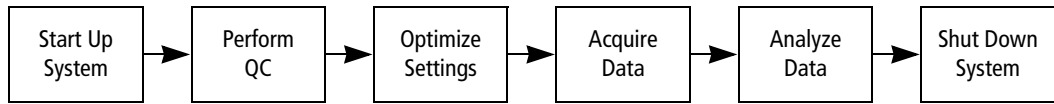
The lab practice is an opportunity for you to practice the skills and apply the knowledge you have obtained thus far. While performing the lab practice you are encouraged to use your training workbook, notes, and any other materials provided to you in class. You can also use any of the files that you have saved in your student folder.

Software

- Use BD FACSComp software to perform QC.
- Use BD CellQuest Pro software for optimization, and acquiring and analyzing data.

Overview

- You will perform startup, QC, optimization, acquisition, analysis, and shutdown.
- You will acquire and analyze two tubes:
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC
- You will be given additional tubes to use for optimizing instrument settings. Do not acquire the optimization tubes.
- The procedure you should use is outlined on the following pages.
- Print your BD FACSComp™ software Summary Report and a BD CellQuest™ Pro software Experiment document for each tube. Record your results on the Analysis Results Worksheet on page 352.
- When you are finished, give the completed worksheet and your printouts to one of the instructors for review.
- You will be allotted approximately two hours to complete this exercise.



Instructions—Lyse/Wash Manual Tube Loading

For more detailed instructions, see the topics referenced at the start of each major section.

See System Startup on page 33.



Steps	Hints
<ol style="list-style-type: none"> 1 Power on. 2 Check sheath. 3 Empty waste. 	

See Performing QC and Instrument Setup on page 71.



Steps	Hints
<ol style="list-style-type: none"> 1 Prepare BD Calibrite beads. 2 Run LW assay in BD FACSCComp software. 3 Print summary report. 	

See Optimizing Settings on page 100.



Steps	Hints
<ol style="list-style-type: none"> 1 Set up Experiment document. 2 Adjust FSC amp gain and SSC detector voltage. 3 Adjust FSC threshold setting. 4 Gate on the population of interest. 5 Adjust fluorescence detector voltages. 6 Adjust fluorescence compensation. 7 Save instrument settings 	<ul style="list-style-type: none"> • Modify your 4-Color TBNK Experiment document for optimizing data. • See FSC vs SSC plot on page 147 for lymphocyte identification.

**Acquire
Data**

See Acquiring Data on page 114, and Using a Reagent Panel on page 143.

Steps	Hints
<ol style="list-style-type: none">1 Specify data file names and location. Save data files to your student folder.2 Specify acquisition and storage settings. Acquire at least 2500 lymphocytes per tube.3 Use your 4-color TBNK panel.4 Acquire. Adjust lymphocyte region on first tube.	<ul style="list-style-type: none">• Use the 4-Color TBNK Experiment document that you created in Using a Reagent Panel on page 143.• Display counters.• Check tube order in browser.• Flow rate set to HI.

**Analyze
Data**

See Analyzing Data on page 122.

Steps	Hints
<ol style="list-style-type: none">1 Create an analysis experiment Document.2 Gate on the lymphocytes.3 Set quadrant markers on fluorescence plots.	<ul style="list-style-type: none">• Show quadrant statistics for fluorescence plots.• Optional: use expression views.

**Shutdown
System**

See System Shutdown on page 36.

Steps	Hints
<ol style="list-style-type: none">1 Clean with 10% bleach.2 Rinse with DI water.3 Power off.	

Analysis Results Worksheet

Record the percentage of lymphocytes for the following populations from your analysis.

CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC Tube

Population	% Lymphocytes
T cells (CD3 ⁺)	
T helper cells (CD3 ⁺ CD4 ⁺)	
T cytotoxic cells (CD3 ⁺ CD8 ⁺)	

CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC Tube

Population	% Lymphocytes
T cells (CD3 ⁺)	
NK cells (CD3 ⁻ , CD16 ⁺ + CD56 ⁺)	
B cells (CD3 ⁻ CD19 ⁺)	

Record the lymphosum = sum of the percentages of lymphocytes for T cells, NK cells, and B cells.

Lymphosum _____

Lab Practice—Lyse/Wash Automatic Tube Loading

In this exercise, you will use the BD FACSCalibur cytometer with the Loader to collect and analyze 4-color TBNK data from a normal peripheral blood sample.

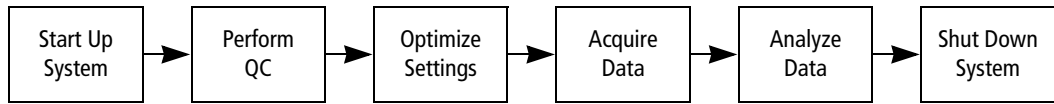
The lab practice is an opportunity for you to practice the skills and apply the knowledge you have obtained thus far. While performing the lab practice you are encouraged to use your training workbook, notes, and any other materials provided to you in class. You can also use any of the files that you have saved in your student folder.

Software

- Use BD FACSComp software to perform QC.
- Use BD CellQuest Pro software for optimization, and prepare or reuse an existing BD CellQuest Pro Acquisition and Analysis Experiment document for acquiring and analyzing data.
- Use BD WorklistManager software to create a worklist and run samples.

Overview

- You will perform startup, QC, optimization, acquisition, analysis, and shutdown.
- You will acquire and analyze two tubes:
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC
- You will be given additional tubes to use for optimizing instrument settings. Do not acquire the optimization tubes.
- The procedure you should use is outlined on the following pages.
- Print your BD FACSComp™ software Summary Report and a BD CellQuest™ Pro software Experiment document for each tube. Record your results on the Analysis Results Worksheet on page 356.
- When you are finished, give the completed worksheet and your printouts to one of the instructors for review.
- You will be allotted approximately two hours to complete this exercise.



Instructions—Lyse/Wash Automatic Tube Loading

For more detailed instructions, see the topics referenced at the start of each major section.

See System Startup on page 33.

Start Up System

Steps

- 1 Power on.
- 2 Check sheath.
- 3 Empty waste.

Hints

See Performing QC and Instrument Setup on page 71.

Perform QC

Steps

- 1 Prepare BD Calibrite beads.
- 2 Run LW assay with the Loader in BD FACSCComp software.
- 3 Print summary report.

Hints

See Optimization on page 207, Preparing a BD CellQuest Pro Experiment Document on page 268, and Using a Reagent Panel on page 143.

Optimize Settings

Steps

- 1 Prepare a BD CellQuest Pro Experiment document to use with BD WorklistManager software.
Acquire at least 2500 lymphocytes per tube.
- 2 Adjust FSC amp gain and SSC detector voltage.
- 3 Adjust FSC threshold setting.
- 4 Gate on the population of interest.
- 5 Adjust FL1, FL2, FL3, and FL4 detector voltages.
- 6 Adjust fluorescence compensation.
- 7 Save instrument settings

Hints

- Modify your 4-Color TBNK Experiment document for optimizing data.
- Use your 4-color TBNK reagent panel.
- Use the Loader in manual mode for optimization.
- Remember to save your modified Experiment doc in:
BD Applications/WorklistManager Folder/CellQuest Experiments Folder.
- Save your optimized instrument settings in BD Files/Instrument Settings Files folder

Acquire
Data

See Acquiring Data on page 271.

Steps	Hints
<ol style="list-style-type: none">1 Enter information in BD WorklistManager Sign In and Set Up views. Be sure to save data files.2 Enter Assay Settings. Link instrument settings to panel. Specify mix settings.3 Enter sample information.4 Assign and load racks.5 Run samples.	<ul style="list-style-type: none">• Print rack manifest.

Analyze
Data

See Analyzing Data on page 122.

Steps	Hints
<ol style="list-style-type: none">1 Create an analysis experiment Document.2 Gate on the lymphocytes.3 Set quadrant markers on fluorescence plots.	<ul style="list-style-type: none">• Show quadrant statistics for fluorescence plots.• Optional: use expression views.

Shutdown
System

See System Shutdown on page 36.

Steps	Hints
<ol style="list-style-type: none">1 Clean with 10% bleach.2 Rinse with DI water.3 Power off.	<ul style="list-style-type: none">• Clean and rinse is done by automatic long clean

Analysis Results Worksheet

Record the percentage of lymphocytes for the following populations from your analysis.

CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC Tube

Population	% Lymphocytes
T cells (CD3 ⁺)	
T helper cells (CD3 ⁺ CD4 ⁺)	
T cytotoxic cells (CD3 ⁺ CD8 ⁺)	

CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC Tube

Population	% Lymphocytes
T cells (CD3 ⁺)	
NK cells (CD3 ⁻ , CD16 ⁺ + CD56 ⁺)	
B cells (CD3 ⁻ CD19 ⁺)	

Record the lymphosum = sum of the percentages of lymphocytes for T cells, NK cells, and B cells.

Lymphosum _____

Lab Practice—Lyse/No-Wash Manual Tube Loading

In this exercise, you will use the BD FACSCalibur cytometer to collect and analyze 4-color TBNK data from a normal peripheral blood sample.

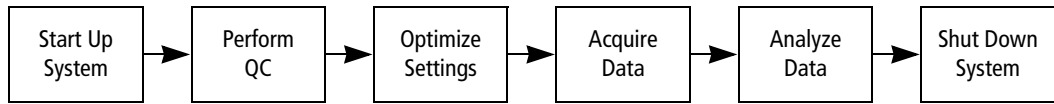
The lab practice is an opportunity for you to practice the skills and apply the knowledge you have obtained thus far. While performing the lab practice you are encouraged to use your training workbook, notes, and any other materials provided to you in class. You can also use any of the files that you have saved in your student folder.

Software

- Use BD FACSComp software to perform QC.
- Use BD Multiset software for optimization, and acquiring and analyzing data.

Overview

- You will perform startup, QC, optimization, acquisition, analysis, and shutdown.
- You will acquire and analyze three samples: BD Multi-check normal and low controls, and a normal peripheral blood sample. Each sample will be run with the following two reagent combinations in BD Trucount tubes for a total of six tubes.
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC
- You will use one of the BD Multi-check control tubes for optimization.
- The procedure you should use is outlined on the following pages.
- Print your BD Multiset software lab report, physician report, and summary reports for each tube and record the results on the Analysis Results Worksheet on page 360.
- When you are finished, give the completed worksheet and your printouts to one of the instructors for review.
- You will be allotted approximately two hours to complete this exercise.



Instructions—Lyse/No-Wash Manual Tube Loading

For more detailed instructions, see the topics referenced at the start of each major section.

See System Startup on page 33.

Start Up
System

Steps

Hints

- 1 Power on.
- 2 Check sheath.
- 3 Empty waste.

See Performing QC and Instrument Setup on page 71.

Perform
QC

Steps

Hints

- 1 Prepare BD Calibrite beads.
- 2 Run LNW assay in BD FACSCComp software.
- 3 Print summary report.

See Preparing to Run Samples on page 199 and Optimization on page 207.

Optimize
Settings

Steps

Hints

- 1 Enter information in BD Multiset Sign In and Set Up views.
Verify Automatic Printing Options preference settings.
Verify absolute count Lot ID and Beads/Pellet setting.
- 2 Enter sample information.
- 3 Load instrument settings.
- 4 Load first tube.
- 5 Run tests.
- 6 Optimize settings on first tube.
Adjust SSC PMT voltage.
Adjust FL3 threshold.

- Verify that tubes are loaded in the correct order.

Acquire
& Analyze
Data

See Acquisition and Analysis on page 209.

Steps	Hints
<ol style="list-style-type: none">1 Acquire first tube.2 Load and acquire remaining tubes.3 Review reports.	<ul style="list-style-type: none">• Verify that tubes are loaded in the correct order.

Shutdown
System

See System Shutdown on page 36.

Steps	Hints
<ol style="list-style-type: none">1 Clean with 10% bleach.2 Rinse with DI water.3 Power off.	

Analysis Results Worksheet

Record the percentage of lymphocytes and the absolute counts for the following populations from your physicians report.

CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC Tube

Population	% Lymphocytes	Absolute Count
T cells (%CD3 ⁺)		
T helper cells (%CD3 ⁺ CD4 ⁺)		
T cytotoxic cells (%CD3 ⁺ CD8 ⁺)		

CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC Tube

Population	% Lymphocytes	Absolute Count
T cells (%CD3 ⁺)		
NK cells (%CD3 ⁻ , CD16 ⁺ + CD56 ⁺)		
B cells (%CD3 ⁻ CD19 ⁺)		

Record the lymphosum = sum of the percentages of lymphocytes for T cells, NK cells, and B cells.

Lymphosum _____

Lab Practice—Lyse/No-Wash Automatic Tube Loading

In this exercise, you will use the BD FACSCalibur cytometer with the Loader to collect and analyze 4-color TBNK data from a normal peripheral blood sample.

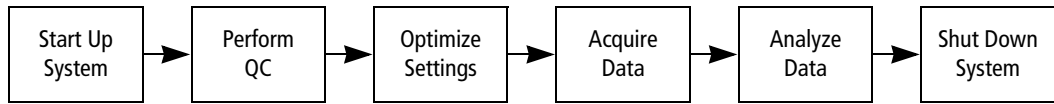
The lab practice is an opportunity for you to practice the skills and apply the knowledge you have obtained thus far. While performing the lab practice you are encouraged to use your training workbook, notes, and any other materials provided to you in class. You can also use any of the files that you have saved in your student folder.

Software

- Use BD FACSComp software to perform QC.
- Prepare or reuse an existing BD Multiset schedule (document) template to specify application specific settings needed for acquiring and analyzing data. You will use BD Multiset software for optimization.
- Use BD WorklistManager software to create a worklist and run samples.

Overview

- You will perform startup, QC, optimization, acquisition, analysis, and shutdown.
- You will acquire and analyze three samples: BD Multi-check normal and low controls and a normal peripheral blood sample. Each sample will be run with the following two reagent combinations in BD Trucount tubes for a total of six tubes.
 - CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC
 - CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC
- You will use one of the BD Multi-check control tubes for optimization.
- The procedure you should use is outlined on the following pages.
- Print your BD Multiset software lab report, physician report, and summary reports for each tube and record the results on the Analysis Results Worksheet on page 364.
- When you are finished, give the completed worksheet and your printouts to one of the instructors for review.
- You will be allotted approximately two hours to complete this exercise.



Instructions—Lyse/No-Wash Automatic Tube Loading

For more detailed instructions, see the topics referenced at the start of each major section.

See System Startup on page 33.



Steps	Hints
1 Power on.	
2 Check sheath.	
3 Empty waste.	

See Performing QC and Instrument Setup on page 71.



Steps	Hints
1 Prepare BD Calibrite beads.	
2 Run LNW assay with the Loader in BD FACSCComp software.	
3 Print summary report.	

Optimize
Settings,
Acquire
& Analyze
Data

See Preparing a BD Multiset Schedule Template on page 228 and Optimizing Settings and Acquiring and Analyzing Data on page 231.

Steps	Hints
<ol style="list-style-type: none"> 1 Prepare a BD Multiset schedule document to use with BD WorklistManager software. Verify Automatic Printing Options preference settings. Verify absolute count Lot ID and Beads/Pellet setting. 2 Enter information in BD WorklistManager Sign In and Set Up views. 3 Enter Assay Settings. Link instrument settings to panel. Specify mix settings. 4 Enter sample information. 5 Assign and load racks. 6 Run samples. 7 Pause at first tube to optimize settings. Adjust SSC PMT voltage. Adjust FL3 threshold. 8 Acquire samples. 9 Review reports. 	<ul style="list-style-type: none"> • Use the MultiSET.WM.sch schedule document you saved in your student folder. • To optimize settings, you must pause the first tube during the 5-second countdown before acquisition begins.

Shutdown
System

For detailed instructions, see System Shutdown on page 36.

Steps	Hints
<ol style="list-style-type: none"> 1 Clean with 10% bleach. 2 Rinse with DI water. 3 Power off. 	<ul style="list-style-type: none"> • Clean and rinse is done by automatic long clean

Analysis Results Worksheet

Record the percentage of lymphocytes and the absolute counts for the following populations from your physicians report.

CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC Tube

Population	% Lymphocytes	Absolute Count
T cells (%CD3 ⁺)		
T helper cells (%CD3 ⁺ CD4 ⁺)		
T cytotoxic cells (%CD3 ⁺ CD8 ⁺)		

CD3 FITC/CD16 + 56 PE/CD45 PerCP/ CD19 APC Tube

Population	% Lymphocytes	Absolute Count
T cells (%CD3 ⁺)		
NK cells (%CD3 ⁻ , CD16 ⁺ + CD56 ⁺)		
B cells (%CD3 ⁻ CD19 ⁺)		

Record the lymphosum = sum of the percentages of lymphocytes for T cells, NK cells, and B cells.

Lymphosum _____

Appendix C

BD CellQuest Pro Reference

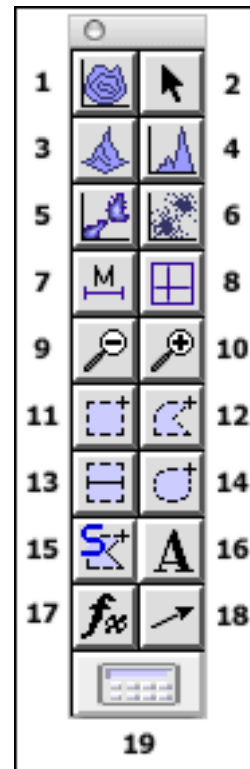
The following topics are discussed in this appendix:

- BD CellQuest Pro software tool palette
- BD CellQuest Pro software files
- BD CellQuest Pro software instrument controls
- BD CellQuest Pro software views

BD CellQuest Pro Software Tool Palette

The Tool Palette allows you to create plots, regions, markers, and text. Tools are selected by clicking them. The tool becomes highlighted and is functional.

- 1 Contour-Plot tool**—Select the tool, click in an unused section of the Experiment document, and drag diagonally until the plot outline is the desired size.
- 2 Selection tool**—Select the tool, select one or more items in the Experiment document, and resize or move the items.
- 3 3D-Plot tool**—Select the tool, click in an unused section of the Experiment document, and drag diagonally until the plot outline is the desired size.
- 4 Histogram-Plot tool**—Select the tool, click in an unused section of the Experiment document, and drag diagonally until the plot outline is the desired size.
- 5 Density-Plot tool**—Select the tool, click in an unused section of the Experiment document, and drag diagonally until the plot outline is the desired size.
- 6 Dot-Plot tool**—Select the tool, click in an unused section of the Experiment document, and drag diagonally until the plot outline is the desired size.
- 7 Histogram Marker tool**—Select the tool, click in a histogram plot to position the left edge of the marker, and drag to position the right edge.
- 8 Quadrant Marker tool**—Select the tool, click in a dot plot, density plot, or contour plot, and drag to set the markers.
- 9 Zoom-Out tool**—Select the tool, then click the plot. Returns a zoomed-in plot to normal size.
- 10 Zoom-In tool**—Select the tool, then click and drag diagonally to designate the area of the plot you want to enlarge.
- 11 Rectangular-Region tool**—Select the tool, click in a dot plot, density plot, or contour plot, and drag diagonally until the region outline is the desired size.
- 12 Polygonal-Region tool**—Select the tool, click in a dot plot, density plot, or contour plot to establish the starting point, move and click the cursor to make the vertices of the polygon, and click the first vertex to complete the region.
- 13 Histogram-Region tool**—Select the tool, click in a histogram plot to position the left edge of the region and drag to position the right edge.



- 14 Elliptical-Region tool**—Select the tool, click in a dot plot, density plot, or contour plot, and drag diagonally until the region outline is the desired size.
- 15 Snap-To-Region tool**—Select the tool, click a population cluster on a dot, density, or contour plot. The region is drawn to automatically encompass a distinct population.
- 16 Text tool**—Select the tool, click to position the insertion point, and type the text. Click anywhere to complete the entry.
- 17 Expression Editor tool**—Opens a dialog where you can enter custom mathematical expressions (formulas). The results of your calculated expression appear on the Experiment document.
- 18 Arrow tool**—Select the tool, click within the Experiment document, and drag the line out.
- 19 Calculator tool**—Click the tool to recalculate data if Auto Recalculate is turned off.

BD CellQuest Pro Software Files

The two common file types created by BD CellQuest Pro software are:

- Experiment documents
- FCS list-mode data files

Experiment Documents

Experiment documents contain plots, regions, gates, statistics, markers, text, and colors. Experiment documents do NOT contain data files. When an Experiment document is saved, each plot includes a reference to the data file currently displayed.

NOTE The references from an Experiment document to data files can be broken if a file is moved or deleted. A missing data file can be relinked by placing it in the same folder as the Experiment document. If only the Experiment document is moved, referenced data files will be found when opening the Experiment document.

Experiment document features include:

- An Experiment document can be saved, closed, and then reopened at a later time to resume an acquisition or analysis.
- An Experiment document can be saved as a template that you can reuse for routine acquisition or analysis work.
- An Experiment document can be converted into a stationery pad that you can use repeatedly as a master for creating similar documents. When you open the stationery pad document, a copy of the document is created and opened, leaving the original unchanged.
- Three types of plots can appear in an Experiment document: Acquisition, Analysis, and Acquisition to Analysis. Experiment documents can contain a combination of these plot types. However, an Acquisition plot cannot be used to analyze data, and an Analysis plot cannot be used to display acquisition data.

Default Experiment Documents and File Menu Commands

BD CellQuest Pro software creates a new Experiment document named *Untitled-1* each time you start the program.

The File menu provides commands for creating, opening, closing, saving, and printing Experiment documents.

File Menu Command	Description
New	Opens a new, untitled Experiment document.
Open	Opens an existing Experiment document.
Close	Closes the current Experiment document.
Save	Saves the entire contents of the Experiment document, including the plots, regions, and quadrant markers, etc. The FCS data file is not saved with the Experiment document.
Save As	Allows you to save the entire contents of the Experiment document, including the plots, regions, and quadrant markers, under a new name. The FCS data file is not saved with the Experiment document.

FCS Data Files

BD CellQuest Pro software saves acquisition data as Flow Cytometry Standard (FCS) 2.0 files. These files are saved automatically when the Acquisition Controls Setup checkbox is not selected.

FCS is a standard format for flow cytometer data files. (For a detailed description of FCS 2.0, refer to the Data file standard for flow cytometry, *Cytometry*. 1990;11:323-333.)

A typical FCS data file consists of 2,000 to 10,000 events. A data file with 10,000 events with four parameters each requires about 45 kB of storage at 256-channel resolution and 85 kB of storage at 1024-channel resolution.

BD CellQuest Pro Instrument Controls

BD CellQuest Pro software provides controls for adjusting and monitoring instrument performance:

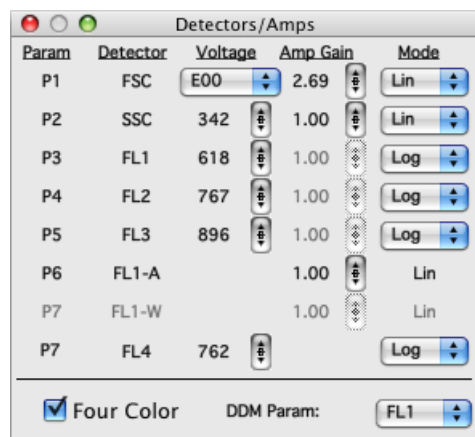
- Detector and amplifier controls allow you to adjust the amplitude of signals produced by flow cell events.
- Threshold controls allow you to specify a signal that must be exceeded for an event to be processed.
- Compensation controls allow you to adjust signals to correct for spectral overlap between fluorochromes.
- Additional controls include Test Pulses, Laser Power, Laser Current, Sample Voltage, Sheath Fluid, Waste Tank.

Detectors

There are two types of detectors in the flow cytometer: photodiodes and photomultiplier tubes (PMTs). Photodiodes are less sensitive to light signals than the PMTs.

FSC Photodiode

A photodiode is used to detect FSC because FSC is a strong signal. The FSC Detector setting is adjusted by clicking the FSC Voltage menu control and selecting one of the settings shown in the following table.



FSC Detector Value	Multiplies the FSC signal by
E-1	$10^{-1} = 0.1$
E00	$10^0 = 1.0$
E01	$10^1 = 10.0$
E02	$10^2 = 100.0$
E03	$10^3 = 1000.0$

E-1 reduces the signal of large events. E01, E02, and E03 increase the signal of small events.

PMTs

PMTs are used to detect side scatter (SSC), FL1, FL2, FL3, and FL4 fluorescence because these signals are weak. The light signals are multiplied by applying a voltage to the PMT. As the voltage is increased, the signal increases, and the data appears at a higher channel number. As the voltage is decreased, the signal decreases, and the data appears at a lower channel number. The SSC, FL1, FL2, FL3, and FL4 Detector Voltages are adjusted by clicking the up and down arrows or by clicking the icon between the arrows to display a slider. The up and down arrows increment or decrement the voltage one volt at a time. The slider can be dragged appropriately.

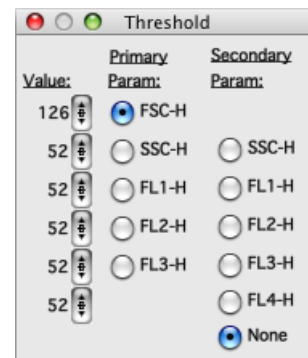
Amplifiers

Amplifiers allow you to make fine adjustments to the signals. The Amplifier Mode and Amp Gain allow you to set amplifier settings for FSC, SSC, FL1, FL2, FL3, and FL4. If linear (Lin) is selected in the Mode menu, the amplifier gain can be adjusted between 1.00 and 9.99. This amplification is adjusted by clicking the up and down arrows or by clicking the icon between the arrows to display a slider. If logarithmic (Log) is selected in the Mode menu, the amplifier gain cannot be adjusted. The scale defaults to four log decades. Log amplification is often used to separate negative from dim positive signals.

Threshold

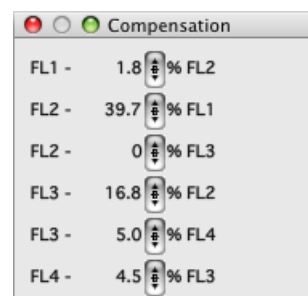
Threshold allows you to specify a signal intensity below which data will not be processed. Threshold can be set for only one parameter (FSC, SSC, FL1, FL2, or FL3) at a time if you have a single laser system. Dual thresholding is available on dual laser systems. Only signals with an intensity greater than or equal to the threshold channel number will create an event to be processed by the computer.

The threshold parameter is specified by selecting a parameter button. The threshold value is set by clicking the up and down arrows or clicking the icon between the arrows to display a slider and dragging to the appropriate value.



Compensation

Compensation allows you to adjust for spectral overlap when the samples are stained with two or more fluorochromes. Fluorochromes emit light over a range of wavelengths; therefore, a signal from one fluorochrome may overlap in a detector used for another fluorochrome. FITC appears primarily in the FL1 detector but some of its fluorescence overlaps into the FL2 detector. PE appears primarily in the FL2 detector but some of its fluorescence overlaps into the FL1 and the FL3 detectors.



Compensation lets you eliminate this spectral overlap electronically. The compensation networks consist of:

FL2-%FL1—Subtracts overlapping FL1 signal from the FL2 detector.

FL1-%FL2—Subtracts overlapping FL2 signal from the FL1 detector.

FL3-%FL2—Subtracts overlapping FL2 signal from the FL3 detector.

FL2-%FL3—Subtracts overlapping FL3 signal from the FL2 detector.

FL3 -%FL4—Subtracts overlapping FL4 signal from the FL3 detector.

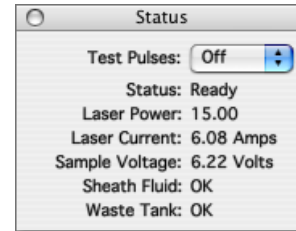
FL4 -%FL3—Subtracts overlapping FL3 signal from the FL4 detector.

The amount subtracted for compensation is dependent on the detector voltage. Compensation adjustments are correct when the compensated populations are aligned with the negative population. An explanation of these adjustments is found in the acquisition exercises. The amount subtracted is adjusted by clicking the up and down arrows or clicking the icon between the arrows to display a slider.

Status

The operating status of the flow cytometer can be checked anytime during acquisition. The Status window can be used as a troubleshooting resource.

Test Pulses: Test pulses are electronically generated signals. On the BD FACSCalibur cytometer, you turn them on and off from the Status window. The test pulse choices are FSC, (signals generated only in the FSC detector) ALL (signals generated in all parameter detectors), or OFF.



Status: Lets you know the instrument operating mode.

- Not Ready—the laser is warming up, the sheath fluid reservoir is empty or the waste reservoir is full.
- Ready—a sample tube is on the Sample Injection Port (SIP) with the arm under the tube, the tube is pressurized, and the cytometer is in RUN mode.
- Standby—either the cytometer is in STNDBY mode (referred to as hard standby) or the cytometer is in RUN mode but there is no sample on the injection port, the arm is not under the tube, or the tube is not fully pressurized. The laser power is reduced in Standby.

Laser Power: The laser power is expressed in milliwatts (mW). In RUN mode, the laser power is about 15 mW. In STNDBY mode, the laser power is about 3 to 6 mW.

Laser Current: This is the amount of amperage needed to get the laser up to power. If this current goes above a certain amperage, a message will appear on the computer screen.

Sample Voltage: This is the measure of the differential relationship between the sheath pressure and the sample pressure. When a tube is placed on the SIP, the voltage decreases as the pressure differential increases.

Sheath Fluid: Indicates if the sheath fluid reservoir is EMPTY or OK.

Waste Tank: Indicates if the waste reservoir is FULL or OK.

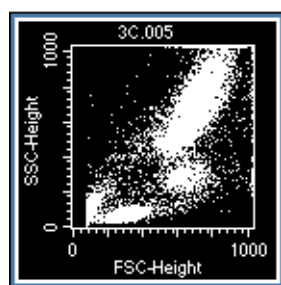
BD CellQuest Pro Software Views

Plots and statistics are referred to as views in the Experiment document. Views appear in three different states: active, selected, or deselected. A frame surrounds the view. The appearance of the frame indicates the state of the view.

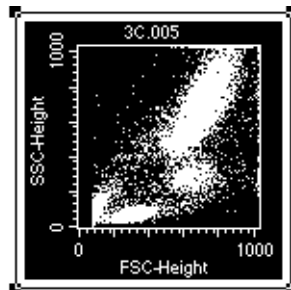
Active views: When the frame is gray, the plot is active. To activate a view, click anywhere in the view except on the frame. Only one view can be active at a time, and any command affects that view only.

Selected views: When black handles appear on each corner, the view is selected. To select a view, click its frame. More than one view can be selected at a time. This can be done by shift-clicking the frames or by clicking in the Experiment document and dragging diagonally to create a rectangle encompassing them. You can select all views by selecting Select All from the Edit menu. Selected views are affected by any command. Only selected views can be resized, deleted, and moved.

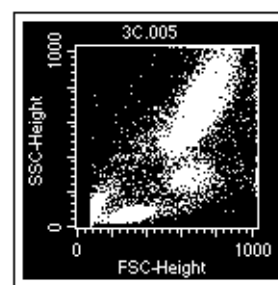
Deselected views: When the frame is clear with no handles, the view is deselected. To deselect a view, click anywhere off the view. Deselected views are unaffected by any command.



Active view



Selected view



Deselected view

Quick Reference

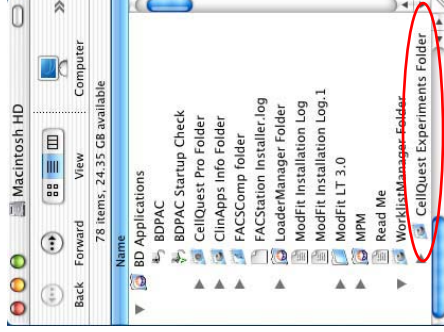
BD FACS Loader with BD CellQuest Pro Software



Preparation

- A** Create a BD CellQuest Pro acquisition document for EACH panel you will use.
Reminders:
 - Make Acquisition->Analysis or Acquisition plots.
 - Select Event Count or Time in Acquisition & Storage and specify count and time.
 - Select a panel in the Acquisition Browser.

- B** Save the documents in the CellQuest Experiments Folder.

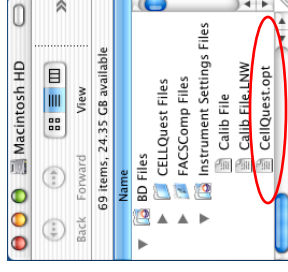


Instrument QC

- 1** Perform QC for the application.
 - Run the appropriate Assay in BD FACSComp software.

Optimization

- 2** Optimize instrument settings using a cellular sample.
 - Optimize in BD CellQuest Pro software.
 - Save optimized settings in the Instrument Settings Files folder.
 - Exit BD CellQuest Pro and ensure that all BD applications are closed.



Acquisition

- 3** Enter information in BD WorklistManager software
 - Sign in.
 - In the Set Up view, specify:
 - File Name Prefix
 - Summary Report
 - Sample Label
 - Save a Worklist (optional)
 - Report and export file locations.
 - Assay settings: for each panel—link to optimized instrument settings and specify mix settings.
 - Worklist
 - Enter sample info.
 - Select panel for each sample.
 - Assign rack.

- 4** Acquire samples.
 - Install rack.
 - Install cover.
 - Place cytometer in Run mode.
 - Click Run Tests.



Quick Reference

BD FACS Loader with BD Multiset Software

Preparation

- 1 Create a BD Multiset Schedule document.

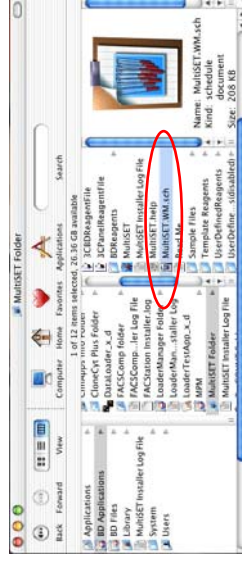
TIP: Customize the default MultiSET.WM.sch document.

Schedule document information used by WorklistManager:

- Preferences: export, printing, panels, events, banner, etc.
- Data source
- Time to view reports
- Test preferences information

Reminders:

- Enter current Lot IDs (eg, Trucount beads/pellet) in BD Multiset software.
- Save your BD Multiset Schedule Template document to any location.



Instrument QC

- 1 Perform QC for the application.

- Run the Lyse/No Wash Assay in BD FACSComp software.



Set Up Worklist

- 2 Enter information in BD WorklistManager software

- Sign In
- In the Set Up view, specify:
 - File Name Prefix
 - Summary Report
 - Sample Label
 - Save a Worklist (optional)
 - Report and export file locations.
 - Multiset Schedule document template.



- Assay settings: for each panel—link to optimized instrument settings and specify mix settings.

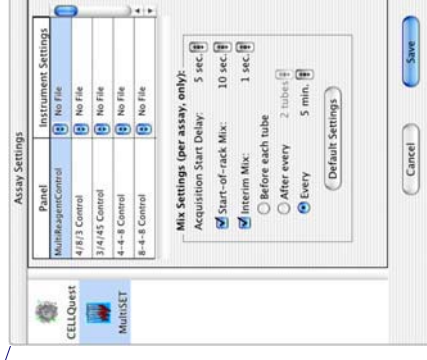
- Worklist
 - Enter sample info.
 - Select panel for each sample.
 - Assign rack.

Optimization

- 3 Optimize instrument settings using the first sample.
 - Install rack.
 - Install cover.
 - Place cytometer in Run mode.
 - Click Run Tests.
 - If needed, adjust SSC PMT and FL3 threshold on first tube.

Acquisition

- 4 Acquire samples.





Quick Reference

BD FACScalibur High Throughput Sampler (HTS)

BD CellQuest Pro

Preparation

A Prepare Acquisition Document in BD CellQuest Pro Software

- Draw plots / regions / stats as needed for acquisition
- Set acquisition and storage criteria
- Save to any location on computer

B Prepare Analysis Document in BD CellQuest Pro Software

- Draw plots, regions, and stats as needed for analysis
- Save to any location on computer

C Prepare Instrument Settings

- Instrument settings to use as starting point for optimization (Calib file or other settings)

BD Plate Manager Software

Layout

1 Create Plate Layout

- Sampler Parameters Mode = Standard vs High Throughput
- Plate type

2 Specify Setup Wells

- Select wells — click setup standard box
- Specify sampler parameters:
- Acq. Document
- Instrument Settings
- Sample Volume

3 Specify Sample Wells

- Select wells—click sample box
- Specify sampler parameters:
 - Acquisition Document
 - Analysis Document
 - Instrument Settings
 - Sample Volume
 - Sample Rate
 - Mixing Volume

Setup

4 Optimize Instrument Settings

- Select setup wells
- Prime HTS 2X
- Acquire
- Save Protocol dialog
- BD CellQuest started
- Optimize settings with appropriate wells
- Manually stop acquisition in PlateManager (pause)

5 Save Instrument Settings in BD CellQuest

Acquire

6 Acquire Samples

- For Acquisition to Analysis plots:
 - Select Print CellQuest Pro Report if desired
 - Select Export stats if desired
- Click Acquire





7 Analyze Data Files

- Select File > Save to save protocol and link data files
- Select Acquired wells
- Click Analyze to begin batch analysis

Analyze

Quick Reference

BD CellQuest Pro File Types

Icon	Description	How to Save	How to Open
 untitled	Experiment document —contains plots, regions, quadrant markers, gates, acquisition setup, and so on.	Select File > Save Document.	Select File > Open Document or double-click the document icon.
 FCS Norm.001	FCS data file —contains raw data acquired from the cytometer, as well as the instrument settings used to acquire the data. Settings can be retrieved and used for other experiments, if needed.	Clear the Setup checkbox in the Acquisition Control window or in the Acquisition Browser.	Select an analysis plot. In the inspector, click the File menu control and select Select File.
 InstrSettings	Instrument settings file —contains PMT voltage, Amp Gain, Compensation, and Threshold settings.	Select Cytometer > Instrument Settings. Click Save.	Select Cytometer > Instrument Settings. Click Open. Select the settings file, click Open, then click Set, and click Done.
 untitled stats	Export file —is a text file containing statistical values (example: quadrant statistics) that can be used to transfer data into other applications such as a spreadsheet or database.	Select the Export statistics checkbox in the Batch Setup window. Alternatively, you can save an export file by selecting File > Export Statistics.	Launch a spreadsheet application. Example: Microsoft® Excel—select File > Open and specify the export file to open.

