



guava easyCyte™ HT System

User's Guide

For systems running guavaSoft software, version 2.7



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Patents	<p>The guava easyCyte HT System is the subject of issued and pending US patents and foreign equivalents, including the following US patents:</p> <p>5,798,222 – Apparatus for monitoring substances in organisms</p> <p>6,403,378 – Cell Viability assay reagents</p> <p>6,710,871 and 6,816,257 – Method and apparatus for detecting microparticles in fluid samples</p> <p>7,320,775 – Exchangeable flow cell assembly with a suspended capillary</p> <p>7,410,809 – Particle or cell analyzer and method</p> <p>8,184,271 and 7,847,923 – Differentiation of flow cytometry pulses and applications</p> <p>The InCyte analysis and guava RapidQuant Human and Mouse IgG Assays are the subjects of pending patent applications.</p>

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Preface

The guava easyCyte™ HT System is the first automated, easy-to-use, desktop single-cell analysis system that can perform a wide range of multi-color cellular and bead assays. The system accommodates both a 96-well plate and microcentrifuge tubes. The current applications include:

- guava ViaCount® Software Module for cell counting and viability
- guava InCyte for assays needing up to six colors and two scatter parameters, or if you wish to analyze any guava FCS 3.0 data files
- guava® ExpressPlus Software Module for any one-, two-, or three-color assay
- guava® ExpressPro Software Module for assays needing up to six colors and two scatter parameters
- guava Nexin®, guava® Caspase, guava® TUNEL, and guava® MitoPotential Software Modules for apoptosis analysis
- guava® Cell Cycle Software Module for DNA analysis
- guava® CellPaint Software Module for tracking target cells in mixed populations, or for mAb screening in mixed cultures
- guava® CellToxicity Software Module for cell-mediated cytotoxicity determinations
- guava® CellGrowth Software Module for cell proliferation studies
- guava RapidQuant™ Software Module for quantification of human and mouse IgG antibodies

The guava ViaCount Cell Counting and Viability Software Module, automatically analyzes the fluorescence of cells stained with the ViaCount Reagent and quantifies the number of viable and non-viable cells in the sample. The guava ExpressPlus Software Module was designed to allow you the flexibility to perform any one-, two-, or three-color assay. The guava ExpressPro Software Module is designed for assays of up to six colors using both the red and blue lasers. It allows you to save a time parameter, as well as perform area, and/or width measurements. The guava InCyte application offers the same features as ExpressPro. It has a unique interface with drag and drop capabilities for acquisition and analysis. Guava InCyte allows you to open and analyze files acquired with any other guava assay. The guava Nexin, guava Caspase, guava TUNEL, and guava MitoPotential Apoptosis Software Modules allow you to identify apoptotic cells and differentiate them into stages. The guava Cell Cycle Software Module allows you to identify and estimate the number of cells within the various phases of the cell cycle. The guava CellPaint Software Module allows you to track “painted” cells when mixed with other “unpainted” cells, which is useful in screening mAbs from hybridoma cell supernatants. The guava

CellToxicity Software Module allows you to paint target cells to differentiate them from effector cells while monitoring cell-based cytotoxicity. The guava CellGrowth Software Module allows you to determine the number of proliferating cells in culture following mitogenic stimulation, as well as to assess the number of live versus dead cells. The guava RapidQuant Software Module allows you to quantify human and mouse IgG antibodies, typically found in hybridoma supernatants, using protein-bound capture beads. All assays allow you to view the data points and analyze further, if necessary, using gates and markers to generate the appropriate statistics.

About This Guide

The *guava easyCyte HT System User's Guide* provides detailed information on operating and maintaining the guava easyCyte HT Instrument, and using guavaSoft™ Software for data acquisition and analysis. This manual is intended for the guava easyCyte 5HT, 6HT, 6HT-2L, and 8HT instruments. Any reference to NIR, Red 2, and NIR 2 parameters, may not apply to your specific instrument. Refer to “easyCyte HT Lasers and Parameters” in Specifications for a complete list of the parameters available with each system.

This guide assumes you have a working knowledge of Microsoft® Windows®. If you have any questions regarding the Dell™ computer or the operating system, refer to the appropriate manufacturer's documentation.

For information on preparing samples, refer to the appropriate guava package insert that was shipped with your reagents.

Conventions Used in This Guide

- **NOTE:** Points out additional information that may be helpful.
- ◆ **WARNING:** Alerts you to situations that could result in bodily harm, instrument damage, failure in a procedure, or incorrect results.

Bold: Indicates buttons to click or options within the software to select.

Italics: Used for names of user's guides and package inserts, as well as messages that appear on the screen.

Help

- 1 Read through the section of the guide specific to the operation you are performing. Refer to the table of contents and index to locate information. A glossary is included to assist you with any unfamiliar terms.
- 2 See the troubleshooting section at the end of each assay chapter for a list of problems and suggested solutions. The troubleshooting for all assays is also repeated in Chapter 17, Troubleshooting.
- 3 Refer to the technical support contact information listed below:
 - For ordering information or technical support, call toll-free:
USA and Canada, Phone: +1 (800) 645-5476 Fax: +1 (951) 676-9209
 - For additional contact information, visit www.millipore.com/flowcytometry

Safety

The guava easyCyte HT System is equipped with safety features for your protection. Use the system only as directed in this user's guide. Do not perform instrument maintenance or service except as specifically stated. Please read the following safety information before using the system.

General Safety

- ◆ **WARNING:** If this instrument is not used in the manner indicated by the instructions in this guide, the safety features of the instrument may be impaired. Follow these guidelines:
 - To avoid damage to the instrument, be sure to remove the shipping restraint before plugging in the instrument.
 - Always use the Eject Tray button in guavaSoft Software to open the sample tray. Click **Pause** first, if necessary. Never open the tray with your fingers. Keep the area in front of the tray clear while it loads or ejects.
 - Ensure the sample tray is loaded before you turn off the power to the easyCyte HT System.
 - The use of tubes or plates other than those specified may result in damage to the instrument.
- ◆ **WARNING:** Do not run any other programs, including Internet Explorer, on the laptop while using guavaSoft Software to acquire data. guavaSoft Software requires the full resources of your laptop during data acquisition. Running other programs during a run may interfere with acquisition or interrupt the run.

Biological Safety

- ◆ **WARNING:** All biological specimens and materials that come into contact with them can transmit potentially fatal disease. To prevent exposure to biohazardous agents, follow these guidelines:
 - Handle all biological specimens and materials as if capable of transmitting infection. Dispose of waste using proper precautions and in accordance with local regulations. Never pipette by mouth. Wear suitable protective clothing, eyewear, and gloves.
 - Add 10 mL of bleach to the waste vial after emptying it. Dispose of waste in accordance with federal, state, and local regulations.
 - Always place empty tubes when indicated by the software (for example, tube locations w6 and w3 in Worklist Editor software). Empty tubes capture the water during a spin/dry after mixing.

Electrical Safety

- ◆ **WARNING:** Turn off the main power switch at the back of the instrument and disconnect the power cord before replacing fuses.

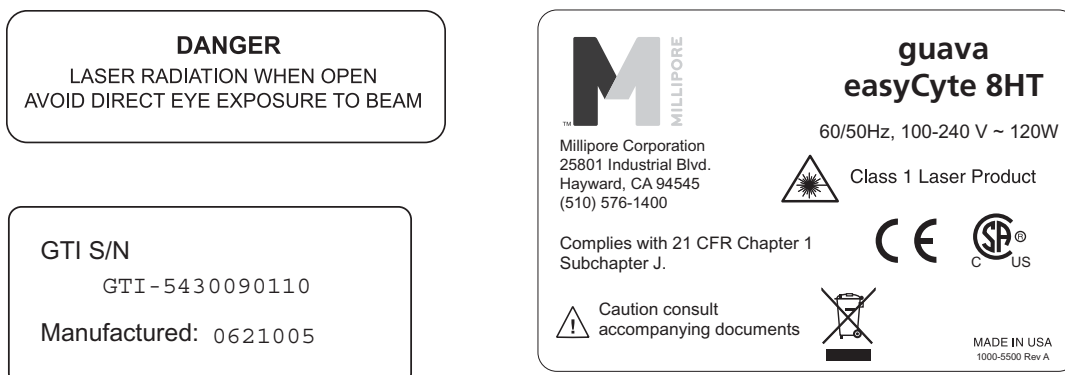
Laser Safety

The guava easyCyte HT System contains a Class IIIb laser operating at 488 nm in CW mode and a Class IIIb laser operating at 640 nm in CW mode. Light shields within the instrument enclose the path of laser radiation. Additionally, the instrument enclosure provides secondary protection from any laser radiation.

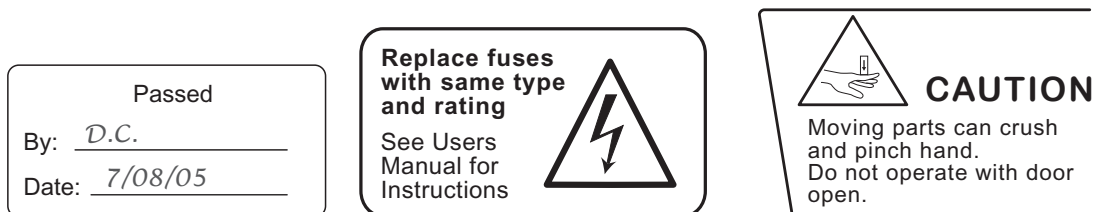
- ◆ **WARNING:** To avoid exposure to laser radiation or electric shock, follow these guidelines:
 - *Do not* open the instrument or attempt to perform any internal maintenance. There are no user-serviceable parts.
 - Turn off the power to the guava easyCyte HT System before attempting to remove the flow cell.

Precaution Labels

Following are examples of the labels affixed to the guava easyCyte HT Instrument:



Example of easyCyte 8HT label



Limitations

- The guava easyCyte HT System is for research use only; not for use in diagnostic procedures.
- The results of the assays are dependent upon the proper use of the reagents and instrument. Refer to the appropriate package insert for specific instructions and limitations.

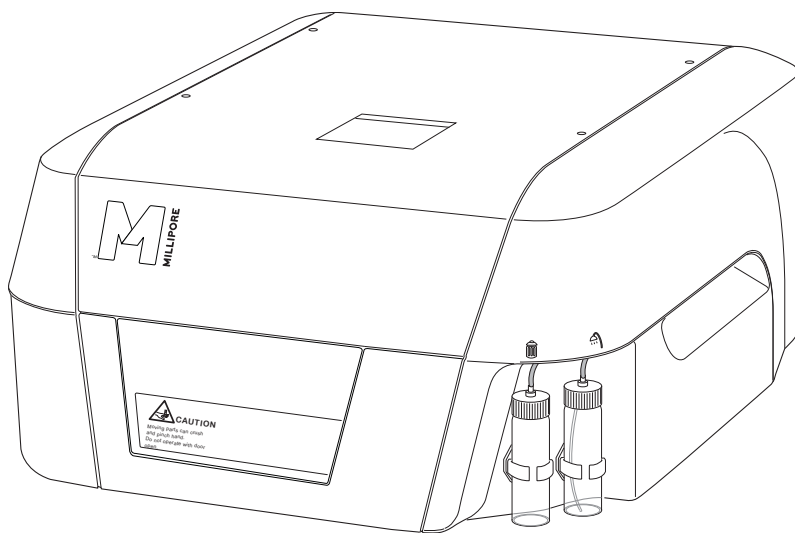
CHAPTER 1

Introduction

guava easyCyte HT System

The guava easyCyte HT System streamlines drug discovery, cell culture monitoring and screening by providing turnkey assays for a wide range of single-cell-based applications. The system includes the guava easyCyte HT Instrument, a laptop computer with pre-installed software for data acquisition and analysis, and optimized reagents and protocols.

guavaSoft™ Software includes dedicated modules for cell- and bead-based assays, minimizing training requirements. In addition, the software includes the guava® easyCheck Module, which verifies that the system is performing optimally, and guava® Clean, a cleaning module that allows you to run an automated cleaning procedure.

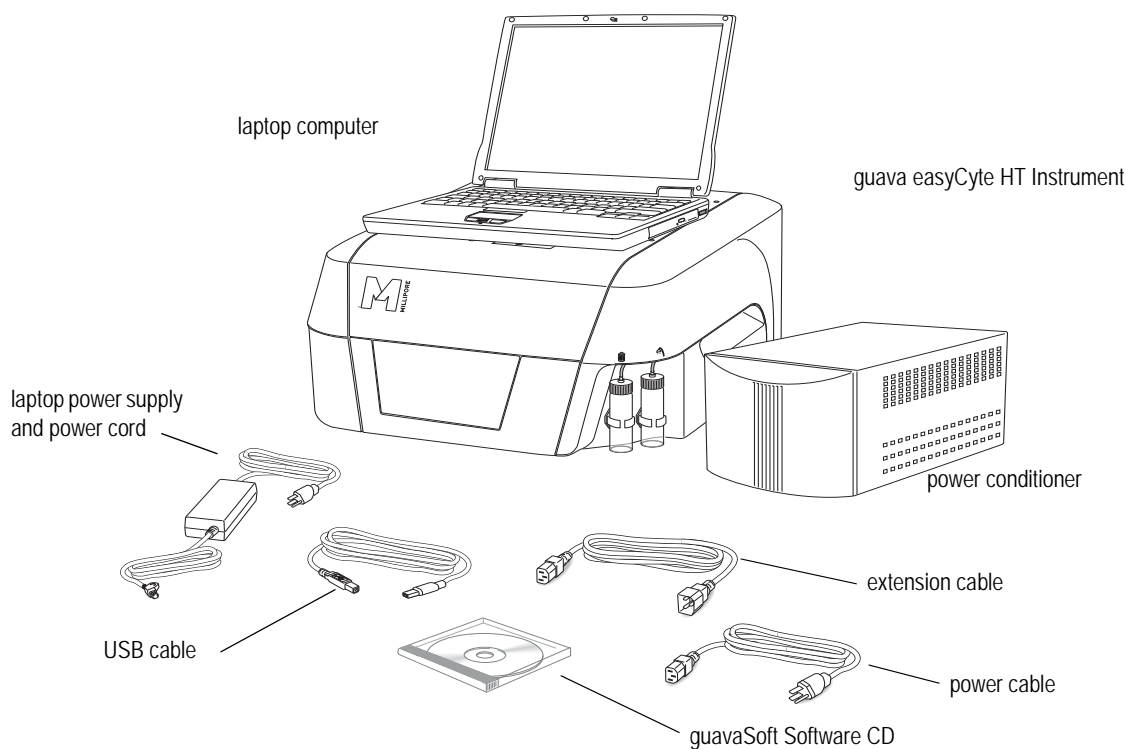


guava easyCyte HT System

guavaSoft Software automatically saves data files, which can be recalled later for offline analysis. In addition, data are exported to a spreadsheet file.

System Components

The guava easyCyte HT System is shipped with the following components:

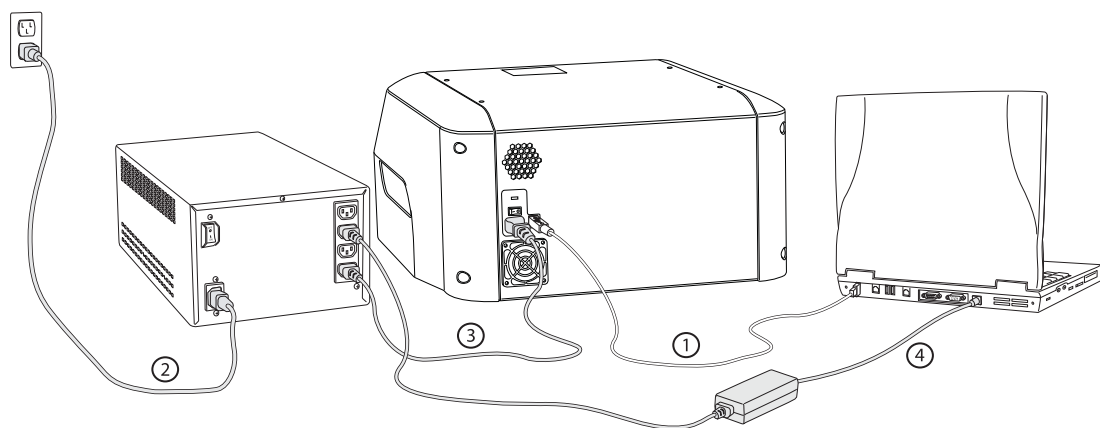


Hardware Connections

Although the guava easyCyte HT System is a portable unit, it contains precisely aligned optical components that are sensitive to jarring movements. Place the instrument on a stable surface in a dedicated location in the laboratory. Allow at least 4 inches between the back of the instrument and the wall for proper ventilation. Maintain easy access to the power cord in case the instrument needs to be disconnected in an emergency. The initial installation will be performed by an EMD Millipore field service representative.

- ◆ **WARNING:** To avoid damage to the instrument, be sure to remove the shipping restraint before plugging in the instrument.
 - **NOTE:** If the instrument needs to be moved to a new location in the lab area or building, always use two people to lift and a sturdy transport such as a cart. If a longer distance move requiring pickup is required, contact EMD Millipore. A Relocation and Installation service is available for a fee. See Appendix D: Order Information for details.
- 1 Connect the cytometer to the laptop computer with the USB cable.
 - 2 Connect the power cable between the power conditioner and a grounded (three-prong) AC power outlet.

- 3 Connect the extension cable between one of the four power outlets on the back of the power conditioner and the power input on the cytometer.
 - 4 Connect the laptop power supply to the laptop. Plug the power supply into the power cord, then plug the power cord into the power conditioner.
- ◆ **WARNING:** The power conditioner is not a continuous power supply. Ensure that the instrument is powered on during acquisition.



- 5 You can connect the computer to a local network or the internet. Refer to Appendix B: Administrator Features for more information. Contact your network administrator for assistance. You can also connect a printer to the computer. If you connect a printer, you must install the appropriate print drivers.
- 6 Refer to “System Startup” on page 2-1 for the correct procedure to start the laptop computer, guava easyCyte HT System, and guavaSoft Software.
- 7 Prime the fluid system by clicking **Cleaning** from the guavaSoft Software main menu and following the instructions to load tubes of deionized water and cleaning solution on the guava easyCyte HT System. Refer to “Running Guava Clean” on page 3-4.

guava easyCyte HT System and Laptop Communication Troubleshooting

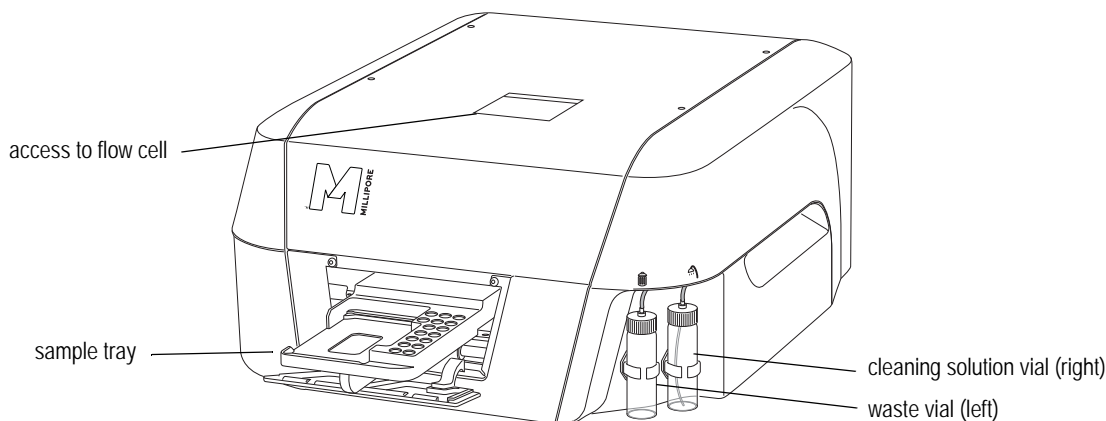
Problem	Possible Causes	Solutions
Laptop prompts for user ID or password.	Laptop is set up for authorization.	Do not enter password. Click OK or Cancel to continue. Contact your IT department for assistance with any modifications. The original laptop setup does not require a password.
During start-up, laptop freezes on particular screen.	System may be searching for directory during startup.	Press Enter to continue. Reboot computer, if necessary.
Message: <i>The instrument appears to be either off or not connected. You can run in Analysis mode only.</i>	<ol style="list-style-type: none"> 1. guava easyCyte HT System is not turned on or is not getting power. 2. Cable connection between easyCyte HT System and laptop is loose. 3. easyCyte HT System and laptop were not powered on in correct sequence or have lost communication. 	<ol style="list-style-type: none"> 1. Ensure easyCyte HT System power cord is properly plugged in and system is turned on. 2. Ensure USB cable is securely connected to laptop. Reboot computer, if necessary. 3. Turn off easyCyte HT System, exit guavaSoft Software, restart laptop, turn on easyCyte HT System, start guavaSoft Software.
guavaSoft Software launches, but only Analysis mode is available when an assay is launched.	Registration code not entered or not entered correctly.	Enter registration code and ensure all characters are correct.
Laptop keeps shutting down.	<ol style="list-style-type: none"> 1. Power supply to laptop is faulty. 2. Screen saver is interfering. 	<ol style="list-style-type: none"> 1. Ensure laptop is plugged in correctly. Use surge protector and ensure it is plugged in and turned on. 2. Adjust power scheme screen saver options. Click Start> Settings>Control Panel. Double-click Display, select Screen Saver tab, click Settings under <i>Energy saving features of monitor</i>. Make sure "Setting for Always On power scheme" are all set to Never. Laptop should not be allowed to "sleep." guavaSoft Software will stop acquiring data until the laptop is woken up.

Problem	Possible Causes	Solutions
For InCyte only Message: <i>Sorry - this unlock is invalid.</i>	Incorrect or no unlock key.	Ensure the correct unlock key is entered. If necessary, contact customer support to obtain unlock key.
Noise occurring during sampling.	<ol style="list-style-type: none"> 1. Mixer paddle is making contact with the plate. 2. Mixer paddle may be misaligned or bent. 	<ol style="list-style-type: none"> 1. Ensure you are using a compatible plate. See "Compatible Microplates" on page D-6. 2. Contact EMD Millipore Technical Support.
Message: <i>The tray door is open.</i> appears when the door is shut.	The door sensor switch is damaged.	Contact EMD Millipore Technical Support.
Message: <i>TRAY HOLD OFF STATE.</i>	<ol style="list-style-type: none"> 1. The program is waiting for the automation to reset. 2. The tray door was opened. 3. The program lost detection of automation position or status. 4. There is a mechanical or electrical problem with the automation or mixer function. 	<ol style="list-style-type: none"> 1. Wait approximately 30 seconds for re-initialization. 2. Keep tray door closed during acquisition. 3. Exit guavaSoft, turn the instrument off, then on again. Restart guavaSoft, then open the worklist. 4. Contact EMD Millipore Technical Support.

- ◆ **WARNING:** Do not run Excel®, Internet Explorer®, or any other program on the laptop while using guavaSoft Software to acquire data from the guava easyCyte HT Instrument. guavaSoft Software requires the full resources of your laptop during data acquisition. Running other programs (even if you are not actively using them) during a run may interfere with acquisition or interrupt the run.

guava easyCyte HT Instrument

The guava easyCyte HT Instrument was designed for easy operation and minimal maintenance. Other than the power switch, the sample tray and waste vial are the only components that you will routinely handle. The power switch is located on the back-right corner of the instrument.



guava easyCyte HT System components

Sample Tray


Sample aliquots are placed in a microplate or sample tube, then loaded in the sample tray. The tray holds one 96-well microplate and up to 10 sample tubes. A minimum sample volume of 100 μ L is required for wells, 150 μ L for 0.5-mL microcentrifuge tubes, and 900 μ L for 1.5-mL microcentrifuge tubes.


- ◆ **WARNING:** Do not attempt to open the sample tray door with your fingers. Always use the **Eject Tray** button in guavaSoft Software to open the door, pausing the system first, if necessary. If you attempt to manually open the door, a warning message appears and the worklist automatically stops. If this happens, exit guavaSoft Software, then restart the program.
- ◆ **WARNING:** On rare occasions, the software and instrument may pause to resynchronize with each other. If this occurs, the system will pause for 20–30 seconds and a message will appear in the status bar at the bottom of the screen indicating a “Tray Hold-Off.” Although this is not serious and the system will resume again after synchronizing, be aware that after the tray hold-off occurs, the tray may move without warning.

For a list of microplates and tubes supported, refer to Appendix D: Order Information at the back of this manual.

- **NOTE:** Snap-cap tubes can be used in place of the 1.5-mL screw-cap tubes (for washing and cleaning) if the caps are cut off.
- ◆ **WARNING:** The use of tubes or plates other than those specified may result in damage to the instrument.

Cleaning Solution and Waste Vials

The cleaning solution vial (located on the right ) can be filled with guava Instrument Cleaning Fluid (ICF), for easy system cleaning. For information on filling the vial, refer to “Filling the Cleaning Solution Vial” on page 3-6.

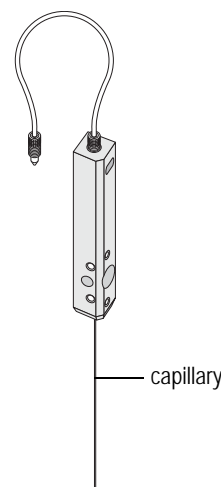
The waste vial (located on the left ) captures the sample fluid after it exits the fluid system. Empty the waste vial at the end of each day or more often, if necessary. Add 5 mL of bleach to the vial after you empty it (see page 3-7 for information).

Fluid System

Sample uptake occurs through a capillary and is regulated by a variable-speed fluid pump. The pump does not require sheath fluid or other supplementary fluids for operation.

Because the system’s sampling precision depends on the integrity of the fluid pathway, it is important to maintain a clean system. Do not allow samples to remain in the capillary for extended periods of time. Perform frequent cleaning cycles to prevent the build-up of cell debris that may restrict sample flow. If a clog does occur, you can clear it by using the backflush feature, which reverses the flow of fluid and flushes it out of the flow cell at a high speed. Refer to “Backflushing the Fluid System” on page 3-2 for instructions on using the backflush feature.

Some assays allow you to select the sample flow rate for acquisition. For most assays, the Medium flow rate is the default. This works well when the sample concentration is approximately 500 particles/μL. If the sample concentration is higher, dilute the sample or use a lower flow rate. For assays where the peak CV is critical, such as Cell Cycle, use the Low or Very Low flow rate. Always use the same flow rate for acquisition that you use for adjusting the settings.



Laser

The blue laser turns on when you turn on the easyCyte HT System; the red laser turns on when you open the guava easyCheck, guava ExpressPro, or guava InCyte Software Module. Both lasers turn off when you turn off the instrument.

For optimal laser performance allow the lasers to warm up for approximately 10 minutes before acquiring samples. If you will not be using the system for an extended period (6 hours or more), turn off the instrument. Managing the time the lasers are on will help extend laser life.

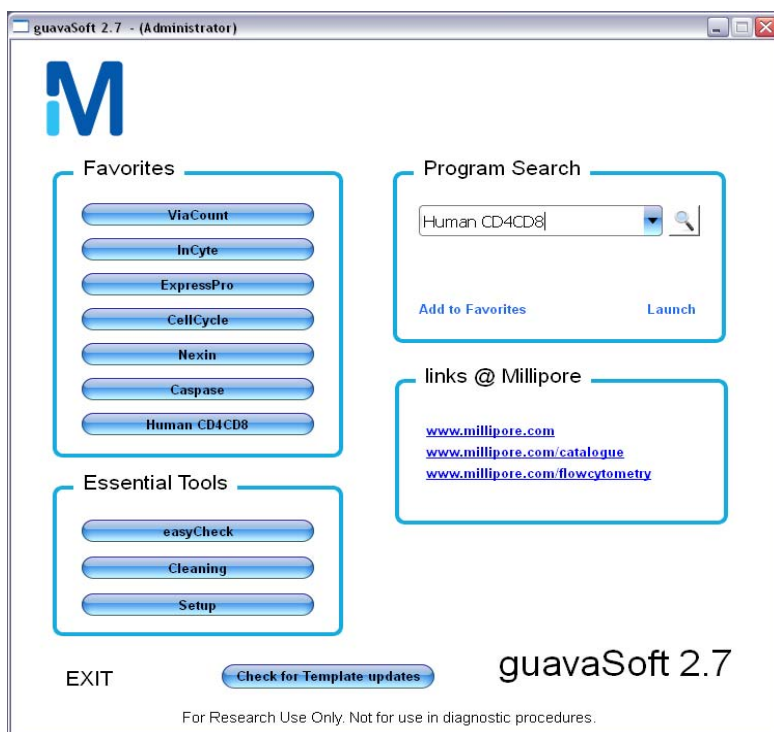
- **NOTE:** For information on the easyCyte HT lasers, refer to easyCyte HT Lasers and Parameters in Specifications.

guavaSoft Software Overview

Use guavaSoft Software for acquiring and analyzing data.

Main Menu

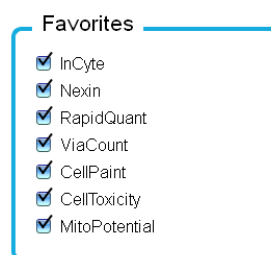
The guavaSoft Software main menu allows you to select an assay from either a list of favorites or the program search list. Essential Tools allows you to run easyCheck, clean the instrument, or select Setup, where you can customize your list of favorites. Use the links @ Millipore to quickly access Millipore websites for information.



Favorites

Favorites allows you to quickly select an assay. You can have up to seven assays at a time in your Favorites list. To add a favorite assay, select the assay from the Program Search list and click **Add to Favorites**. The new assay will be added to the bottom of the list. And if the list already consists of seven assays, the first assay in the list will be removed.

To remove a favorite assay, click **Setup** under Essential Tools. Remove the check from the check box for the assay you wish to remove. Click **DONE**.



Program Search List

The Program Search List allows you to select an assay to run or open a template in InCyte. You can create a Favorites list to quickly select the assay later. The main menu allows you to select from the following list of assays:

- **guava ViaCount** Assay for performing cell counting and viability assays
- **guava ExpressPlus** Assay for performing one-, two-, and three-color assays
- **guava ExpressPro** for performing assays with up to six colors, or where time, area, and/or width parameters are necessary
- **guava InCyte** for performing assays with up to six colors, or where time, area, and/or width parameters are necessary
- **guava Nexin**, **guava Caspase**, **guava TUNEL**, and **guava MitoPotential** Assays for performing apoptosis assays
- **guava Cell Cycle** Assay for performing cell cycle assays
- **guava CellPaint** Assay for tracking target cells in mixed populations, or for monoclonal antibody (mAb) screening in mixed cultures
- **guava CellToxicity** Assay for cell-mediated cytotoxicity determinations
- **guava CellGrowth** Assay for cell proliferation studies
- **guava RapidQuant** Assay for quantifying human and mouse IgG antibodies

Essential Tools

easyCheck allows you to check the system's counting, fluorescence, and scatter performance prior to running samples. The guava easyCheck Bead is a standard reference particle used with this function.

Cleaning allows you to run Guava Clean, an automated cleaning cycle that cleans the fluid system.

Setup allows administrators to:

- configure certain software features for specific users (see "Appendix B: Administrator Features" for more information)
- remove assays from your Favorites list
- enter assay registration codes (see "Registering a guava Assay" on page 1-10)

■ **NOTE:** The Setup button is available only when an administrator is logged onto the system.

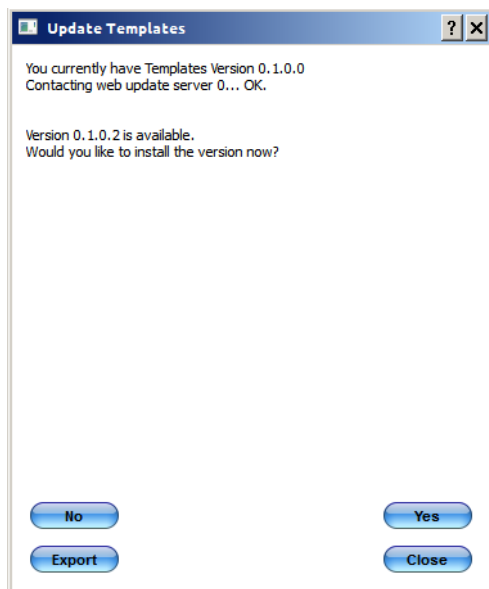
links @ Millipore

This section allows you to access commonly used EMD Millipore websites.

Check for Updates

Select **Check for updates** to search online, or if you are not connected to the internet, any available USB drives, for the latest templates. The system will notify you if you currently have the latest templates installed.

- 1 If updated templates are available, the following dialog appears. Click **Yes** to install the latest template package.
Click **Export** to download the templates to a USB drive.



- 2 Click **Close** when you receive confirmation that the latest templates were installed. If you chose to export the templates to a USB drive, select the drive from the drop-down menu and select **Export**.

Exit

Closes guavaSoft Software

Registering a guava Assay

Using a guava assay to acquire data requires a registration code. In most cases, those codes are already entered. Should you need to enter a registration code, follow the steps below.

- **NOTE:** InCyte requires an unlock key. If you get a message containing your computer code and stating that the unlock is invalid, e-mail EMD Millipore Technical Support and they will provide you with an unlock key for InCyte.

- 1 Click **Setup** in the Essential Tool section of the main menu.
The Customize guavaSoft window appears.



- 2 Enter the registration code under Assay Registration for the assay you wish to use.
The registration codes are case-sensitive.
■ NOTE: You cannot enter the registration code for the guava InCyte assay.
- 3 Click **Save** at the bottom of list.
- 4 Click **DONE**.
You can now launch the assay and acquire data.

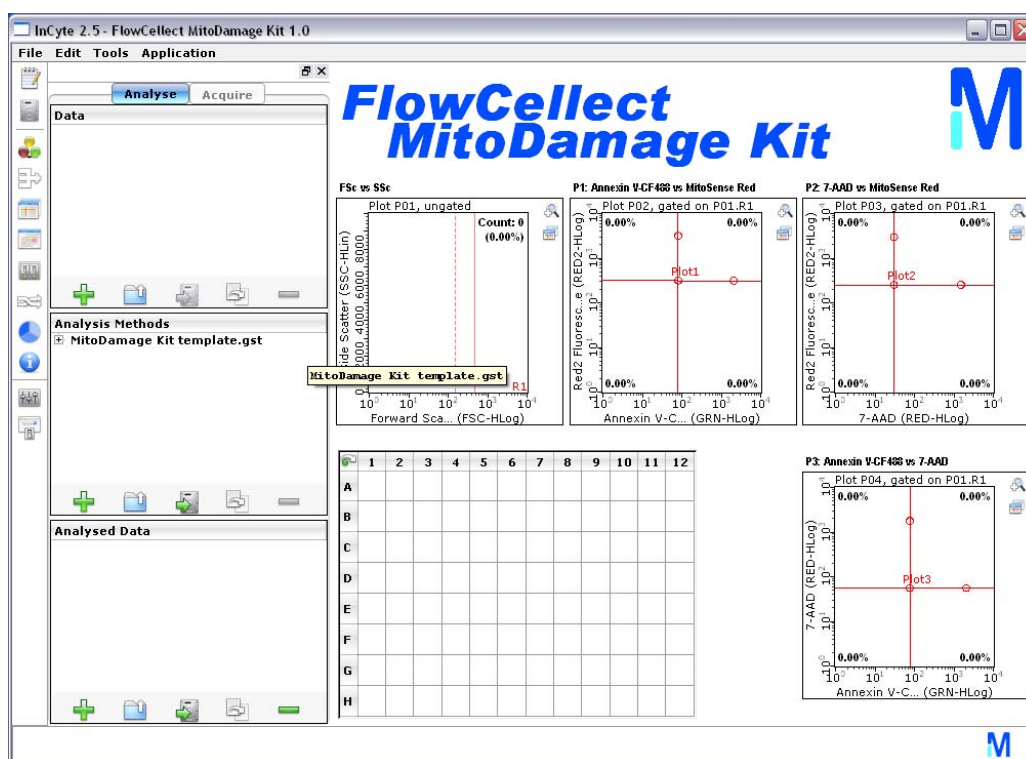
Templates

InCyte software templates are available for all FlowCelect™ assays. The templates can be used for acquisition and/or analysis. Open a template before acquiring your samples in InCyte. The instrument settings, plots, gates, and markers for the assay are contained in the template. If you already acquired data using either InCyte or another guavaSoft module, open the data file in the template and perform analysis using the Analysis Method provided.

For more information on using the templates to analyze data, refer to the user's guide for the individual assay kit.

- 1 To open a template, select it from the Program Search list and click **Launch**.
The assay template opens in InCyte.
- 2 Select the Analyse or Acquire button at the top of the control panel. Perform acquisition or analysis according to the instructions outlined in the kit user's guide.

- If you are acquiring samples, the instrument settings should be close to what you need. Check the settings and fine tune, if necessary.
- If you are analyzing InCyte-acquired data, but you acquired the data without the use of a template, you will need to drag the template Analysis Method to the Analysed Group. Check the gates and markers and fine tune, if necessary.
- If you are analyzing a data file acquired using a program other than InCyte, open the data file. The assay template will contain the appropriate Analysis Method. You will need to create an Analysed Group and drag the data file and the Analysis Method to the Analysed Group. Check the gates and markers and fine tune, if necessary.



Acquisition Screen

- ◆ **WARNING:** Do not run Excel, Internet Explorer, or any other program on the laptop while using guavaSoft Software to acquire data from the guava easyCyte HT Instrument. guavaSoft Software requires the full resources of your laptop during data acquisition. Running other programs (even if you are not actively using them) during a run may interfere with acquisition or interrupt the run.

The Acquisition screen appears when you enter the assay. Use the Acquisition screen to acquire data from samples you run on the guava easyCyte HT System. You can also perform data analysis from the Acquisition screen immediately following acquisition.

- **NOTE:** The guava InCyte acquisition screen differs from the other applications. Refer to Chapter 7, guava InCyte Assay for information on guava InCyte acquisition.



guava ViaCount Module Acquisition screen

Acquisition Buttons

The acquisition buttons are found on the individual assay Acquisition screens. These buttons perform the same functions regardless of the assay you are running, except as noted below.

Acquisition Button	Description
Worklist Editor	Launches Worklist Editor Software from within the guava application. Applies to the guava InCyte, ExpressPro, RapidQuant, and Caspase Assays only.

Acquisition Button	Description
Start Worklist / Stop Worklist	Start Worklist opens the sample tray, then prompts you to load samples, select the worklist file, enter a file name for the data set, select a settings function, and select a well/tube for adjusting settings. Stop Worklist stops the assay after finishing the acquisition for the current sample. After stopping you cannot resume the worklist.
Settings	Presents a dialog box with options to save, retrieve, and adjust instrument settings. Save Settings allows you to save the current instrument settings and gates and markers to a separate file. Retrieve Settings allows you to recall instrument and analysis settings from a settings file and download the settings to the easyCyte HT System. Adjust Settings allows you to adjust instrument settings for the assay you are running, using the appropriate sample.
Pause / Resume	Pauses the assay allowing you to select Eject Tray , Settings , Quick Clean , Clean & Rinse , or Backflush , or to access the Analysis screen where you can view data from previously acquired samples. The acquisition of the current sample will finish before the system pauses, and the button will read Pause requested . Resume restarts the assay where it left off.
Next	Proceeds to the next step in the data acquisition process. The data already acquired is saved. Next is not available during certain functions, for example during a washing or mixing step.
Abort	Stops the worklist and ejects the sample tray. The data for the current sample is not saved. You cannot resume an assay after you abort. You must reload the sample tray and start the assay from the beginning.
Backflush	Reverses the flow of fluid out of the flow cell. Perform a backflush if the acquisition rate declines and you suspect a clog. During acquisition, click Pause before Backflush to pause the assay. Follow a backflush with a Quick Clean. Click Resume to restart the assay.
Clean & Rinse	Thoroughly cleans the fluid pathway with a series of Quick Clean cleaning cycles and Backflush cycles. During acquisition, click Pause before Clean & Rinse to pause the assay. Click Resume to restart the assay. The system will automatically run a Clean & Rinse cycle at the end of the ExpressPro worklist, unless you selected Quick Clean for the Cleaning Option in Worklist Editor software.

Acquisition Button	Description
Quick Clean	Cleans the fluid pathway. During acquisition, click Pause before Quick Clean to pause the assay. Click Resume to restart the assay. The system automatically performs a Quick Clean at the end of each guava easyCheck Cycle. If you selected Quick Clean for the Cleaning Option in Worklist Editor, the system will automatically run a Quick Clean at the end of the each assay. You may perform it more frequently, if needed.
Eject Tray / Load Tray	Eject Tray opens the sample tray allowing you to add or remove a plate or tube. Load Tray retracts the sample tray.

Status Bar

The status bar, located at the bottom of the application window, indicates the user currently logged on and the instrument power status.

Analysis Screen

The Analysis screen allows you to analyze data from samples that were previously acquired. When you open a data set, the data for the first sample appears. The samples within the file are listed in the Analysis Sample List control panel. Click any sample to view the data for that sample. You can also click the Prev or Next button on the Unit Control panel or Sample Information control panel, or use the up/down arrows on the keyboard to select samples.

- **NOTE:** guava InCyte Analysis differs from the other applications. Refer to Chapter 7, guava InCyte Assay for information on guava InCyte analysis.

Click to switch to Acquisition screen.

Analysis results (vary with type of analysis)

Lists all samples within data set

Analysis buttons

guava ViaCount Module Analysis screen showing analysis buttons

You can access the Analysis screen during data acquisition by clicking **Pause**, then **Go to Analysis**. You can view or print data for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis settings (gates and markers) from the analysis screen during an acquisition session. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen. When the data set is complete, the data from the acquisition session is no longer accessible from the Analysis screen. To view the data, open the saved data file from the Analysis screen.

Analysis Buttons

The analysis buttons are found on the individual assay Analysis screens. These buttons perform the same functions regardless of the assay you are running. The results displayed will vary for each application. Refer to the specific application for details.

Access the Analysis screen to:

- analyze existing data files from previously acquired samples
- print results
- export data to FCS 2.0 files or spreadsheet files
- log comments for a particular sample or the data set
- view the event log
- access Worklist Editor (for guava InCyte, ExpressPro, RapidQuant, and Caspase Assays only)

Analysis Button	Description
Worklist Editor	Launches Worklist Editor Software allowing you to create a worklist. Applies to guava InCyte, ExpressPro, RapidQuant, and Caspase Assays only. The Worklist Editor button is at the top of the screen for these assays. To run a worklist, you must switch to the Acquisition screen.
Log Comment	Allows you to enter comments related to a sample or the data set. These comments are saved to the event log.
View Event Log	Click to display the Event Log window, which lists all errors, warnings, statuses, and actions that occurred during the run.
Apply Current Settings to Selected Samples	Allows you to transfer analysis settings from one sample to other selected samples within the Analysis Sample List. Available during analysis mode only.
Open Data Set	Allows you to open an FCS 3.0 data file for analysis. The data for the first sample in the file appears. If you currently have a data file open, you will be prompted to save any changes before opening the new file.
Export to FCS 2.0	Exports the current sample data to FCS 2.0 format, which is compatible with third-party analysis programs. Available during analysis mode only.

Analysis Button	Description
Export to Spreadsheet	Exports the results from the current FCS 3.0 file to a CSV (comma-separated values) file for later analysis using a spreadsheet program. This happens automatically if you make changes to and resave the FCS file during analysis. Available during analysis mode only.
Export Std Curve CSV	Exports the standard curve and results from data table to a CSV (comma-separated values) file. Applies to RapidQuant Assay only.
Print Preview	Click this box if you wish to preview the analysis plots and statistics for the current data.
Print	Prints the results for the current sample.

Files

For each assay, guavaSoft Software saves the following files:

- a flow cytometry standard (FCS) 3.0 data file, which is saved automatically
- an spreadsheet results file, which is saved automatically
- a worklist file, which you create and save before you can run an assay

Additionally, you can optionally choose to save a separate file of instrument and analysis settings, as well as FCS 2.0 files for individual samples.

■ **NOTE:** To keep your computer performing optimally, periodically clear old files from your hard drive by archiving the files to a back-up storage location.

Flow Cytometry Standard (FCS) 3.0 Data Files

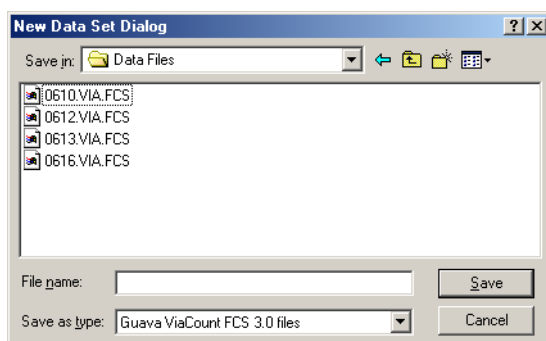
FCS files are data files saved in a format compatible with standard flow cytometry analysis applications as defined by the Society for Analytical Cytology [*Cytometry*. 1990;11(3);323–332]. One FCS 3.0 file is saved for all samples acquired within a data set.

When you start a new data set, you can enter a new file name or select an existing file. The file name assigned to the data file when you start a new data set is also assigned to the spreadsheet results file. An extension is automatically appended to the file name you enter. The first three characters represent the assay type, followed by .FCS.

Assay	File name
guava ViaCount	<i>filename.VIA.FCS</i>
guava ExpressPlus	<i>filename.EP5.FCS</i>
guava ExpressPro	<i>filename.PRO.FCS</i>
guava InCyte	<i>filename.FCS</i>
guava Nexin	<i>filename.NEX.FCS</i>
guava Caspase	<i>filename.CAS.FCS</i>
guava TUNEL	<i>filename.TUN.FCS</i>
guava MitoPotential	<i>filename.MPL.FCS</i>

Assay	File name
guava Cell Cycle	<i>filename.CCY.FCS</i>
guava CellPaint	<i>filename.CPT.FCS</i>
guava CellToxicity	<i>filename.CTX.FCS</i>
guava CellGrowth	<i>filename.CGT.FCS</i>
guava RapidQuant	<i>filename.RPQ.FCS</i>

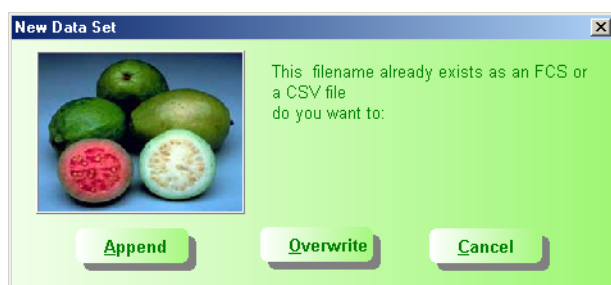
- **NOTE:** Always save guavaSoft Software's data files directly to the laptop's hard drive during acquisition. You may copy the file(s) to another location when acquisition is complete.



Select a directory for file storage. Enter a file name for the data file. Or, select an existing file and either append to it or overwrite it—whichever method you select for saving the data file applies to the spreadsheet file as well.

Appending and Overwriting Existing Files

When you save a data set and you select an existing data file name, you are prompted to either overwrite or append to the file. The corresponding spreadsheet file is also overwritten or appended, and in the case of append, the sample # defaults to the next available number in the existing data file.



If you append data to an existing file, the instrument settings and analysis gates and markers are automatically updated to reflect the settings for the last sample in the file.

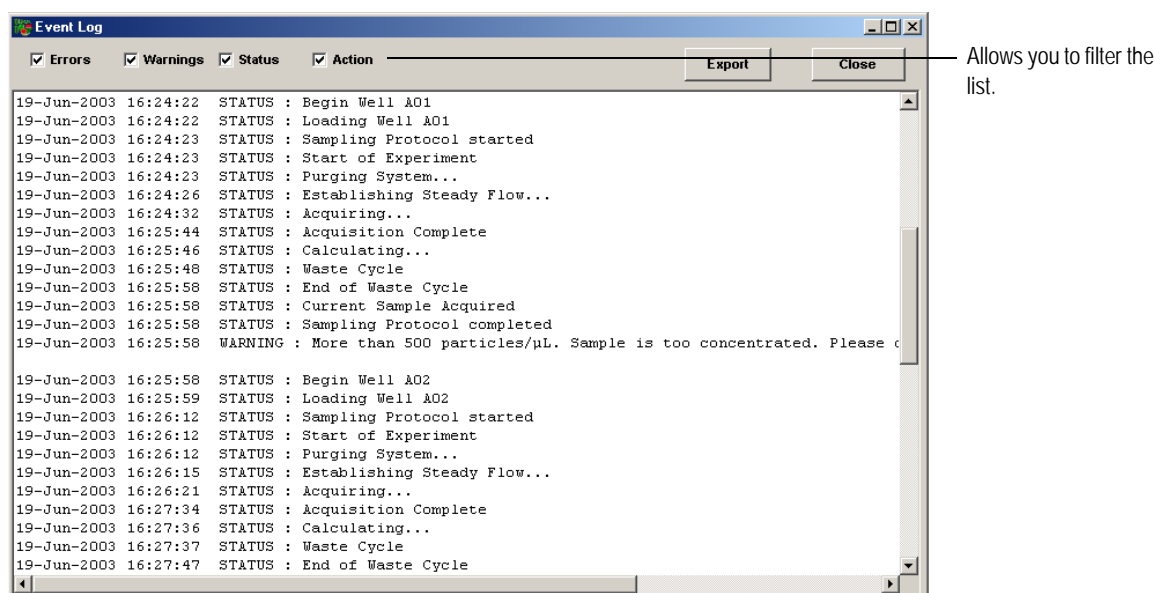
- **NOTE:** Your system administrator may have configured guavaSoft Software to disable appending and/or overwriting files. If appending only is disabled, you may create a new file or overwrite an existing file. If overwriting only is disabled, you may create a new file or append to a copy of an existing file. If both appending and overwriting are disabled, you must create a new file.
- **NOTE:** You cannot append files when using guava InCyte. The append feature is not available.

Exporting to FCS 2.0 Format

To save FCS 2.0 files, click the AutoSave FCS 2.0 Files checkbox in the WorkEdit and WorkList Editor Software. This check box is selected, by default, for the Cell Cycle Assay. You may also export data to FCS 2.0 format from the Analysis screen for subsequent analysis using a third-party software program. FCS 2.0 files contain the data for an individual sample per file.

Event Log

Each time you run an assay, guavaSoft Software saves a log containing a list of all events that occurred during the assay. This information is contained within the FCS 3.0 data file. To view this list, click **View Event Log** from the Analysis screen. A list of all events appears with the date and time the event occurred. The Event Log window automatically appears at the end of the run if an error or warning occurred.



You can filter the list to view errors, warnings, statuses, and/or actions. Click the appropriate check box(es) to display the types of events you wish to view.

Every step the instrument performs, independent of the operator (for example, priming, setting thresholds, performing calculations) is logged. Every step the operator performs (for example, assay buttons clicked, selections, changes to gates and markers, logging comments) is also logged. During data analysis, although changes to settings are logged, the specific details of the change may not be.

If errors or warnings occur during a run, a message appears in red in the status bar indicating that errors/warnings have been logged and how many times they have occurred.

Warnings include:

- *Less than 10 (25 for the Cell Cycle Assay only) particles/μL. Sample is too dilute. Accuracy may be compromised.*

- *More than 500 (1200 for the Cell Cycle Assay and 2500 for the RapidQuant Assay) particles/μL. Sample is too concentrated. Please dilute or accuracy may be compromised.*
In the Guava ExpressPlus Assay, the high concentration warning limits are based on the flow rate selected—the default values are 500 particles/μL for Medium, 800 for Low, and 1200 for Very Low. The default values for the guava ExpressPlus, guava ExpressPro, guava InCyte, guava RapidQuant, and guava Caspase Assays can be changed in WorkEdit Software, and the warnings will reflect the new user-defined concentrations. However, EMD Millipore does not recommend entering values higher than the default values.
- *The run timed out before enough events were acquired.*
- *Adjust Settings timed out. Please re-enter Adjust Settings if necessary to complete the instrument set-up.*
- *Maximum velocity exceeded for “x” events.* (applies only to area/width parameters)
- *Your system has only “xx” MB of free disk space left.* (appears when trying to save a guava ExpressPro Assay data file with 500 MB or less of free disk space)

Errors include:

- *The tray door was opened. When you click OK, worklist will be halted but your acquired data will be saved. To continue, either restart the worklist or create a new worklist beginning from the well that was aborted when the door was opened.*
- *Too few events for EasyFit Analysis.* (for ViaCount Assay only, if EasyFit Analysis is enabled)
- *Too much debris for EasyFit Analysis.* (for ViaCount Assay only, if EasyFit Analysis is enabled)
- *EasyFit Analysis could not be performed on this sample.* (for ViaCount Assay only, if EasyFit Analysis is enabled)
- *Error occurred while trying to generate FCS 2.0 file for the sample no. x.*
- *Error occurred while updating gate definitions.* (for guava ExpressPlus and ExpressPro Assays only)

To export the log to a text file, click **Export**. Select the storage location and enter a file name. Then, click **Save**.

Spreadsheet Files

guavaSoft Software creates a spreadsheet file containing data for all samples within a data set. This file can be opened and analyzed using a spreadsheet program such as Microsoft® Excel. The file name you assign to the FCS 3.0 data file is also used for the spreadsheet file. The extension .CSV is appended to the spreadsheet file name. The spreadsheet file is saved in the same directory as the data file and contains all the relevant statistical results, sample information, and instrument settings for each sample run within a data set. For the guava ExpressPro and guava RapidQuant Assays, the sample information and instrument settings are automatically exported, but you must manually select the statistical results for each sample within a data set. For information on selecting the statistics to export to the spreadsheet file for these assays, refer to “Selecting Statistics for the Spreadsheet File” on page 6-22 and “Selecting Statistics for

the Spreadsheet File” on page 16-17.

- **NOTE:** When you analyze a data set in Analysis mode and save the changes, a spreadsheet file is automatically created. If you click **Stop** in the Export Spreadsheet dialog box before all sample data is exported, only the sample data that was exported before you clicked Stop will appear in the spreadsheet file. If you wish to create a spreadsheet file to include all data, use the Export to Spreadsheet option (see page 1-17).

The guava Excel Utility program allows you to extract the viability and concentration information from the CSV file and save it to a separate Excel file. For information on this utility, which is installed on your guava laptop computer, refer to “Guava Excel Utility” on page 4-21.

Worklist Files

A worklist file contains all of the information for each well/tube entered at the WorkEdit (or WorkList Editor) screen. You must save a worklist file before you can run a guava assay, except in the guava ExpressPro, InCyte, Caspase, and RapidQuant Assays where a worklist can be started immediately. The extension .gwl is appended to the worklist file name, except for InCyte worklists, which are .xml files. Refer to “Creating a Worklist” on page 1-29 for more information on worklists.

Settings Files

guavaSoft Software allows you to save settings files. These files contain instrument settings as well as analysis gates and marker settings. An extension is automatically appended to the file name you enter. The first three characters represent the assay type, followed by .GST for guava Settings.

Assay	File name
guava ViaCount	<i>filename.VIA.GST</i>
guava ExpressPlus	<i>filename.EP5.GST</i>
guava ExpressPro	<i>filename.PRO.GST</i>
guava InCyte	<i>filename.GST</i>
guava Nexin	<i>filename.NEX.GST</i>
guava Caspase	<i>filename.CAS.GST</i>
guava TUNEL	<i>filename.TUN.GST</i>
guava MitoPotential	<i>filename.MPL.GST</i>
guava Cell Cycle	<i>filename.CCY.GST</i>
guava CellPaint	<i>filename.CPT.GST</i>
guava CellToxicity	<i>filename.CTX.GST</i>
guava CellGrowth	<i>filename.CGT.GST</i>
guava RapidQuant	<i>filename.RPQ.GST</i>

Settings

guavaSoft Software allows you to save the current instrument settings and analysis gates and markers to a file. You can recall this file later to:

- download the instrument settings to the guava easyCyte HT System for acquisition
- apply the gates and markers to data during acquisition

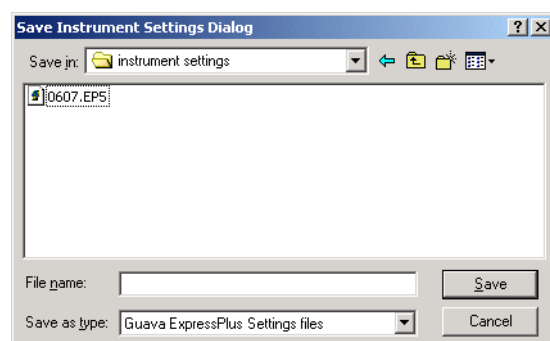
Saving Instrument Settings and Analysis Markers

You cannot save settings until you have performed the adjust settings step.

- 1 Click **Settings** from the Acquisition screen.
The following dialog box appears.

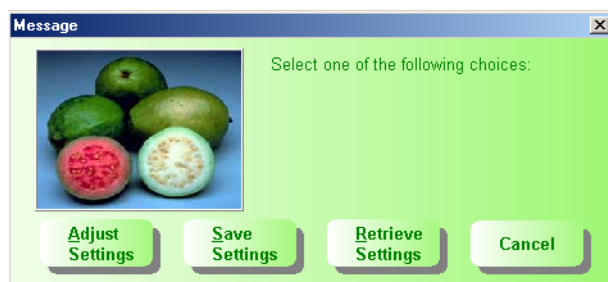


- 2 Click **Save Settings**. Enter a name for the file, select the directory where you wish to save it, and click **Save**.



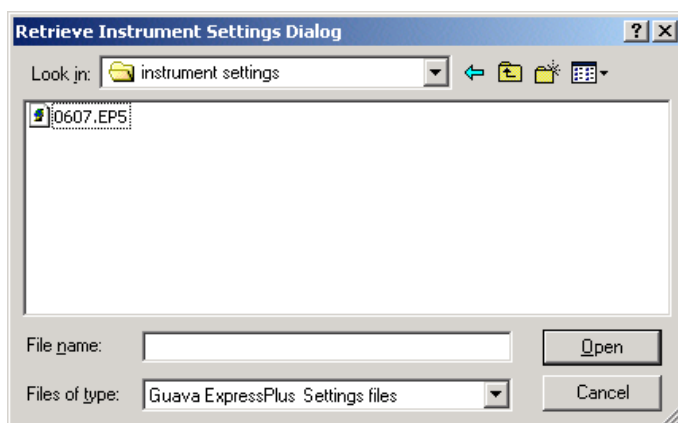
Retrieving Instrument Settings and Analysis Markers

- 1 Click **Settings** from the Acquisition screen.
The following dialog box appears.



- 2 Click **Retrieve Settings**.

- 3 Locate the file and click **Open**.



The settings are downloaded to the guava easyCyte HT System and the software automatically proceeds to the settings dialog box.

- **NOTE:** If you retrieve instrument settings after you perform the adjust settings step, you will be required to repeat the adjust settings step to ensure that the thresholds are set correctly.

Worklists

WorkEdit Software

WorkEdit™ Software allows you to set up the acquisition commands to acquire samples from a microplate and/or 10 tube locations in the sample tray.

WorkEdit Software applies to the following assays: guava ViaCount, guava ExpressPlus, guava Nexin, guava TUNEL, guava MitoPotential, guava Cell Cycle, guava CellPaint, guava CellToxicity, or guava CellGrowth.

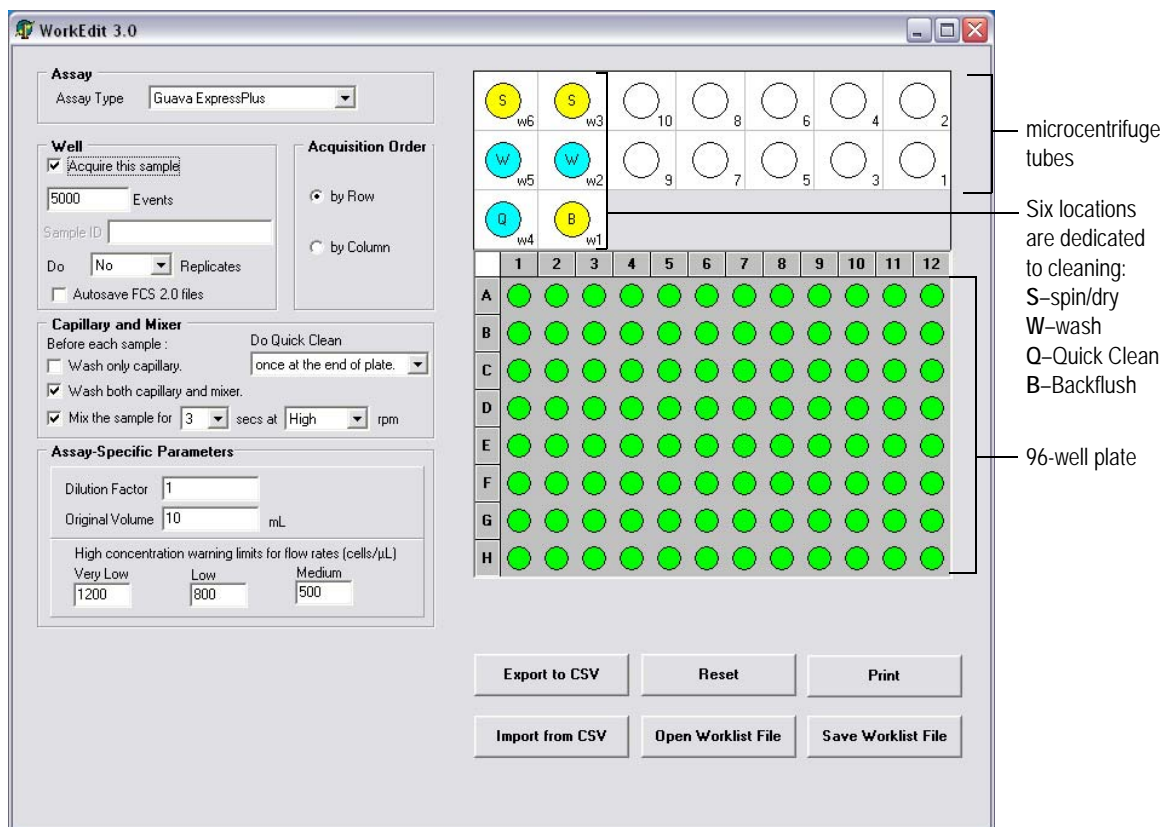
The right side of the screen displays a map for the microplate, allowing you to select wells for acquisition. Above the plate map are locations for up to 10 tubes for samples and six tubes for washing and cleaning functions. The left side of the screen allows you to select the assay, the number of events to acquire for each selected well/tube, the number of acquisition replicates, and the acquisition order. You can also select to save FCS 2.0 files for each sample, wash the mixer and/or capillary, and choose to mix samples before acquisition.

You can also create a CSV file that can be used as a template and modified using Excel or other spreadsheet program to quickly create subsequent worklists. See "Creating a Worklist Template" on page 1-33.

The information entered in WorkEdit Software will appear in the Sample Information control panel in guavaSoft Software during the assay. However, you cannot modify the information within guavaSoft Software.

See "Worklist Editor Software" on page 1-27 for more information on how Worklist Editor Software functions with the guava InCyte, ExpressPro, RapidQuant, and Caspase Assays.

NOTE: The options you select within WorkEdit Software will always supersede the settings from a file that you retrieve settings from or append.



guava WorkEdit Software screen

WorkEdit Fields

Field	Description
Assay	Choose guava ViaCount, guava ExpressPlus, guava Nexin, guava TUNEL, guava MitoPotential, guava Cell Cycle, guava CellPaint, guava CellToxicity, or guava CellGrowth. You may select only one assay per worklist.

Field	Description
Well	<ul style="list-style-type: none"> Click Acquire this sample after selecting a well/tube for acquisition. Wells/tubes selected for acquisition appear in green. Enter the number of Events to acquire for the selected well/tube. The default is 1000 for the ViaCount and CellToxicity Assays, 3000 for the three-color guava Caspase Assay, 5000 for the guava ExpressPlus, guava InCyte, guava ExpressPro, and Cell Cycle Assays, 500 for the guava RapidQuant Assay, and 2000 for all other assays. The ranges are 100 to 100,000 (CellToxicity Assay), 200,000 (guava ExpressPlus, guava ExpressPro, guava RapidQuant, and guava Caspase Assays), and 20,000 (all other assays). For the guava ExpressPlus, guava ExpressPro, guava RapidQuant, and guava Caspase Assays, you may also set an acquisition duration (1–600 seconds), depending on the flow rate selected. Acquisition ends when the first limit (number of events or time limit) is reached. Enter an optional Sample ID for each individual well or tube. If you do not enter an ID, the ID defaults to the well/tube number. Select the number of Replicates (2–8 for wells; 2–15 for tubes). If you wish to acquire the sample only once, select No. Check Autosave FCS 2.0 files to save files in FCS 2.0 format. This option is selected as the default for the Cell Cycle Assay. <p>■ NOTE: All well features can be applied to selected wells.</p>
Acquisition Order	<p>Choose by Row or by Column to acquire selected wells by row or column. The recommended acquisition order is by Row.</p> <p>Acquiring by row starts with row A (wells A1, A2, etc) and ends with row H. Acquiring by column starts with column 1 (wells A1, B1, etc) and ends with column 12. Only the wells selected in the worklist will be acquired. If tubes and wells are selected for acquisition, samples in tubes will be acquired in numerical order before samples in wells.</p>
Capillary and Mixer	<p>Select Wash only capillary to wash the capillary between samples. Select Wash both capillary and mixer if you choose to mix samples and wish to wash the capillary and mixer between samples. All washing functions apply to the entire run (tubes and wells). Select Mix the sample for (1–10) seconds, then select Low, Med, or High mixing speed. All mixing functions apply to the entire run (tubes and wells), except for guava ExpressPlus, guava ExpressPro, and guava InCyte Assays, which allow you to choose mixing times and speeds for individual samples.</p> <p>Select an option to perform a Quick Clean cleaning cycle during the run. Choose once at the end of the plate, every 48 samples, every 24 samples, every 12 samples. Regardless of the option selected, the system will always perform a Quick Clean after the last sample.</p> <p>For the guava ExpressPro Assay only, you can select to perform a Clean & Rinse during the run for a more thorough cleaning. Choose once at the end of the plate (default if Clean & Rinse is selected), every 48 samples, or every 24 samples.</p>

Field	Description
Assay-Specific (Sample Specific) Parameters	<p>This information applies to guava ViaCount, guava ExpressPlus, guava ExpressPro, and guava InCyte Assays.</p> <ul style="list-style-type: none"> For ViaCount Assay, enter the dilution factor and original volume (defaults are 20 and 10, respectively). Select Enable EasyFit if you would like the software to calculate viable and dead/apoptotic cells for you. The EasyFit Analysis feature will apply to the entire run (tubes and wells). For guava ExpressPlus, guava ExpressPro, and guava InCyte Assays, enter the dilution factor and original volume (defaults are 1 and 10, respectively). You may lower the high concentration warning limits from the default values shown, if necessary. EMD Millipore does not recommend entering values higher than the default values. The software will display a warning message during acquisition if the cell concentration exceeds the concentration value you enter for the selected flow rate.

Use the buttons at the bottom of the WorkEdit screen:

- **Export to CSV** to save a CSV template file for an assay. Select the assay and click Export to CSV. Enter a filename and click Save.
 - **Reset** to clear all changes and revert back to the original screen when WorkEdit was first opened.
 - **Print** to print the WorkEdit screen.
 - **Import from CSV** to import values from a CSV file, for example, a template file that you exported, into a worklist file
 - **Open Worklist File** to open a saved worklist file. WorkEdit software worklist files are saved with the extension .gwl.
 - **Save Worklist File** to save a worklist file.
- **NOTES:** When you export a worklist to a CSV file, you can then edit the file using Microsoft Excel or other third-party spreadsheet program.
- The Sample ID, Sample Well, and General settings fields are required. If fields are blank or values are erroneous, a default value will appear and a message will indicate this.
 - If all values for a particular column are identical, the item and value can be added to General settings. If an item is mentioned in both the column and General settings, the column values are given preference.
 - The Assay Type field in General settings is required.
 - A sample position can be designated for acquisition by making any alphanumeric entry in the Sample ID field.
 - Columns and the values in General settings can be reordered.

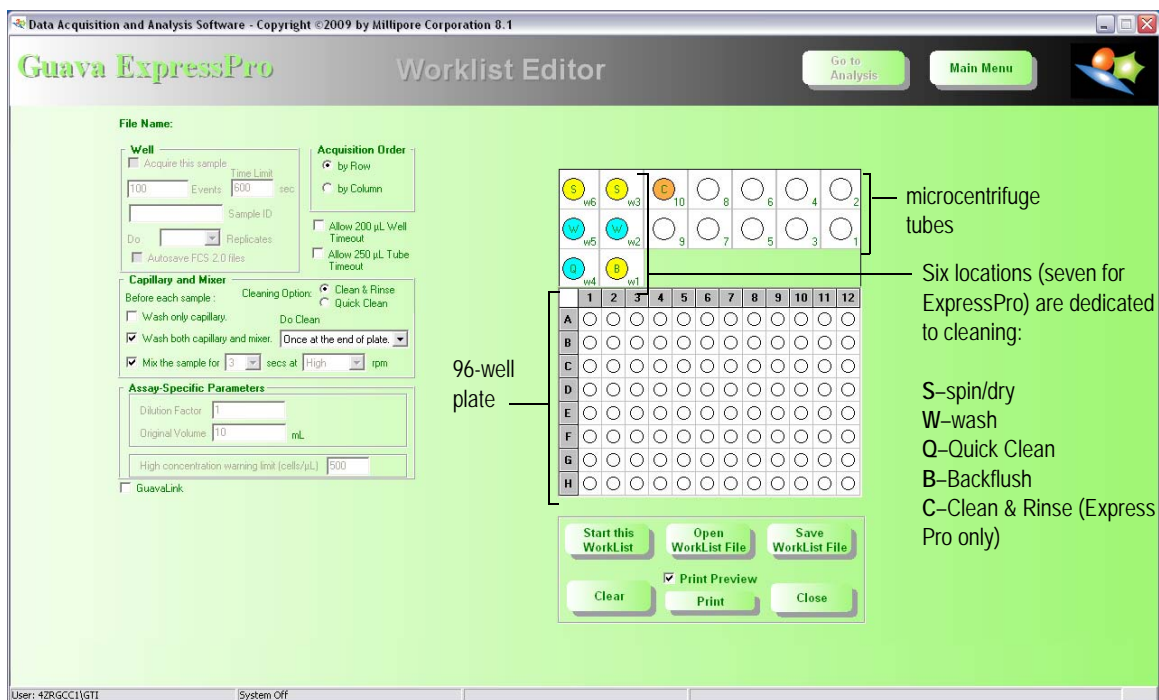
Worklist Editor Software

Worklist Editor Software applies to the guava ExpressPro, RapidQuant, Caspase, and InCyte Assays

If you are using the guava ExpressPro, guava RapidQuant, guava Caspase, or guava InCyte Assay for data acquisition, you can open Worklist Editor Software directly from the assay screen by clicking **Worklist Editor** in the acquisition button panel. Worklist Editor launches within each software module. You can also open Worklist Editor from the Analysis screen, however, only to generate, modify, and save worklists. To run a worklist you must be in Acquisition mode. The basic layout of the Worklist Editor window is the same as WorkEdit Software with a few additional features.

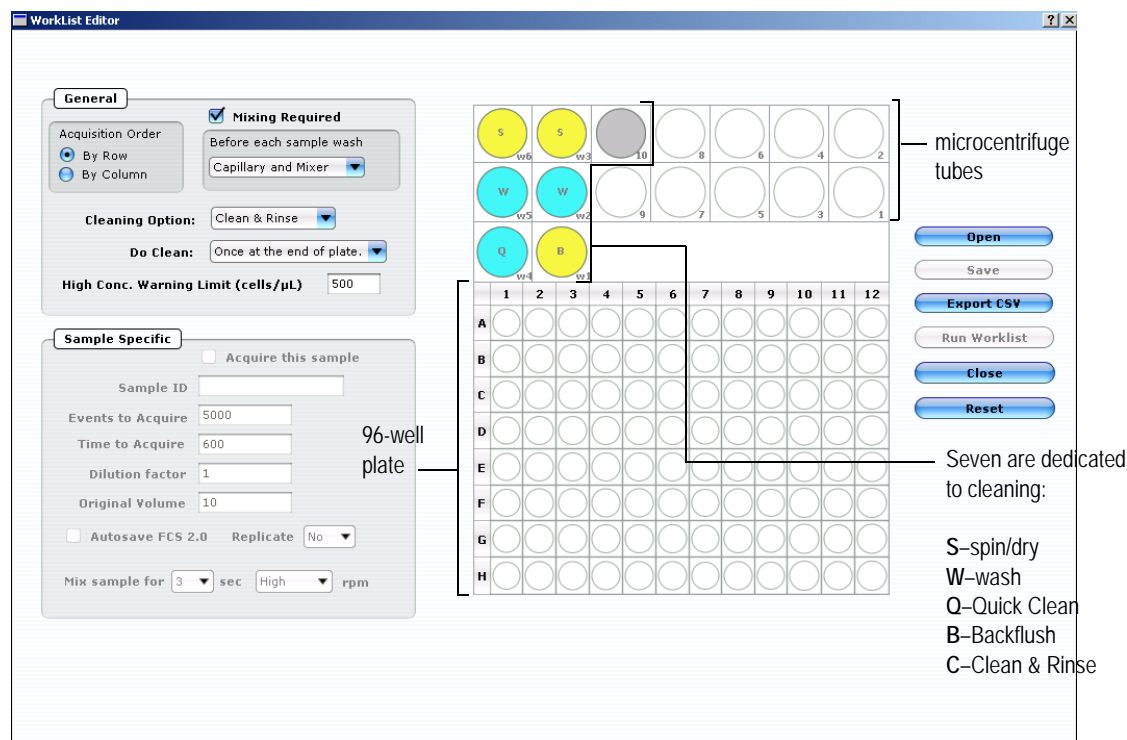
The information entered in Worklist Editor will appear in the guava ExpressPro, guava RapidQuant, guava Caspase, or guava InCyte Sample Information control panel during the assay. However, you cannot modify the information once you start the worklist.

For guava InCyte you can create a CSV file that can be used as a template and modified using Excel or other spreadsheet program to quickly create subsequent worklists. See “Creating a Worklist Template” on page 1-33.



guava Worklist Editor screen from within guava ExpressPro Assay

The Worklist Editor for guava InCyte varies slightly from the Worklist Editor for the other four assays, mainly in the button panel.



guava Worklist Editor screen from within guava InCyte Assay

Follow the same instructions for creating a worklist found on page 1-29. Once you have selected the tubes/wells and entered the acquisition information, click **Start this Worklist** (**Run Worklist** for InCyte) to start the worklist.

You can also click:

- **Open Worklist File** (Open in InCyte) to open a saved worklist file
- **Save Worklist File** (Save in InCyte) to save the worklist to a file
- **Clear** (Reset in InCyte) to clear all changes made and revert back to the original screen
- **Print** to print the Worklist Editor screen
- **Close** to close the window and return to the assay
- **Export to CSV** for WorkList Editor launched from guava InCyte, to export a CSV file. See notes below.

■ NOTES:

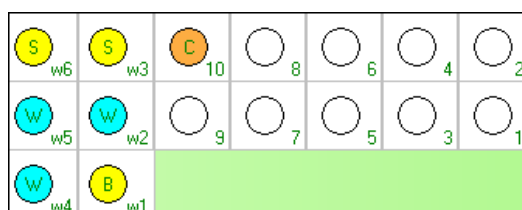
- You cannot print the worklist screen from InCyte's WorkList Editor.
- You cannot import or export CSV files from ExpressPro, Caspase, and RapidQuant WorkList Editor.

Creating a Worklist

Use WorkEdit Software to select wells and/or tubes for acquisition, and program specific acquisition commands.

- 1 Open WorkEdit Software by double-clicking the WorkEdit icon on the desktop.
For the guava ExpressPro, guava InCyte, guava RapidQuant, and guava Caspase Assays, click **Worklist Editor** from the assay window.
 - **NOTE:** Do not open WorkEdit Software while guavaSoft Software is running, except when in the guava ExpressPro, guava InCyte, guava RapidQuant, or guava Caspase Assay.
- 2 Select the **Assay Type**.
You can select one assay per worklist file. Assay-Specific Parameters will appear at the bottom-left corner of the WorkEdit screen for the ViaCount, ExpressPlus, ExpressPro, and RapidQuant Assays.
 - **NOTE:** This step does not apply to the guava ExpressPro, InCyte, RapidQuant, and Caspase Assays.
- 3 Select wells/tubes for acquisition.
You may select wells/tubes all at once, or you can select groups of wells that will have the same acquisition criteria applied to them (see steps 5–7).
 - **NOTE:** Tube locations w1 through w6 are designated for capillary/mixer washes, instrument cleaning, and backflushing. Tube location 10 is designated for washing if you select Clean & Rinse for the guava ExpressPro and InCyte Assays only.
 - Click an individual well/tube to select it. To select groups of wells/tubes, click and drag, or press the Shift key and click. To select non-adjacent wells/tubes, press the Ctrl key and click the wells/tubes.
 - Click letters A–H on the microplate map to select a row.
 - Click numbers 1–12 on the microplate map to select a column.
 - Click the white box in the upper-left corner of the plate map to select the entire plate.
 - To deselect, press the Ctrl key while clicking the well/tube, column, or row.
- 4 Click **Acquire this sample** to mark the selected wells/tubes for acquisition.
The wells/tubes selected for acquisition appear green.
- 5 Enter the number of events to acquire for the selected wells/tubes.
The default is 500 for the RapidQuant Assay, 1000 for the ViaCount and CellToxicity Assays, 3000 for the three-color (SR and FAM with 7-AAD) guava Caspase Assay, 5000 for the guava ExpressPlus, guava ExpressPro, InCyte, and Cell Cycle Assays, and 2000 for all other assays. The range is 100 to 2×10^4 (100 to 1×10^5 for the CellToxicity Assay, 100 to 2×10^5 for the guava ExpressPlus, guava ExpressPro, guava InCyte, guava RapidQuant, and two-color guava Caspase Assays, and 3×10^3 to 2×10^5 for the three-color guava Caspase Assay).
 - **NOTE:** Acquiring events significantly above these recommended ranges, especially with a high sample number, may eventually overload the computer's system memory and cause errors.

- 6 For guava ExpressPro, InCyte, RapidQuant, and Caspase Assays, select a time limit. This time is the maximum time that sample will be acquired, up to the maximum time allowed for a given flow rate.
- 7 Enter the number of acquisition replicates for the selected wells/tubes.
For wells, you may select 2–8 replicates; for tubes, you may select 2–15 replicates. To select only one acquisition per well/tube, select **No**.
 - **NOTE:** Although you can acquire replicates using guava InCyte, you cannot analyze replicates.
- 8 Check the Autosave FCS 2.0 files check box if you want to save files in FCS 2.0 format for the selected wells/tubes.
 - **NOTE:** Keep in mind FCS files can take up significant space on your hard drive. Back up data regularly to keep your computer performing optimally.
- 9 Select the Acquisition Order. Choose either by Row or by Column.
If you selected tubes for acquisition, samples in tubes will be acquired before the samples in wells. The recommended acquisition order is by Row.
- 10 Select the appropriate wash and mix features.
The options you select apply to the entire run (tubes and wells).
 - If you choose to wash the capillary only, place a 1.5-mL microcentrifuge tube filled with deionized water in tube position w5.
 - If you choose to wash the capillary and the mixer, place two 1.5-mL microcentrifuge tubes filled with deionized water in tube positions w2 and w5, and place two empty tubes in positions w3 and w6.
 - **NOTE:** Select to wash the mixer only if you are mixing samples.
 - **NOTE:** To minimize carryover, EMD Millipore recommends changing the wash water in w2 after every plate.
 - **NOTE:** When washing the capillary and mixer, change the tube in w3 at the end of every plate if it's more than a third full.



For washing capillary and mixer, load tubes filled with water in positions w2 and w5. Load empty tubes in positions w3 and w6.

Load a tube with 100 µL bleach in position w1 for backflushing.

Always load a tube filled with water in position w4 for the Quick Clean, which is run automatically at the completion of the assay.

For ExpressPro only, when running Clean & Rinse, load tubes filled with guava ICF in position 10, filled with water in position w4, and containing 100 µL bleach in position w1.

- You can choose to mix each sample before acquiring it. Then, select the length of time and speed you wish to mix each sample. For the guava ExpressPlus, guava

ExpressPro, guava InCyte, and guava RapidQuant Assays, the mixer speed and mix time can be applied to individual wells or tubes.

- **NOTE:** EMD Millipore recommends mixing at high speed for 3 seconds; although certain cell types may require a longer mixing time at the same or lower speed.
- Select the frequency of performing a Quick Clean during the assay. For the guava ExpressPro Assay only, you can choose one of two cleaning options—Quick Clean or Clean & Rinse. If you choose Clean & Rinse, place a 1.5-mL microcentrifuge tube filled with guava Instrument Cleaning Fluid (ICF) in position 10. Ensure that the tube in w1 contains 100 μ L of bleach for backflushing and the tube in w4 is filled with DI water for both Clean & Rinse and Quick Clean.
- **IMPORTANT:** You can use Clean & Rinse a total of four times before you need to refill the w4 tube with water and tube 10 with ICF, and empty then add 100 μ L of bleach to the w1 tube for backflushing. Always remember to fill and/or empty these tubes after every four Clean & Rinse cycles or at the end of each run.
- **NOTE:** EMD Millipore recommends performing a Quick Clean at least every 24 samples for the guava Cell Cycle and guava MitoPotential Assays.

11 If applicable, proceed to the assay specific parameters at the bottom of the screen for the guava ViaCount, guava ExpressPlus, guava ExpressPro, guava InCyte, and guava RapidQuant Assays.

- For the ViaCount, guava ExpressPlus, guava ExpressPro, and guava InCyte Assays enter the dilution factor and the original volume. The original volume is the volume of the cell suspension, from which you took your sample aliquot, before you diluted or stained it.
- For ViaCount Assay only, select **EasyFit** if you want the software to calculate viable cells and dead/apoptotic cells. The placement of the viability and FSC threshold markers will be ignored by the EasyFit Analysis algorithm during analysis. If you select EasyFit, all samples within the entire run will be analyzed and saved with the EasyFit Analysis method.

Once you select EasyFit you cannot change the setting during acquisition. However, you can turn off the feature later (for selected samples) during analysis from the Analysis screen and manually analyze the data by setting the viability marker and apoptosis marker, if you wish. You can also acquire data without the EasyFit Analysis feature enabled, and enable it later (for selected samples) during analysis.

For more information on using EasyFit Analysis, refer to “EasyFit Analysis” on page 4-10. For examples of when to use EasyFit Analysis versus manual analysis, refer to Appendix A: EasyFit Case Study Examples.

12 For the guava ExpressPlus, guava ExpressPro, guava InCyte, and guava Caspase Assays, lower the high concentration warning limits from the default values shown, if necessary. The software will display a warning message during acquisition if the cell concentration exceeds the concentration value you enter for the selected flow rate.

There is also a high concentration warning limit in the RapidQuant Assay; however it should not be adjusted under normal conditions.

High concentration warning limits for flow rates (cells/ μ L)		
Very Low	Low	Medium
1200	800	500

For guava ExpressPlus Assays

High concentration warning limit (cells/ μ L)	500
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For guava ExpressPro and guava Caspase Assays

High Conc. Warning Limit (cells/ μ L)	500
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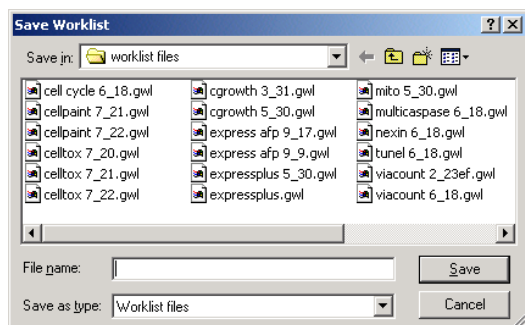
For guava InCyte Assay

High concentration warning limit (beads/ μ L)	2500
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For guava RapidQuant Assay

- **NOTE:** If you are acquiring in the ExpressPlus, InCyte, or ExpressPro Software Module and your samples are heterogeneous in size or granularity, such as lysed whole blood, or do not contain cells simultaneously positive for two fluorochromes, such as PBMCs stained with CD3 and CD19, consider lowering the default high concentration warning limits (and acquiring your cells at lower concentrations). This will minimize the number of coincident events, which could adversely affect your results. EMD Millipore recommends a concentration of less than or equal to 200 cells/ μ L for these types of samples.

- 13 Click **Save Worklist File** to save the file.
Worklist files are saved as .gwl, except for InCyte worklist files, which are saved as .xml or .csv files.



- 14 Click **Print** if you wish to print the WorkEdit screen.
- 15 Close the WorkEdit window before starting guavaSoft Software by clicking the close box in the upper-right corner of the window.
If you launched Worklist Editor from within the guava InCyte, ExpressPro, RapidQuant, or Caspase Assay, click **Start this Worklist** if you are ready to begin the assay.

Creating a Worklist Template

You can create a worklist template for WorkEdit software and Worklist Editor software for InCyte only. The template can be modified and updated using Microsoft Excel or similar spreadsheet program.

To create the template, you will export a worklist, then use Excel or other spreadsheet program to edit the fields. Then, simply import the file back into WorkEdit (or Worklist Editor for InCyte) Software to run your worklist.

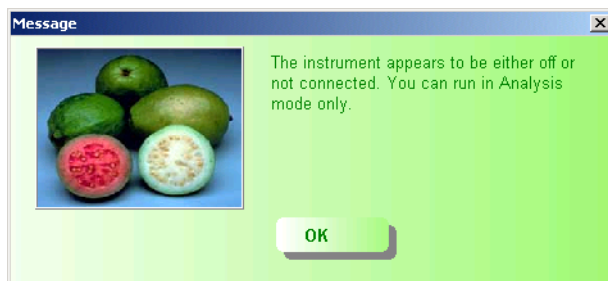
- **NOTE:** You cannot create a CSV file if you are using Worklist Editor Software for ExpressPro, RapidQuant, or Caspase Assays.
- 1 Open WorkEdit or Worklist Editor and select the desired settings to create a worklist.
- 2 Export to a CSV file. This CSV file will serve as your template.
 - If you are using WorkEdit software, select **Export to CSV**.
 - If you are using Worklist Editor Software for InCyte, select **Export CSV**.
- 3 Open the CSV file in Excel or compatible spreadsheet software.
 - Edit the Sample ID column, as necessary (up to 40 characters).
 - Do not modify the Sample Well column. This information pertains to the sample well or tube location.
 - The Sample ID, Sample well, Assay Type, and General settings headers are required.
 - To designate a sample for acquisition, enter a value in the Sample ID field.
 - If values are not entered for certain fields, default values may apply.
 - If values entered are not valid (for example, non-numerical values in fields that require numbers), default values are applied. For InCyte, an error message appears, indicating the applicable cells.
 - Columns positions (headings) can be changed, as can General settings values.
 - Templates for InCyte and ExpressPlus are unique in that they include columns for Mix speed and Mix time. These assays allow sample-specific Mix Speed and Mix Time settings.
- 4 Save the file as .csv. Do not save it in Excel format (for example, .xls).
- 5 Open the file in Worklist Editor or WorkEdit software for the application that you are running.
 - If you are using WorkEdit software, select **Import from CSV**.
 - If you are using Worklist Editor Software for InCyte, select **Open**.

CHAPTER 2

Getting Started

System Startup

- 1 Turn on the power conditioner if it is not already on. Once it is powered on, it can remain on.
- 2 Turn on the laptop computer.
- 3 When the computer is finished booting up, turn on the guava easyCyte HT System. The power switch is located half-way up on the right side at the back of the instrument (see figure of “guava easyCyte HT System components” on page 1-6).
- 4 Start guavaSoft Software by double-clicking the **guavaSoft 2.7** application icon on the desktop.
You can also click the **Start** button, point to Programs, point to Millipore, point to guavaSoft 2.7, then click **guavaSoft**.
 - **NOTE:** If the software detects a communication problem with the easyCyte HT System or that the system is not turned on, the following message appears.



guavaSoft Software will start but you will be able to access an assay's Analysis mode only. If you wish to perform acquisition, exit guavaSoft Software. Ensure that the USB cable between the computer and the instrument is securely connected before restarting the computer. When the computer is finished booting up, turn on the guava easyCyte HT System and start guavaSoft Software.

Running the easyCheck Procedure

Run the easyCheck Procedure at the start of each day you use the guava easyCyte HT System to ensure the system is performing properly. easyCheck averages the results from three acquisitions of a guava easyCheck Bead sample to determine if the results are within the expected range.

- **NOTE:** Before running the easyCheck Procedure, perform a Quick Clean to prime the fluid system. If it has been more than a day since you used your guava system, perform two Quick Cleans using water to prime the fluid system.
- 1 Prepare a 1:20 dilution of the guava® easyCheck Bead Reagent. Refer to the *guava easyCheck Kit* package insert for information.
 - 2 Click **easyCheck** from the main menu.
Allow the instrument to warm up for 10 minutes before acquiring the beads.

The screenshot shows the 'easyCheck' software interface. On the left, there's a sidebar with 'Unit Control' and 'Sample Information'. The main area is titled 'Acquisition'. It features input fields for 'Bead Lot #', 'Bead Expiration Date', and 'Expected Particles/mL'. A table titled 'easyCheck Results' shows data for three replicates across parameters: Sample Information, Particles/mL, FSC Intensity, SSC Intensity, GFP-MFI, YLW-MFI, RED-MFI, NIR-MFI, PD2-MFI, and N2-MFI. The 'Average % CV' row shows 0.00% for all parameters. On the right, there's a 'Main Menu' button and a 'Start' button. A status bar at the bottom indicates 'Ready to Start' and '00:00 of 00:00'.

Enter bead lot #, expiration date, and particles/mL.

- 3 The first time you run the easyCheck Procedure, enter the **Bead Lot #** and **Bead Expiration Date** (found on the guava easyCheck Bead Reagent vial label) and **Expected Particles/mL** in the appropriate fields. Thereafter, enter any necessary changes to these values.
Optionally, you may enter the guava® easyCheck Kit lot number and expiration date (found on the side of the guava easyCheck Kit box).
The Expected Particles/mL is approximately 50,000; however, check the information card that comes with the guava easyCheck Kit for the actual particle count for each new lot. The particles/mL corresponds to the concentration of beads in your prepared sample where the guava easyCheck Bead Reagent was diluted 1:20 with Guava® Check Diluent.
- **NOTE:** Your system administrator may have configured guavaSoft Software to require that you enter values in these fields each time you run the easyCheck Procedure. If the fields are blank when you access the easyCheck screen, you must enter the current information.

4 Click **Start**.

◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

5 The sample tray opens and a dialog box appears prompting you to load DI water, and empty tubes, and select the tube/well containing the beads.

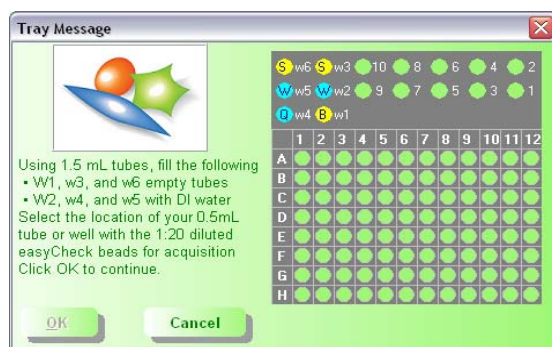
- Load tubes filled with DI water in w2, w4, and w5.

■ **IMPORTANT:** Always load tube position w4 with water (for Quick Clean).

- Load empty tubes in w3 and w6.
- Load a tube containing 100 μ L of bleach in position w1 (for disinfecting backflushed fluid)
- Load the easyCheck sample in a 0.5-mL tube.
- Click to select the tube/well with beads.
- Click **OK**.

◆ **WARNING:** If you click **Cancel** in the dialog box, the tray will automatically load. Keep the area clear as the tray loads.

■ **NOTE:** If you are using a microplate, make sure well A1 of the plate is in the top-right corner of the tray.



The system then automatically performs the adjust settings function and acquires three replicates of the bead sample and displays the Particles/mL, FSC Intensity, SSC Intensity, and GRN, YLW, RED, NIR, RED2, and NIR2 (if applicable) mean fluorescence intensity (MFIs) for each replicate. The averages and %CVs for all results are also displayed.

■ ACQUISITION NOTES

- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 1 minute.
- If the acquisition rate appears to slow dramatically, the fluid pathway may be blocked or the sample volume may be low. If you have sufficient sample, click **Stop** and wait for the system to stop, then click **Backflush**. When the backflush is complete, click **Quick Clean**. When the cleaning is complete, click **Start**.

easyCheck Results

The software displays the %CVs and the averages for the particles/mL (bead count), FSC and SSC intensities, and GRN, YLW, RED, NIR, RED2, and NIR2 (if applicable) mean fluorescence intensity (MFI) for the three replicates.

- If any result for Particles/mL falls outside $\pm 10\%$ of the expected value, the result is outside the acceptable range and appears in red. For example, if the actual particle count is 50,000, the acceptable Particles/mL range ($\pm 10\%$) is 45,000 to 55,000.
- If the %CV for Particles/mL is $> 10\%$, it appears in red.
- The %CV for FSC and SSC Intensities, and GRN/YLW/RED/NIR/RED2/NIR2 MFI for the three replicates should be $< 5\%$.

easyCheck Results									
Sample Information	Particles/mL	FSC Intensity	SSC Intensity	GRN MFI	YLW MFI	RED MFI	NIR MFI	RD2 MFI	NIR2 MFI
Replicate 1	49670	861	3344	200	791	508	196	507	802
Replicate 2	52178	862	3405	203	802	514	199	513	813
Replicate 3	52672	860	3417	205	818	508	197	494	797
Average	51507	861	3389	202	803	510	197	505	804
% CV	3.13%	0.14%	1.17%	1.21%	1.67%	0.74%	0.86%	1.93%	0.99%

NOTE: Example of results from instrument with all six detectors installed.

Check Results displays the Particles/mL for each replicate. Results appearing in red are outside the $\pm 10\%$ acceptance range.

FSC and SSC Intensities and GRN/YLW/RED/NIR/RED2/NIR2 MFI results can be used to monitor instrument performance over time.

Refer to the information card that comes with the guava easyCheck Kit for the acceptable intensity ranges for each parameter. This information may change from lot to lot.

To monitor instrument performance, look at the average and %CV values for FSC and SSC Intensity and GRN, YLW, RED, NIR, RED2, and NIR2 (if applicable) MFI. Refer to “Viewing and Exporting easyCheck Results” on page 2-5 for information on displaying past easyCheck results.

If the Particles/mL (count) for a replicate or the average falls outside the acceptance range, or if an intensity value is outside the acceptable range, run Quick Clean (refer to page 3-1) or Clean & Rinse (see page 3-3). Rerun the easyCheck Procedure after cleaning is complete. If values continue to fall outside the acceptance range, refer to “easyCheck Procedure Troubleshooting” on page 2-8, for more information.

If the signal intensity for any of these parameters shows significant drift over time beyond the range listed, and this change is not correlated to a change in the bead lot, a new flow cell, or instrument service, contact EMD Millipore.

Viewing and Exporting easyCheck Results

To display a history of all easyCheck runs and view the results for individual runs, click Show History at the bottom of the easyCheck screen. The History List control panel opens showing a list of all easyCheck runs. To display the results for a particular run, click on the run in the list.

easyCheck Analysis

Unit Control
Sample Information
History List

Dead Lot#
Board Expiration Date

Expected Particles/mL
5000

Log Comment
View Event Log
Show Trend Graph
Print Screen
Export to Spreadsheet

easyCheck Results

Sample Information	Particles/mL	FSC Intensity	SSC Intensity	GRN MFI	YLW MFI	RED MFI	NIR MFI	RD2 MFI	NI2 MFI
Replicate 1	51323	1702	2902	1646	1709	2922	2679	1732	2003
Replicate 2	49136	1779	2894	1643	1767	2911	2670	1732	2796
Replicate 3	49327	1782	2904	1647	1790	2926	2683	1732	2803
Average	49928	1781	2900	1645	1789	2920	2677	1732	2801
% CV	2.42%	0.08%	0.19%	0.11%	0.11%	0.26%	0.24%	0.02%	0.14%

Info:

Voltages and Gain		Threshold	
FSC Gain:	1.0	FSC:	244
SSC Voltage:	250	SSC:	498
GRN Voltage:	250	GRN:	20
YLW Voltage:	250	YLW:	427
RED Voltage:	250	RED:	93
NIR Voltage:	250	NIR:	37
RD2 Voltage:	250	RD2:	0
NI2 Voltage:	250	NI2:	0

Log Comment
View Event Log
Show Trend Graph
Print Screen
Export to Spreadsheet

Last Guava Check: Never

Show History

Ready for Next Sample

00:00 of 00:00

User: 42RGCC1\GTI System Off Laser Off

NOTE: Example of easyCheck Procedure history results from instrument with no SSC detector installed.

- Click **Log Comment** to enter comments related to the run and save these comments to the event log.

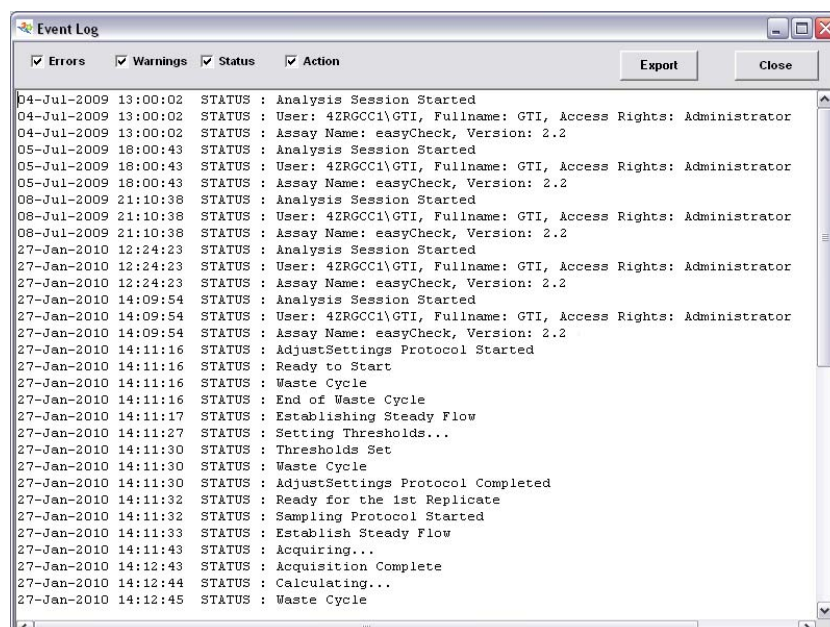
Log Comments

Enter comments to the Event Log:

Clear Save Comments to Log Cancel

- Click **View Event Log** to display the event log, which lists all errors, warnings, statuses, and actions that occurred during the easyCheck run. For more information

on the event log, refer to “Event Log” on page 1-19.

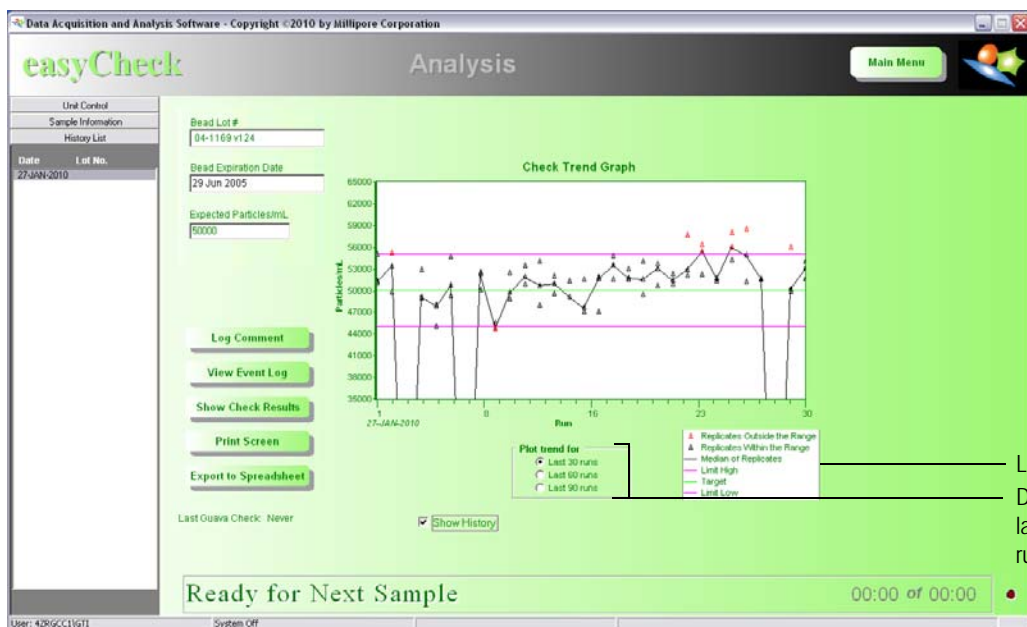


- Click **Show Trend Graph** to display a trend graph of the Particles/mL value from the last 30, 60, or 90 runs. See “Viewing Trend Data” on page 2-7.
- Click **Print Screen** to print the results. A print dialog box appears allowing you to select the printer.
- Click **Export to Spreadsheet** to export the data from all easyCheck runs to a spreadsheet file. The file contains the average and %CV for each parameter, as well as the details for each replicate of all runs.

Viewing Trend Data

To view a trend graph of the Particle/mL data, click Show History at the bottom of the screen, then click **Show Trend Data**.

A trend graph appears showing the Particles/mL results from the last 30, 60, or 90 runs. A data point appears for each of the three replicate values. The date appears for every seventh or eighth time that the easyCheck Procedure was run.



Legend
Display data from
last 30, 60, or 90
runs.

A legend in the lower-right corner of the window lists the information found on the graph. A description of the items in the legend appears in the following table.

Legend Item	Description
Replicates Outside Range	data point appears as a red triangle (value falls outside the high or low, 10% limit lines)
Replicates Within Range	data point appears as a black triangle
Median of Replicates	a black line connects the median values from each triplicate
Limit High	pink line that appears 10% above expected particle count
Target	green line at the expected particle count entered
Limit Low	pink line that appears 10% below expected particle count

easyCheck Procedure Troubleshooting

Problem	Possible Cause	Solutions
No event counts appear for RED2 and NIR2.	<ol style="list-style-type: none"> 1. Wrong beads used. 2. Red laser not operating or problem with the signal. 	<ol style="list-style-type: none"> 1. Use easyCheck beads. Do not use Guava Check beads. 2. Contact EMD Millipore Technical Support.
One or more Particles/mL results falls outside the acceptance range (appear in red).	<ol style="list-style-type: none"> 1. System is not clean. 2. Incorrect information entered in easyCheck fields. 3. Bead suspension incorrectly prepared. 	<ol style="list-style-type: none"> 1. Run Quick Clean, then rerun easyCheck Procedure. If results still fall outside range, run Guava Clean. 2. Ensure correct Bead Lot # and Expected Particles/mL are entered. Refer to easyCheck Beads vial label and information card for values. 3. Prepare fresh bead sample and rerun easyCheck Procedure. Refer to <i>guava easyCheck Kit</i> package insert for preparation instructions.
FSC, SSC, GRN, YLW, RED, NIR, RED2, and/or NIR2 intensity is >10% outside the acceptable range.	<ol style="list-style-type: none"> 1. System is not clean. 2. Problem with detector or laser. 	<ol style="list-style-type: none"> 1. Run Quick Clean. If results are still outside range, run Guava Clean. 2. If problem persists, contact EMD Millipore Technical Support.
Particle counts for FSC, SSC, GRN, YLW, RED, NIR, RED2, and/or NIR2 intensity is not within 100 events of each other.	<ol style="list-style-type: none"> 1. If FSC count is low, capillary may not be seated correctly. 2. If any of the counts is low, possible problem with detector. 	<ol style="list-style-type: none"> 1. Remove metal plate. Unscrew tubing from top of flow cell and firmly push down on top of flow cell assembly. If problem persists, contact EMD Millipore Technical Support. 2. Rerun easyCheck Procedure. If counts are still low, contact EMD Millipore Technical Support.

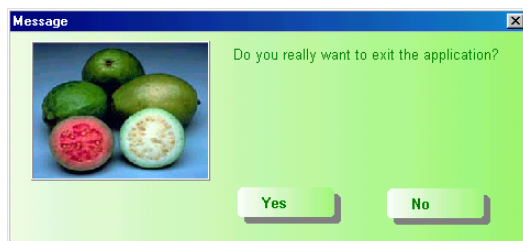
Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells and 150 µL for 0.5-mL tubes. <i>Use 0.5-mL tubes in the tube position, as a higher volume (1 mL) is required for 1.5-mL tubes.</i>
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. No beads in sample. 4. Clogged flow cell. 5. Broken flow cell. 6. Sample pump not working. 7. Laser not operational. 8. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells and 150 µL for 0.5-mL tubes. <i>Use 0.5-mL tubes in the tube position, as a higher volume (1 mL) is required for 1.5-mL tubes.</i> 3. Ensure correct sample is loaded. 4. Perform a Backflush. Follow with Quick Clean. 5. Remove flow cell and inspect for damage, including chipped capillary. Replace, if necessary. 6. Run Quick Clean and watch for fluid in waste vial. 7. Contact EMD Millipore Technical Support. 8. Ensure tubing connector is secure.
<p>Message:</p> <p><i>The login user does NOT have read/write permission to the file GuavaCheckLog.csv in the Log folder. Contact the system administrator for assistance.</i></p>	The user was not assigned access control to the system.	Contact your system administrator for user access to the software.

System Shutdown

- 1 Run a cleaning procedure at the end of the day. See “Running Guava Clean” on page 3-4 for information.
 - 2 Exit guavaSoft Software. Do not shut down the guava easyCyte HT System while guavaSoft Software is running.
 - 3 To shut down the guava easyCyte HT System, press the power switch located half-way up on the right edge at the back of the instrument.
- ◆ **WARNING:** Ensure the sample tray is loaded before you turn off the power to the easyCyte HT System.

Quitting guavaSoft Software

- 1 Click the **Exit** button in the upper-right corner of the main menu. A confirmation dialog box appears.



- 2 Click **Yes** to exit the software.

CHAPTER 3

Cleaning and Maintenance

guavaSoft Software offers three cleaning cycles—Quick Clean, Clean & Rinse, and Guava Clean. Run **Quick Clean** as often as you like throughout an assay to quickly clean the fluid system. Run **Clean & Rinse** to thoroughly clean the system after an assay or before running an assay where instrument sensitivity is critical. Run **Guava Clean** at the end of each day to thoroughly clean the fluid system.

To clean the outside of the instrument, wipe it down with a soft cloth moistened with 70% alcohol. Follow with a cloth moistened with water.

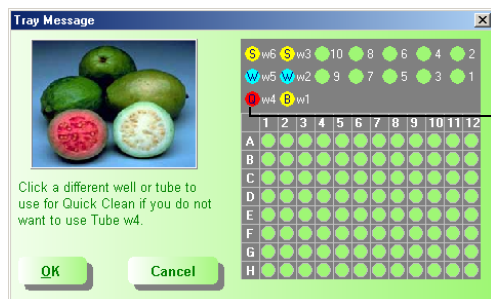
◆ **WARNING:** To avoid exposure to laser radiation or electric shock, *DO NOT* open the guava easyCyte HT System or attempt to perform any internal maintenance. There are no user serviceable parts.

Running Quick Clean

Quick Clean is a short cleaning cycle that allows you to clean the fluid system during or after an assay. The Quick Clean feature is accessible from each guavaSoft Assay screen, as well as from the easyCheck screen. At the completion of an assay, the system automatically performs a Quick Clean. For the guava InCyte and ExpressPro assays, you can choose between Quick Clean or Clean & Rinse. You can also perform additional Quick Clean and Clean & Rinse cycles as often as you like during an assay.

- 1 Place a tube filled with deionized water in tube position w4 when you load the tray at the start of a run.
 - 2 If you wish to perform Quick Clean using a cleaning solution other than water, you can add one of the following to another tube or well. The system will prompt you to select the tube/well.
 - undiluted Guava Instrument Cleaning Fluid (ICF) to clean the system, followed by water to rinse
 - a 10% bleach solution in Guava ICF (1 part bleach in 9 parts Guava ICF; for example, 100 μ L bleach plus 900 μ L Guava ICF) to clean and sanitize, followed by water to rinse
- **NOTE:** You can perform approximately three Quick Clean cycles from a single well and seven Quick Cleans from a 1.5-mL tube containing 1.5 mL of fluid.

- 3 Click **Quick Clean** from the easyCheck screen or the guava InCyte or ExpressPro Assay screen.
A message appears prompting you to select the well or tube used for the Quick Clean. The default tube for Quick Clean is position w4.
- **NOTE:** If the system is acquiring samples, click **Pause**, then click **Quick Clean**.
- 4 Leave the default position w4 selected, or click to select a different well/tube. Click **OK**.



Tube w4 is default Quick Clean position. Click OK to select tube w4, or select a different well/tube and click OK.

(NOTE: The dialog boxes that prompt you to load tubes or select a sample may have slightly different appearances, however their functionality is the same.)

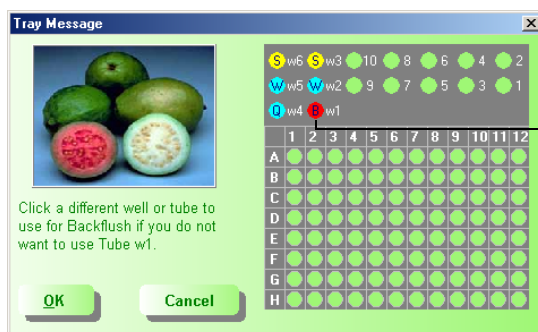
- 5 If you ran water, you are finished. If you ran Guava ICF, either straight or diluted with bleach, click **Quick Clean** again to rinse, select the well/tube containing water, and click **OK**.
- 6 If you paused the run to perform the Quick Clean, click **Resume**.
- **NOTE:** If you will not be using the system for 30 minutes or more, leave the instrument on and leave guavaSoft at the current assay. After a Quick Clean, the capillary tube is left in distilled water to prevent it from drying out.

Backflushing the Fluid System

The backflush feature reverses the flow of fluid out of the flow cell. Click the Backflush button, located on the each assay screen and the easyCheck screen, when you suspect that the fluid pathway is blocked.

- 1 Click **Backflush** from the easyCheck screen or any guava assay screen.
A message appears prompting you to select the well or tube used for backflushing. The default tube for backflushing is position w1, containing 100 μ L of bleach.
- **NOTE:** If the system is acquiring samples, click **Pause**, then click **Backflush**.

- 2 Leave the default position w1 selected, or click to select a different well/tube. Click **OK**.



Tube w1 is default backflush position. Click OK to select tube w1, or select a different well/tube and click OK.

(NOTE: The dialog boxes that prompt you to load tubes or select a sample may have slightly different appearances, however their functionality is the same.)

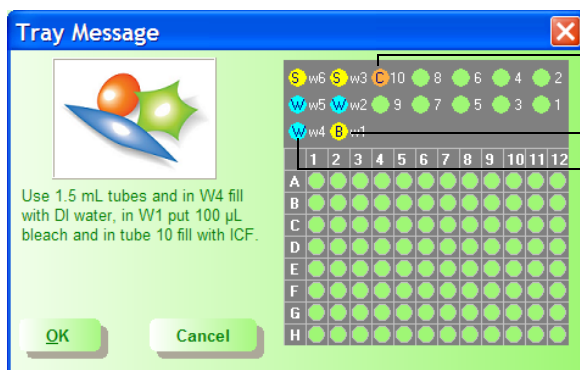
- 3 When the backflush is complete, click **Quick Clean** to rinse the bleach from the capillary. Follow the instructions on page 3-1 to perform a Quick Clean.
- 4 Click **Resume** if you paused the run to perform a backflush.

Running Clean & Rinse

Clean & Rinse automatically performs a series of Quick Clean cleaning cycles with one backflush to thoroughly clean the fluid system. Use Clean & Rinse when optimum system sensitivity is needed. Clean & Rinse is available from Worklist Editor Software, as well as from the guava InCyte or ExpressPro acquisition screen, allowing you to incorporate Clean & Rinse cycles into these assays.

- 1 Click **Clean & Rinse** from the guava InCyte or ExpressPro acquisition screen. You can also select it as a cleaning option in Worklist Editor Software for these applications.

A message appears prompting you to load a tube filled with DI water in position w4, a tube of 100 μ L bleach in position w1, and a tube filled with Guava ICF in position 10.



Load a tube of ICF in position 10.

Load a tube of bleach in position w1.

Load a tube of DI water in position w4.

(NOTE: The dialog boxes that prompt you to load tubes or select a sample may have slightly different appearances, however their functionality is the same.)

- 2 Load a tube filled with DI water in w4, a tube with 100 μ L of bleach in w1, and a tube filled with ICF in tube position 10. Click **OK**.

- **NOTE:** You can perform approximately four Clean & Rinse cycles before you will need to refill the fluid in tubes w4 and 10, and empty then refill tube w1 with bleach.

Running Guava Clean

Run a cleaning cycle at the end of each day to thoroughly clean the fluid system and the outside of the capillary and mixer. If your instrument has not been used for a few days, you may clean it at the start of the day to thoroughly flush the fluid system. You may also clean between assays if a thorough cleaning is needed.

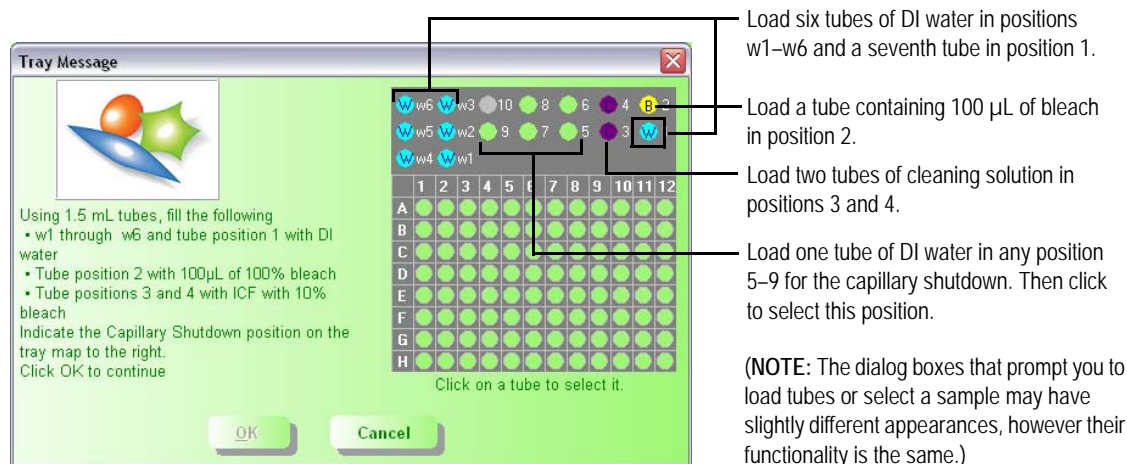
If running samples that have high background, such as lysed whole blood, we recommend running two complete cleaning cycles to flush the system of any residual sample.

- 1 Click **Cleaning** from the guavaSoft main menu.
The Guava Clean screen appears.



- 2 Click **Start Clean**.
The plate is ejected.
- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.
- 3 Load the following tubes on the instrument:
 - Load seven tubes filled with DI water in positions w1, w2, w3, w4, w5, w6, and tube position 1.
 - Load a tube containing 100 μ L of bleach in position 2, indicated by the yellow well.
 - Load two tubes filled with Guava ICF in tube positions 3 and 4. See the following additional notes on cleaning solution.

- Load a tube filled with DI water in any tube position 5–9 for the capillary shutdown. Then, click to select this position on the plate map.



■ NOTES on cleaning solution:

- If you are running a cleaning cycle between assays, run undiluted Guava ICF as the cleaning solution (positions 3 and 4).
- If you are shutting down the instrument at the end of the day or you wish to sanitize the system, run a 10% bleach solution in Guava ICF (1 part 100% bleach in 9 parts Guava ICF) as the cleaning solution (positions 3 and 4).

4 After selecting the tube position for DI water for the capillary shutdown, click **OK**.

◆ **WARNING:** If you click **Cancel** in the dialog box, the tray will automatically load. Keep the area clear as the tray loads.

5 If you are finished using the instrument, click **Eject Tray** and dispose of the used plate and tubes. Then, shut down the capillary to keep it moist while the instrument is not in use. See “Shutting Down the Capillary” on page 3-6.
The instrument shuts down with the capillary in a tube of water to minimize clogging, particularly when the instrument is used infrequently.

■ **NOTE:** If you will not be using the instrument for an extended time, perform the capillary shutdown procedure periodically, using a fresh tube of DI water to ensure that the capillary does not dry out.

6 Click **Main Menu** to return to the guavaSoft Software main menu, or click **Exit** to close guavaSoft Software.

■ **NOTE:** Do not reuse the tubes that were previously used for the Guava Clean procedure. After the cleaning cycle is complete, dispose of the tubes according to your local regulations.

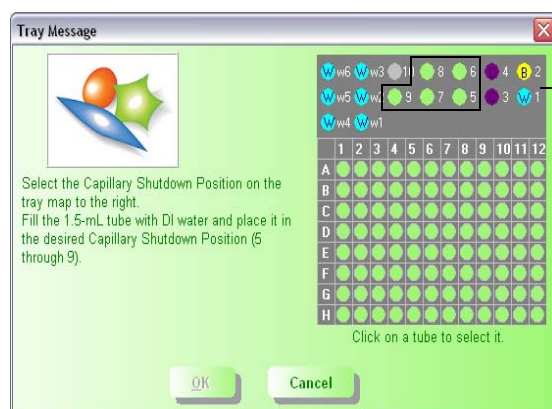
Shutting Down the Capillary

At the completion of Guava Clean, eject the tray and dispose of the used plate and tubes. Then, to keep the capillary wet when you turn off the system, use the Capillary Shutdown feature to place the capillary in a tube of water.

- **NOTE:** If you will not be using the instrument for an extended time, perform the capillary shutdown procedure periodically, using a fresh tube of DI water to ensure that the capillary does not dry out.

1 Click **Capillary Shutdown**.

A dialog box appears prompting you to select the tube for the capillary shutdown position.



Select a tube position (5-9) for the capillary shutdown. Place a tube with 1.5 mL DI water in the selected position.

(NOTE: The dialog boxes that prompt you to load tubes or select a sample may have slightly different appearances, however their functionality is the same.)

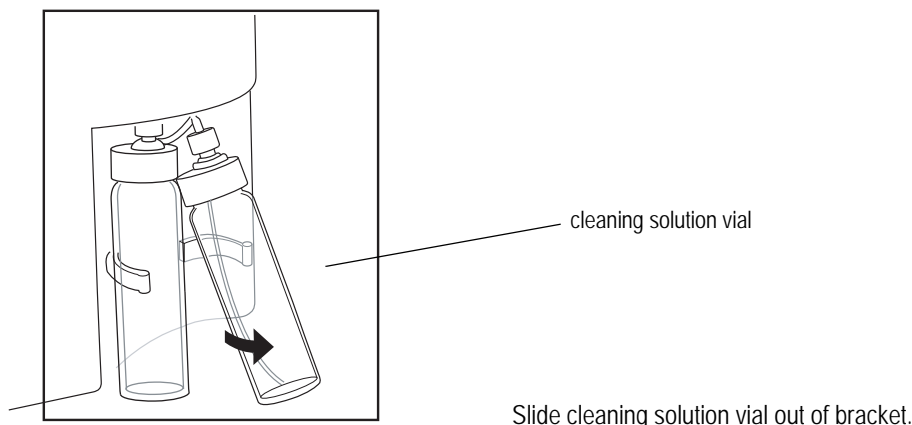
- 2 Click to select a tube position 5 through 9 at the top of the plate map.
- 3 Place a tube containing 1.5 mL DI water in that location and click **OK**.

Filling the Cleaning Solution Vial

Fill the cleaning solution vial with ICF. The solution is aspirated through the tubing in the vial. Do not allow the vial to empty. This will create air bubbles in the fluid system and require that you prime the system with water. When the vial is down to 1/4 full, fill it with ICF. One vial of ICF will allow you to perform approximately 15 cleaning cycles.

- **NOTE:** Check the cleaning solution vial fluid level frequently to ensure it does not run dry. If the vial runs dry air can be aspirated into the system.

- 1 Gently pull up on the vial to remove it from the bracket. The cleaning solution vial is located on the right.



- 2 Unscrew the vial from the cap.
- 3 Fill the vial with ICF to just below the bottom of the cap. Do not overfill the vial.
- 4 Replace the vial on the cap assembly and install the vial on the easyCyte HT System.
- 5 Ensure the tubing that goes into the vial is still attached to the cap.

Emptying the Waste Vial

Empty the waste collection vial at the end of each day, or as needed.

- ◆ **WARNING:** Handle all biological specimens and materials they come in contact with as if capable of transmitting infection. Dispose of these materials using proper precautions in accordance with federal, state, and local regulations.

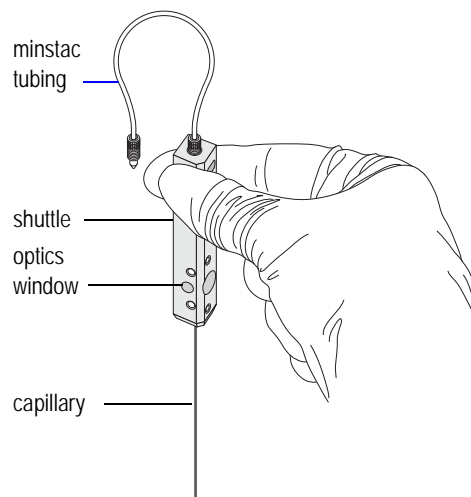
- 1 Gently pull out the vial to remove it from the bracket. The waste vial is located on the left.
- 2 Twist the luer-lock connector to remove the waste tubing from the vial cap.
- **NOTE:** Fluid may seep from the waste tubing while it is disconnected from the vial. To prevent waste fluid from dripping on the work surface, place the free end of the tubing in a small beaker or other container.
- 3 Unscrew the vial cap and empty the contents according to your local and state biohazardous waste disposal guidelines.
- 4 Rinse the waste vial with water.
- 5 Add approximately 10 mL of bleach to the waste vial, replace the cap, and install the vial on the guava easyCyte HT System.

Cleaning the Flow Cell

About the Flow Cell

The flow cell assembly consists of the shuttle, the capillary, and the minstac tubing. The shuttle houses the optical window, where the laser beam intersects the sample. When handling the flow cell assembly, grasp it towards the top of the shuttle, where the minstac tubing is attached to avoid getting fingerprints on the optics window. When installing a flow cell, ensure this tubing connection is tight to avoid fluid leaking from the top of the shuttle.

A flow cell removal/tightening tool is provided and can be used to remove the flow cell from the instrument, as well as tighten the minstac tubing.

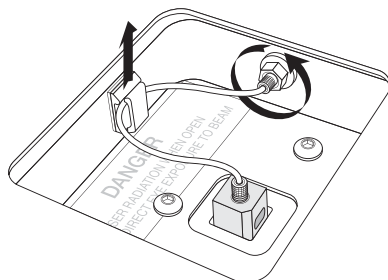


Using the Syringe Tool to Clean the Flow Cell Assembly

Use the syringe cleaning tool to clean the flow cell. When removing the flow cell, handle it with care. The capillary is fragile; avoid touching it unnecessarily. Do not force the flow cell into the receptacle. However, slight pressure may be required to properly seat the flow cell once it has been inserted into the receptacle.

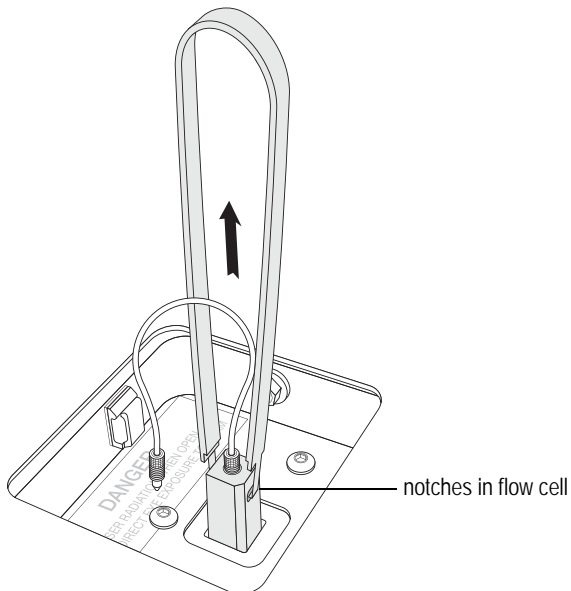
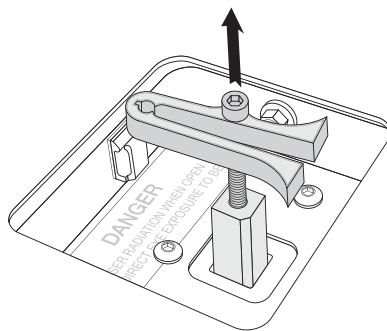
◆ **WARNING:** To avoid exposure to laser radiation, turn off the power to the guava easyCyte HT System before attempting to remove the flow cell.

- 1 Remove the metal plate from the top of the instrument.
- 2 Remove the tubing from the clamp and disconnect it from the instrument.

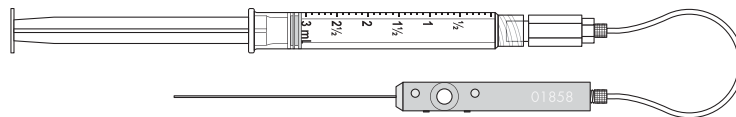


- 3** Remove the flow cell by grasping it at the top with your fingers and pulling straight up. Do not pull up on the tubing. You can also use the flow cell removal tool to remove the flow cell. See the instructions below, depending on the type of flow cell and removal tool you have.

- Unscrew the tubing at the top of the flow cell and set it aside.
 - Screw the threaded stem of the removal tool into the top of the flow cell shuttle.
 - Pull the tool straight up to remove the flow cell, as shown below.
 - Disconnect the removal tool, then reattach the minstac tubing to the top of the flow cell.
- Grasp the flow cell with the removal tool by placing the clamps into the cutouts at the top of the flow cell, as shown.
 - Pull the tool straight up to remove the flow cell.



- 4** Fill the syringe cleaning tool with water or Guava ICF. Connect the syringe to the minstac tubing on the flow cell. Ensure the fitting is tight.

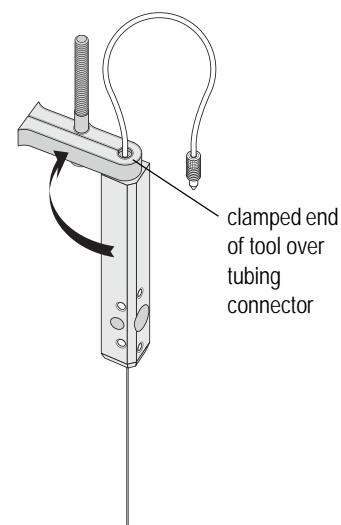


- 5** Use a Kimwipe® to hold the flow cell at the top of the shuttle to capture fluid that may leak. Apply gentle, steady pressure to the plunger and watch as the fluid flows from the tip of the capillary.
- Make sure the fluid stream is straight. If it is not straight, the tip of the flow cell may be chipped or there may be a partial clog in the flow cell.
 - Check the capillary to ensure there are no leaks along the length of it.
 - Make sure fluid is not leaking where the tubing is connected at the top of the flow cell.
- 6** Unscrew the syringe from the minstac tubing. Leave the minstac tubing attached to the flow cell.

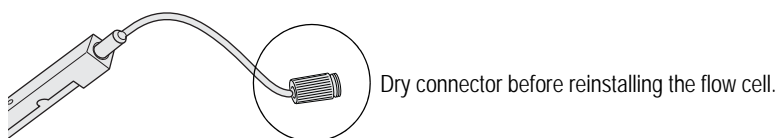
- 7** If you noticed leaks at the tubing connection and you have the tool shown at the right, use the tool to tighten the tubing connection. A tight connection ensures that fluid will not leak from the top of the flow cell.

- Point the tool's threaded stem up (away from the flow cell) and insert the tubing connector into clamp end of the removal tool.
- Squeeze the handles of the tool to secure the flow cell, while tightening.

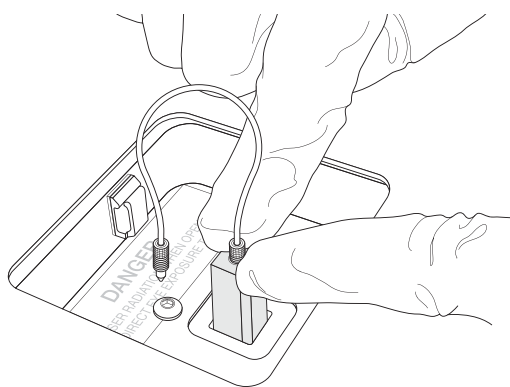
If you don't have the tool shown at the right, use your fingers to hand-tighten the tubing connection.



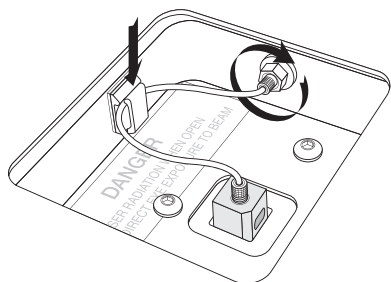
- 8** Use a Kimwipe to dry the end of the tubing that you disconnected from the syringe.



- 9** If the flow cell is clean and intact, reinstall it. If it is damaged or not functioning properly, discard it and replace it with a new flow cell. Install the clean (or new) flow cell by correctly positioning it vertically above the instrument and carefully lowering it into the receptacle. The flow cell fits only one way into the receptacle. Avoid bumping the capillary as you install it. Use your fingers to push down on the top of the flow cell on either side of the tubing until the flow cell clicks into place. Do not press down on the tubing at the top of the shuttle.



- 10 Connect the tubing to the instrument. Make sure the tubing is screwed on tightly by hand tightening only. Then insert the tubing into the clamp.

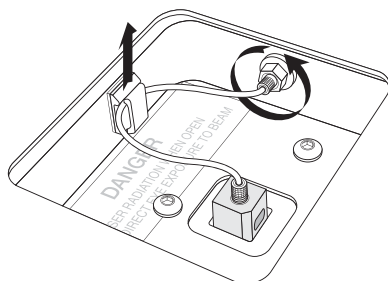


- 11 Ensure that the cleaning solution vial is full, then run Quick Clean to prime the system. If starting the instrument after it has been shut down, run Guava Clean to prime.
- 12 To ensure that the flow cell was correctly installed, run the easyCheck procedure. While the first replicate is being acquired, watch for bubbles in the minstac tubing. If bubbles or leaks are visible, the tubing may not be adequately tightened.

Replacing the Flow Cell

You can replace the flow cell if it becomes damaged or clogged so severely that backflushing the system, running Guava Clean, and using the syringe tool do not fix the problem. When replacing the new flow cell, handle it with care. The capillary is fragile; avoid touching it unnecessarily. Do not force the flow cell into the receptacle.

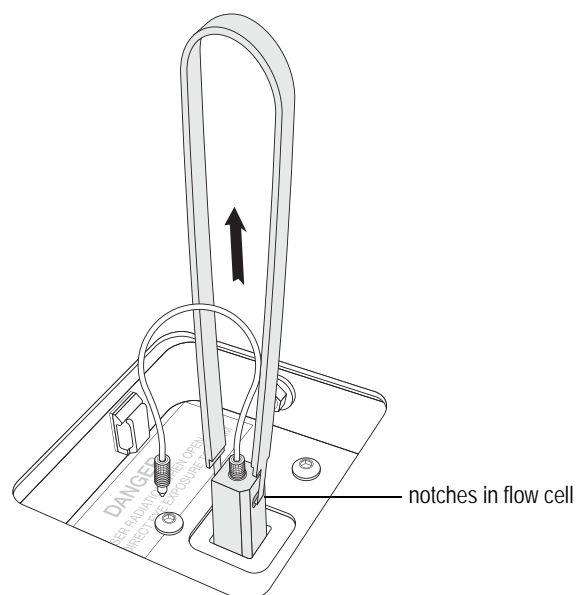
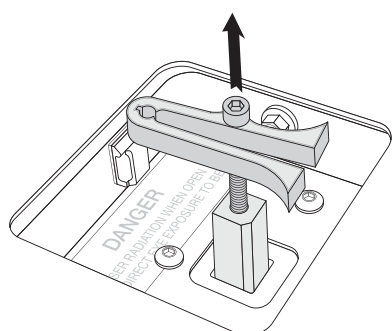
- ◆ **WARNING:** To avoid exposure to laser radiation, turn off the power to the guava easyCyte HT System before attempting to remove the flow cell.
- 1 Remove the metal plate from the top of the instrument.
 - 2 Remove the minstac tubing from the clamp and disconnect it from the instrument.



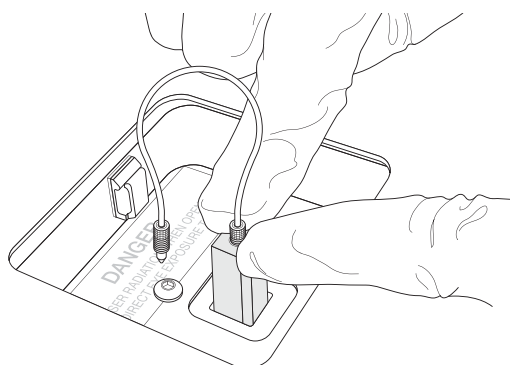
3 Remove the flow cell by grasping it at the top with your fingers and pulling straight up. Do not pull up on the tubing.

You can also use the flow cell removal tool to remove the flow cell. See the instructions below, depending on the type of flow cell and removal tool you have.

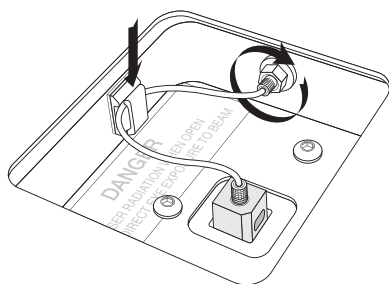
- Unscrew the tubing at the top of the flow cell and set it aside.
- Screw the threaded stem of the removal tool into the top of the flow cell shuttle.
- Pull the tool straight up to remove the flow cell, as shown below.
- Disconnect the removal tool, then reattach the minstac tubing to the top of the flow cell.
- Grasp the flow cell with the removal tool by placing the clamps into the cutouts at the top of the flow cell, as shown.
- Pull the tool straight up to remove the flow cell.



4 Install a new flow cell by correctly positioning it above the instrument and carefully lowering it into the receptacle. The flow cell fits only one way into the receptacle. Keep the flow cell completely vertical and avoid bumping the capillary against the instrument or sides of the receptacle. Use your fingers to press down on the top of the flow cell on either side of the tubing until the flow cell clicks into place. Do not press down on the tubing at the top of the shuttle.



- 5 Connect the tubing to the instrument, then insert the tubing into the clamp.

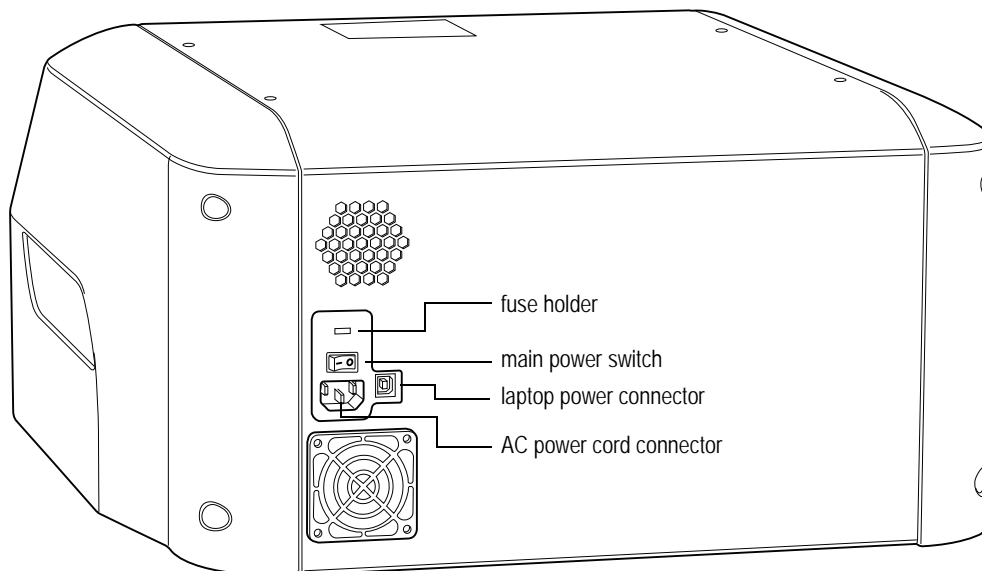


- 6 Ensure that the cleaning solution vial is full, then run Quick Clean to prime the system. If starting the instrument after it has been shut down, run Guava Clean to prime.
- 7 To ensure that the flow cell was correctly installed, run the easyCheck procedure. While the first replicate is being acquired, watch for bubbles in the minstac tubing. If bubbles or leaks are visible, the tubing may not be adequately tightened.

Replacing the Fuses

The AC fuse is located on the rear panel to the right of the AC power cord connector.

- ◆ **WARNING:** Turn off the main power switch at the back of the instrument and disconnect the power cord.



- 1 Remove the fuse holder cover using a small screwdriver and pivot to expose the fuse carrier.
- 2 Pull out the fuse carrier.

- 3 Carefully remove the fuses and replace with new fuses. Use the same type and rating as listed in the Specifications section.
 - **NOTE:** If you have a 110 V supply you will need to replace a single fuse. If you have a 220 V supply you will need to replace two fuses.
- 4 Insert the fuse carrier back into the fuse holder. The fuses will face the inside of the fuse holder.
- 5 Replace the fuse holder cover.
- 6 Reconnect the power cord and turn on the main power switch.

Returning the System for Service

Contact EMD Millipore Technical Support for the Decontamination Form and instructions. The form must be returned via e-mail before you can return the instrument. A return authorization (RMA) number will be issued to you. Write this number on the outside of the shipping box. If you did not save the original shipping box, you can order one. Contact Technical Support for information.

- 1 Perform the Guava Clean procedure (see “Running Guava Clean” on page 3-4).
- 2 Power off the laptop and instrument.
- 3 Disconnect the laptop, instrument, and power conditioner.
- 4 Pack the laptop and power cord in the laptop box.
- 5 Pack the power conditioner in a box.
- 6 Pack the USB cable, extension cable, and power cord in the accessory box.
- 7 Remove the flow cell from instrument. Use the syringe cleaning tool to flush it with water, and then flush with air and store in capillary box. For information on cleaning the capillary, see “Using the Syringe Tool to Clean the Flow Cell Assembly” on page 3-8.
- 8 Put the shipping restraint on instrument.
- 9 Tape the capillary hatch closed.
- 10 Tape the tray door closed.
- 11 Place the instrument in the shipping bag and secure the bag.
- 12 Place the instrument in the shipping box.
- 13 Place the power conditioner box and laptop box to the left of the instrument.
- 14 Place the blue shipping foam over instrument. Place the accessory box on top.
- 15 Place the cover on the box and insert the white locks into the holes.

CHAPTER 4

guava ViaCount Assay

Introduction

The guava ViaCount Assay is a rapid and reliable alternative to trypan blue exclusion for determining cell count and viability. The ViaCount Reagent differentially stains viable and non-viable cells based on their permeability to the DNA-binding dyes in the reagent, allowing the quantitative assessment of viable and non-viable nucleated cells present in a suspension. The system counts the stained nucleated events, then uses the forward scatter (FSC) properties to distinguish free nuclei and cellular debris from cells to determine an accurate cell count.

To run the assay, stain a sample with the Guava ViaCount Flex Reagent according to the instructions in the *Guava PCA™-96 ViaCount Flex Reagent* package insert. Then, acquire the stained sample on the guava easyCyte HT System using guavaSoft Software. You may select the EasyFit Analysis feature to perform data analysis. This feature uses a computer-assisted population analysis method to determine the viable cells and non-viable (dead/apoptotic) cells. The EasyFit Analysis algorithm allows better discrimination of live cells, dead cells, and debris when those populations show overlap in one or more parameters because the EasyFit feature performs analysis in three dimensions. In addition, EasyFit Analysis is much less affected by the presence of contaminating debris, and more accurate results can be obtained.

You may also choose to perform manual data analysis by setting a viability marker, as well as an optional apoptosis marker to divide cells into live, apoptotic, and dead fractions. The software then displays the results based on your marker setting(s). Results include a viable cell count and total cell count (both in cells/mL) and the percent viability of the sample. If the apoptosis marker is enabled, results include the count and percentage of apoptotic and dead cells, as well as viable cells. Additionally, the software calculates the number of total, viable, apoptotic (where applicable), and dead cells in the original sample, based on the dilution factor and original sample volume that you entered in WorkEdit Software prior to acquisition.

The ViaCount data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you selected to save FCS 2.0 files. In addition to the saved data file, all results, instrument settings, and the acquisition summary information for a data set are exported to a spreadsheet file.

Running the guava ViaCount Assay

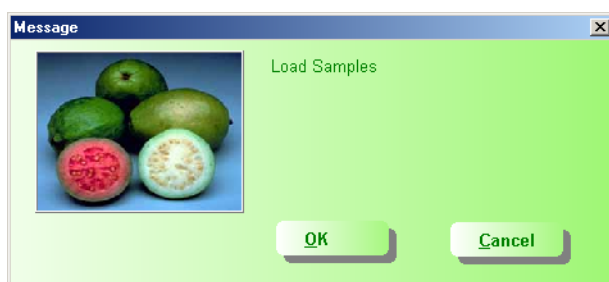
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “ViaCount Software Module Control Panels” on page 4-16.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.
- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.
- 3 Click **ViaCount** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

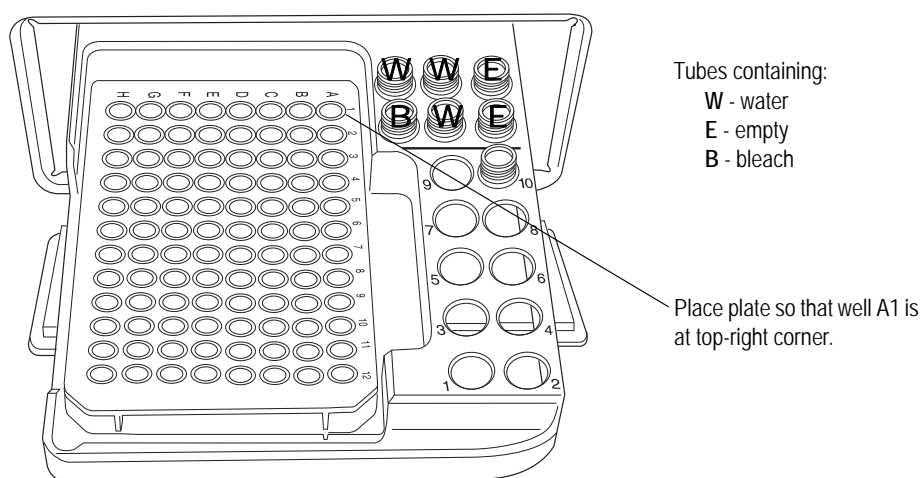


- 4 Prepare samples for analysis in a microplate or 0.5-mL tubes. Refer to the *Guava PCA-96 ViaCount Flex Reagent* package insert for information.
- 5 Click **Start Worklist**.
The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

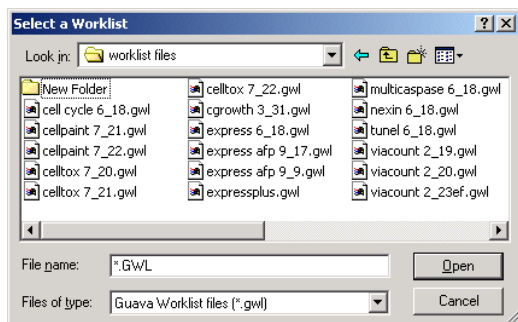


- 6 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

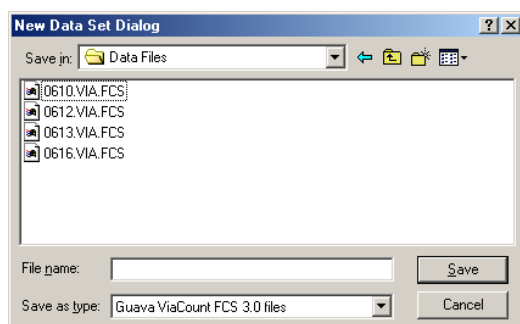
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select a worklist file and click Open.

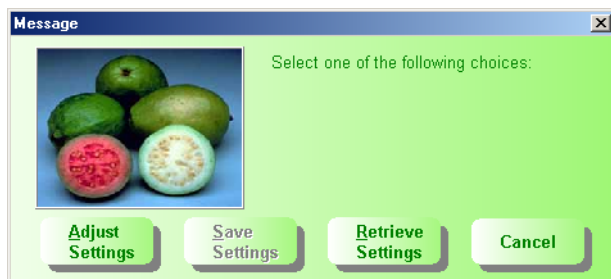
- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



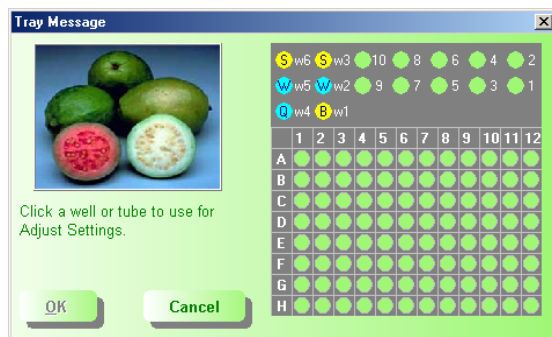
Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.
- To adjust instrument settings, click **Adjust Settings**.
 - To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the easyCyte HT System.

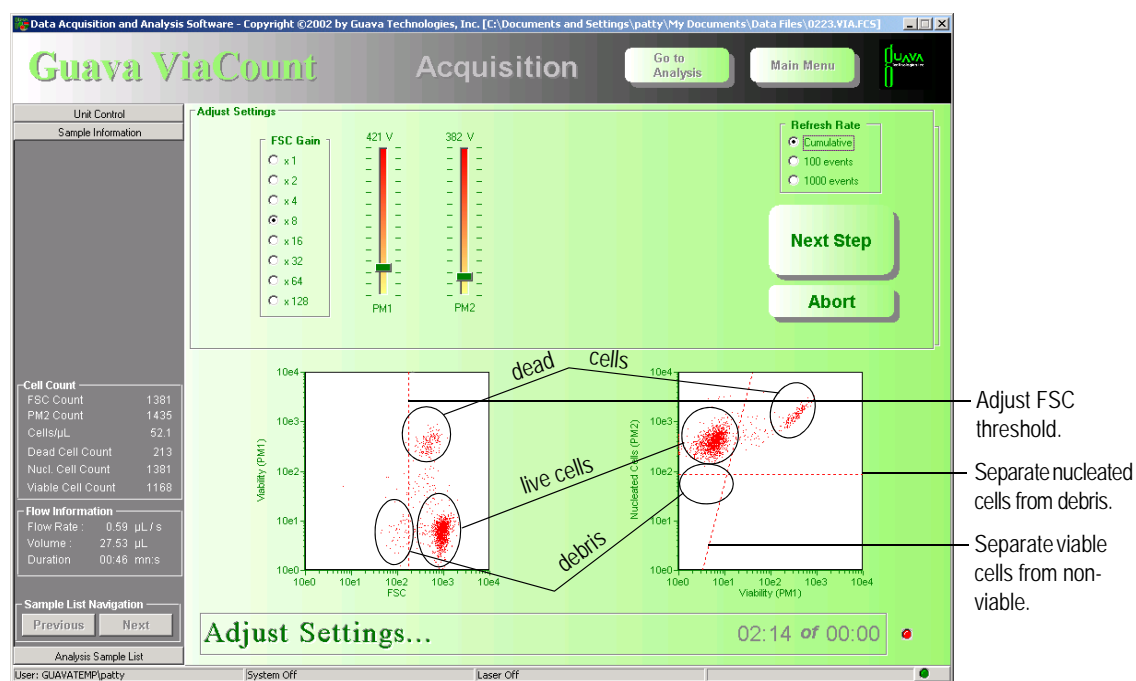


■ **NOTE:** Whether you choose to analyze samples manually or use EasyFit Analysis, you must perform the adjust settings step.

- 11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends running a representative stained sample. Click to select the well/tube used to adjust settings, then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

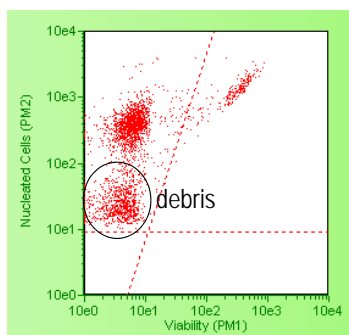


- 12 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is between 10 and 500 cells/ μ L.

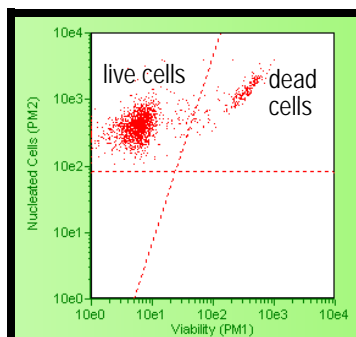
■ **NOTE:** If the value is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with ViaCount Flex Reagent to lower the concentration to <500 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

13 Fine tune the settings only if necessary. You can make the following adjustments once events start to appear on the screen:

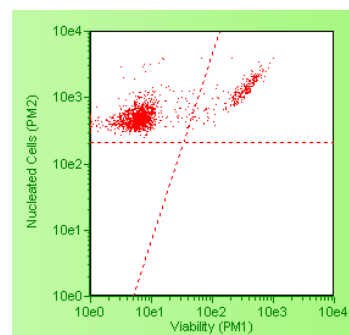
- Set the **Refresh Rate** to the maximum number of events you want to display.
- Use the **FSC Gain** setting to reduce or amplify the FSC signal so that the cells are visible and on scale. For optimal EasyFit Analysis, position the live cells at $10e3$ (as shown on page 4-5 in the dot plot on the left).
- Adjust the voltages (using the PM1 and PM2 sliders or the arrow keys on the keyboard) so that the viable cells are positioned in the upper left and the dead cells are in the upper right of the Viability (PM1) vs Nucleated Cells (PM2) plot. For optimal EasyFit Analysis, position the live cells between $10e0$ and $10e1$ on the Viability (PM1) axis and between $10e2$ and $10e3$ on the Nucleated Cells (PM2) axis (as shown on page 4-5 in the dot plot on the right). All dead cells should be above $10e2$ but below saturation (4000 on the PM2 axis). Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.
- Adjust the horizontal line (PM2 threshold) on the Viability (PM1) vs Nucleated Cells (PM2) dot plot to separate the viable cells in the upper left from the debris in the lower left. Set the line approximately 2–3 mm below the live cell population (as shown below in the center plot). Be sure to exclude all debris.



PM2 threshold set too low—too much debris included

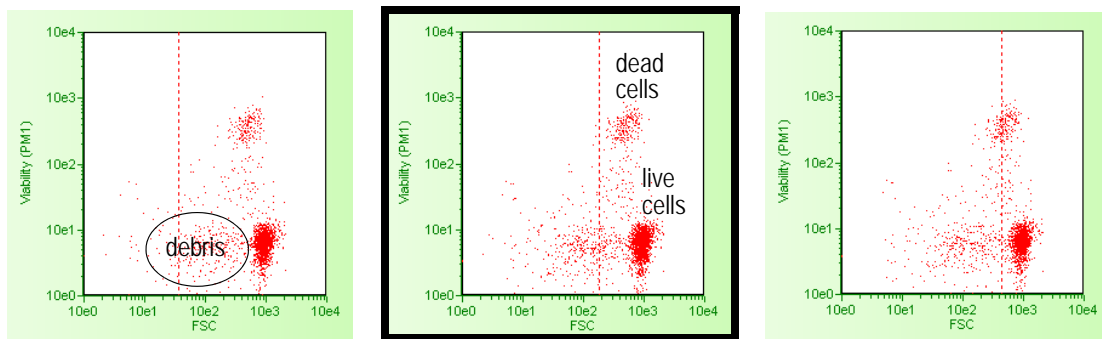


PM2 threshold set correctly



PM2 threshold set too high—live cells eliminated

- Adjust the vertical line (viability discriminator) on the Viability (PM1) vs Nucleated Cells (PM2) dot plot to separate the viable cells in the upper left from the non-viable cells in the upper right. You can adjust the angle of the line. Refer to step 5 on page 4-12 for information.
 - To adjust the FSC threshold, click and drag the vertical marker up or down the FSC axis of the FSC vs Viability (PM1) dot plot until the desired amount of debris is eliminated below the marker.
- **NOTE:** It is important to properly adjust the FSC threshold even if you plan to analyze using the EasyFit feature. Although the FSC threshold is not used during EasyFit Analysis, the FSC threshold is used as a counting gate to determine the total number of events acquired. If you set the threshold too low, a large number of non-cellular events (debris) will be acquired rather than events of interest—live and dead cells.



FSC threshold set too low—too much debris included

FSC threshold set correctly

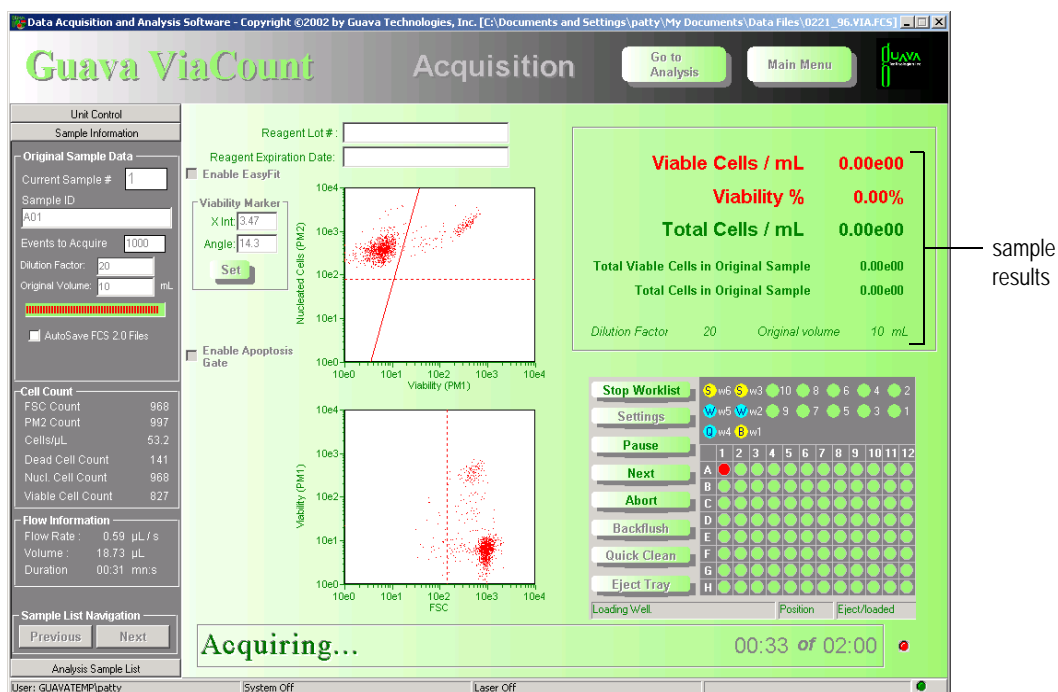
FSC threshold set too high—dead cells eliminated

- 14 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.

If necessary, you can repeat the adjust settings step to ensure that other samples are on scale and appropriately positioned by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

- 15 Click **Resume**.

The system acquires the first sample and displays the results. The tube/well currently being acquired is highlighted in red.



Manual analysis following sample acquisition

If you selected EasyFit, the software groups the events into three uniquely colored populations, corresponding to viable cells, dead/apoptotic cells, and debris. You cannot change to manual analysis during acquisition. However, after the data have been

collected, you can change to manual analysis later from the Analysis screen (see “guava ViaCount Analysis” on page 4-9).



EasyFit Analysis following sample acquisition

■ ACQUISITION NOTES

- If the sample is too dilute or too concentrated, a message appears indicating that the counting accuracy may be compromised. Refer to the *Guava PCA-96 ViaCount Flex Reagent* package insert for the recommended cell concentration and proper dilution instructions.
 - You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
 - If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
 - The progress bar provides an estimate of the target event count during the acquisition period, which times out after 2 minutes.
- 16** If you did not select EasyFit and wish to adjust the viability marker or set an apoptosis marker for any sample, click **Pause** at any time during the run. The system will pause when the acquisition is complete. Once the system is paused, make the necessary adjustments, then click **Resume**. The remaining samples are automatically acquired. Refer to “Manual Analysis” on page 4-12 for details on adjusting these markers. If you selected Enable EasyFit, you cannot make any further analysis adjustments at this point.
- Whether you selected the EasyFit feature or not, you can adjust the FSC threshold, viability marker, and PM2 threshold from the Adjust Settings screen by clicking **Pause**, then **Settings**, then **Adjust Settings**.

The system automatically performs a Quick Clean at the end of the assay.

guava ViaCount Analysis

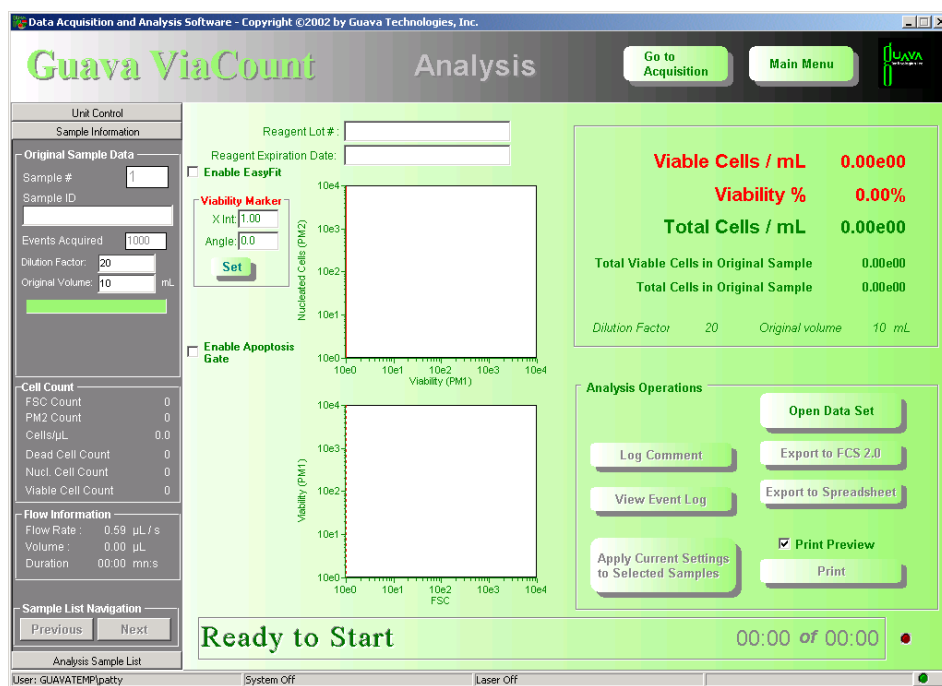
Use the Analysis screen to analyze samples, print results, log comments, or view the event log from a data set that was saved previously. You can also export data to FCS 2.0 format or a spreadsheet file.

You can save changes made to the sample ID, dilution factor, original volume, FSC threshold, or markers within Analysis by overwriting the existing file or saving a new file. You can also change between the EasyFit and manual methods of analysis, using either EasyFit Analysis or manual analysis for individual samples.

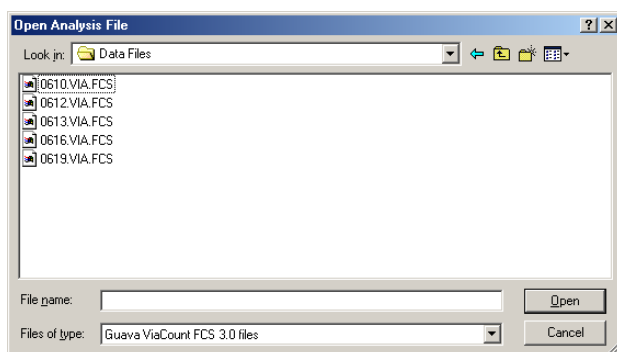
- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis method (EasyFit vs manual) or analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

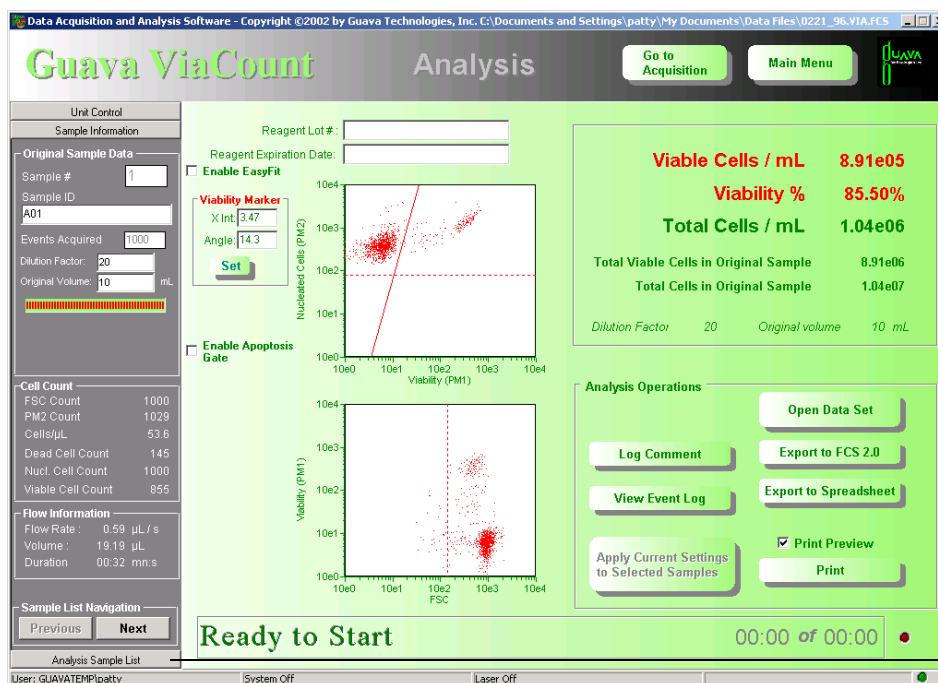
- 1 Click **ViaCount** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- 3 Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker settings appear as they were when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



Analysis
Sample List
control panel

EasyFit Analysis

You may select **Enable EasyFit** and allow the software to calculate the results based on its own internal analysis method. Using a 3-dimensional software algorithm, EasyFit groups the events into three populations—viable cells, dead/apoptotic cells, and debris.

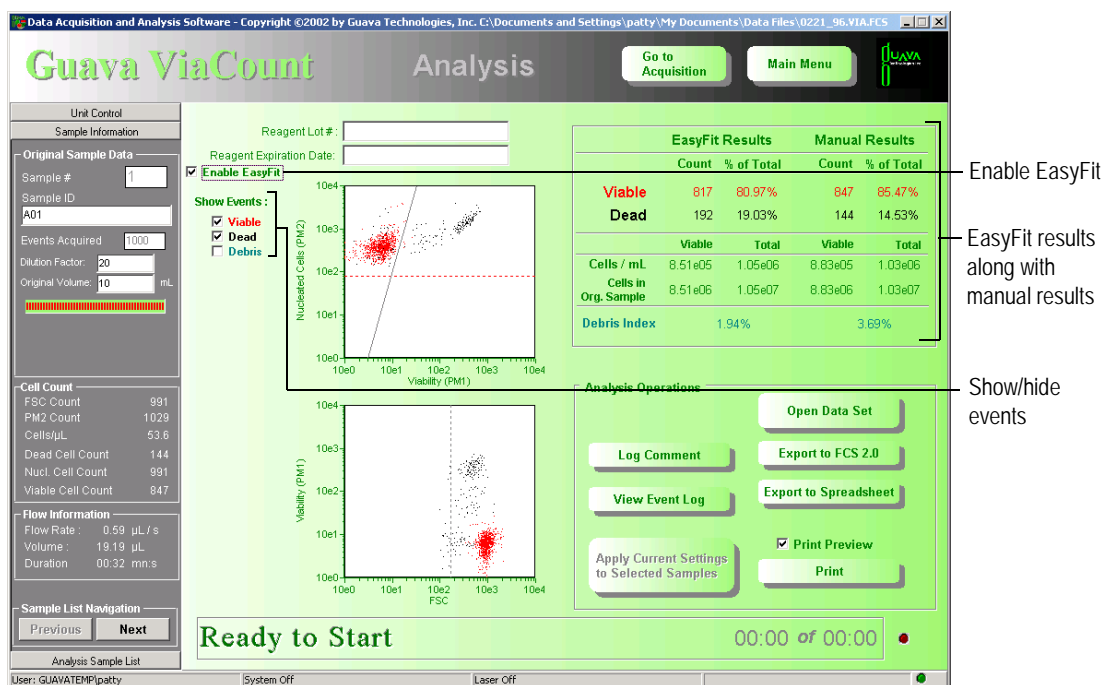
You may switch between EasyFit and manual analysis to see which method may be more appropriate for the given data. When EasyFit Analysis is selected, the FSC threshold and viability marker appear in gray for reference, but they cannot be adjusted. EasyFit Analysis uses its own method of grouping (clustering) data into populations. The threshold and viability marker remain on the plots so that you can compare the EasyFit

results with the manual results, which are also displayed. When the EasyFit feature is disabled, you may, if necessary, adjust the FSC threshold and viability marker to appropriately discriminate debris from cells, as well as live cells from dead/apoptotic cells.

- **NOTE:** The apoptosis gate is not available for EasyFit Analysis. Apoptotic cells are included in the EasyFit dead cell population. If you wish to calculate individual results for dead and apoptotic cells, use the manual analysis method and set an apoptosis gate.

To hide or show the events within specific populations, use the check boxes to the left of the Viability (PM1) vs Nucleated Cell (PM2) dot plot. The selection you make applies to the data while you are viewing it. The selections are not saved to the file.

- **NOTE:** Before routinely using EasyFit for your analysis, you should validate the EasyFit Analysis method for the cells you are testing by running multiple experiments and comparing the EasyFit results to another cell counting and viability test method, such as manual ViaCount analysis. In some cases EasyFit Analysis will provide more accurate results because it uses 3-dimensional gating to identify live and dead/apoptotic cells. However, even after validating EasyFit Analysis, compare the EasyFit results to the results obtained with the manual method, and check the plots for each sample to ensure the analysis is correct.



The manual results—results obtained from the FSC threshold and viability marker, which you left at their defaults or modified—appear next to the EasyFit results.

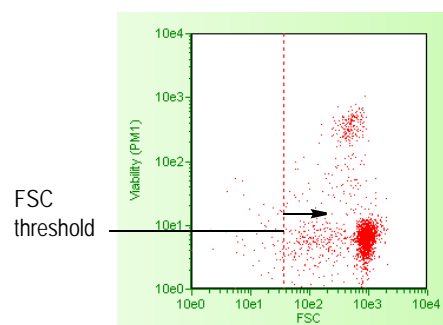
Proceed to step 8 on page 4-13 to continue analyzing sample data.

- **NOTE:** For examples of when to use EasyFit versus manual analysis, refer to Appendix A: EasyFit Case Study Examples.

Manual Analysis

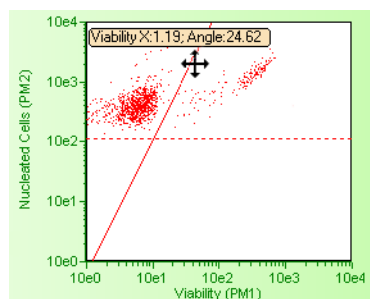
To analyze manually, remove the check mark from the Enable EasyFit check box.

- 4 If necessary, you may adjust the FSC threshold during analysis. During acquisition, the FSC threshold is used as a counting gate—any event that passes the threshold is included in the Events to Acquire. After acquisition is complete, if you move the threshold, the total cell count will change. However, by moving the threshold you may be able to correct for debris that was included or cellular events that were not included during acquisition. You cannot adjust the FSC threshold if EasyFit is enabled.

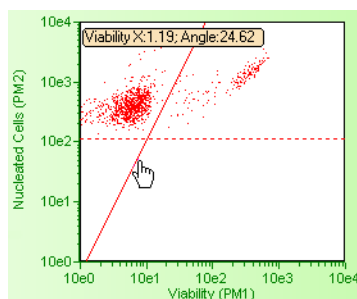


Adjust FSC threshold, if necessary, when analyzing manually.

- 5 If necessary, you may adjust the location and angle of the red viability marker to more precisely discriminate viable from non-viable cells (or to separate dead cells from live and apoptotic cells).
 - **NOTE:** If you are using EasyFit Analysis, viable cells versus dead and apoptotic cells are determined by the software. The viability marker will be ignored.
 - To adjust the angle of the marker, click and drag the top of the line to tilt it to the desired location.
 - To move the entire marker to the left or right, click and drag the bottom of the line to shift it to the desired location.
You may also set the marker by entering the coordinates and angle in the Viability Marker fields and clicking **Set**.



Click and drag top of line to adjust angle.



Click and drag bottom of line to move location.

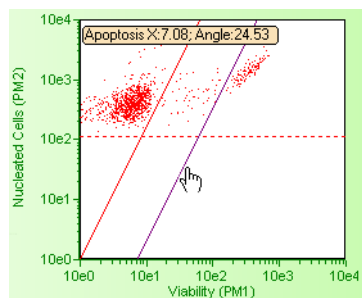


Viability marker adjusted to separate viable cells (left) from apoptotic and dead cells (right).

- 6 To set the apoptosis marker, click Enable Apoptosis Gate. The apoptosis marker is available only during manual analysis. It is not available when EasyFit is enabled.

- 7 To set the purple apoptosis marker, follow the same instructions outlined in step 5 for setting the Viability Marker.

■ **NOTE:** Make sure the apoptosis marker (purple) is to the right of the viability marker (red) and that the markers are not intersecting.



☒ **Enable Apoptosis Gate**

Apoptosis Marker

X Int: 6.20

Angle: 25.3

Set

Click to enable Apoptosis marker.

Click and drag top of line to adjust angle.

Click and drag bottom of line to move location.

Viability marker (red) separating viable cells (left) from apoptotic cells (right). Apoptosis marker (purple) separating apoptotic cells (left) from dead cells (right).

- 8 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 9 You can apply markers settings or EasyFit Analysis from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 10 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
- **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting files.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
- **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 11 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

guava ViaCount Results

ViaCount results appear immediately after the acquisition of the sample is complete. The results displayed vary depending on whether the EasyFit or manual method of analysis was used and if the apoptosis marker was set. Following are examples of results from each type of analysis.

Viable Cells / mL	8.19e06
Viability %	95.6 %
Total Cells / mL	8.56e06
Total Viable Cells in Original Sample	8.19e07
Total Cells in Original Sample	8.56e07
<i>Dilution Factor</i>	20
<i>Original volume</i>	10 mL

Results include statistics for viable and total cells:

- Number of viable cells per mL
- Percentage of viable cells
- Total number cells per mL
- Number of viable cells in original sample
- Total number of cells in original sample

The dilution factor and original volume that you entered are displayed.

ViaCount manual results for viability marker

	Cells/mL	% of Total	Cells in Original Sample
Viable	8.40e05	85.19%	8.40e06
Apoptotic	4.14e04	4.20%	4.14e05
Dead	1.05e05	10.61%	1.05e06
Total	9.86e05	100.0 %	9.86e06
<i>Dilution Factor</i>	20	<i>Original volume</i>	10 mL

Results include statistics for viable, apoptotic, dead, and total cells:

- Number of cells per mL
- Percentage of cells
- Number of cells in original sample

The dilution factor and original volume that you entered are displayed.

ViaCount manual results for viability and apoptosis markers

	EasyFit Results		Manual Results	
	Count	% of Total	Count	% of Total
Viable	817	80.97%	847	85.47%
Dead	192	19.03%	144	14.53%
	Viable	Total	Viable	Total
Cells / mL	8.51e05	1.05e06	8.83e05	1.03e06
Cells in Org. Sample	8.51e06	1.05e07	8.83e06	1.03e07
Debris Index	1.94%		3.69%	

EasyFit results appear along side the manual results. Results include:

- Number of viable and dead cells in sample (count)
- Percentage of viable and dead cells in sample (count)
- Number of viable cells per mL
- Total number of cells per mL
- Number of viable cells in original sample
- Total number of cells in original sample
- Debris index (The debris index is a measure of debris. The higher the index the more debris in the sample.)

$$\frac{\text{\# of debris events}}{\text{\# of total cells} + \text{\# of debris events}} \times 100$$

ViaCount EasyFit results (manual results also included)

Exporting ViaCount Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

- **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

- **NOTE:** The guava Excel Utility allows you to extract the cell concentration and percent viability information from the CSV file and save it to an Excel spreadsheet file. For information on using the program, refer to “Guava Excel Utility” on page 4-21.

Exporting ViaCount Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.
The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

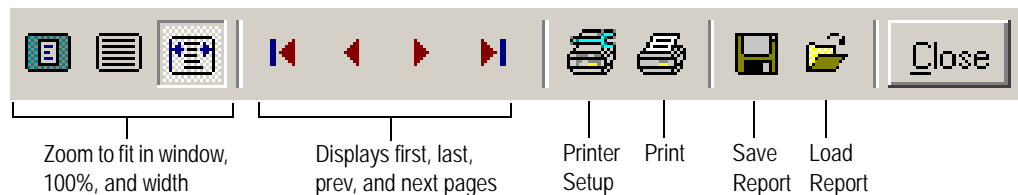
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

Move the cursor over the icon to display the description.



ViaCount Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

The screenshot shows the 'Sample Information' control panel. It has a title bar 'Sample Information'. Below it are four sections: 'Original Sample Data' with input fields for 'Current Sample #' (1), 'Sample ID' (A01), 'Events to Acquire' (1000), 'Dilution Factor' (20), and 'Original Volume' (10 mL), plus an 'AutoSave FCS 2.0 Files' checkbox; 'Cell Count' with a table of counts; 'Flow Information' with 'Flow Rate', 'Volume', and 'Duration' fields; and 'Sample List Navigation' with 'Previous' and 'Next' buttons.

Cell Count	
FSC Count	968
PM2 Count	997
Cells/ μ L	53.2
Dead Cell Count	141
Nucl. Cell Count	968
Viable Cell Count	827

Flow Information	
Flow Rate :	0.59 μ L / s
Volume :	18.73 μ L
Duration	00:31 mn:s

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 1000.
- Displays the dilution factor and the original sample volume. The default values are 20 and 10, respectively.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells detected by the FSC and PM2 detectors, as well as the cell count and the number of dead, nucleated, and viable cells in the volume of sample acquired.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

Unit Control

Detection

PM Power Setting 300 V

PM 1 300 V

PM 2 300 V

FSC Gain: x 16

Pump Status

End of Stroke

Waste

Pump Reset

Position 0.0 Steps

Actual Speed 0 Steps/s

Pump Action

Pump on

Waste

Pump Reset

Purge

Sample

Speed 0 Steps/s

Threshold parameters

	Offset	Threshold
FSC	0.65 V	194 mV
PM 1	0.46 V	8 mV
PM 2	0.57 V	111 mV

Detection

Displays laser status and the PM1 and PM2 voltage settings and the FSC gain.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold settings for each parameter.

■ **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field. Do not change the voltages from this panel. Use the sliders to adjust the PM1 and PM2 voltages.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava ViaCount Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save ViaCount spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save ViaCount FCS file to another directory or give it a new name.
ViaCount Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
<p>Message: <i>Less than 10 particles/μL. Sample is too dilute. Accuracy may be compromised.</i></p>	<ol style="list-style-type: none"> 1. Sample concentration is too low. 2. Clogged flow cell. 3. Settings were not adjusted correctly. 	<ol style="list-style-type: none"> 1. Recommended concentration range for accurate counting is 1×10^4 to 5×10^5 cells/mL. Refer to <i>Guava PCA-96 ViaCount Flex Reagent</i> package insert for proper dilution instructions. 2. Perform a Backflush. Follow with Quick Clean. 3. Adjust Settings and make necessary changes.
<p>Message: <i>Sample is too concentrated. Please dilute or accuracy may be compromised.</i></p>	<p>Sample is too concentrated.</p>	<p>Recommended concentration range for accurate counting is 1×10^4 to 5×10^5 cells/mL. Refer to <i>Guava PCA-96 ViaCount Flex Reagent</i> package insert for proper dilution instructions.</p>
<p>Few events, as indicated in Particle Count section of Sample Information control panel.</p>	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 4. Cells were lysed during staining. 5. Sample was not properly stained. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software. 4. Check sample. If necessary, restain sample from original suspension. 5. Check buffers used to process cells. Check expiration date of reagent.

Problem	Possible Cause	Solutions
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Events appear off scale in dot plots.	FSC gain or PM1 and /or PM2 voltages set incorrectly, or samples staining brightly.	Adjust gain setting or voltage settings so positive populations appear on scale.
Viability is significantly lower than expected.	<ol style="list-style-type: none"> 1. Sample was not mixed properly before acquisition. 2. Residual bleach in flow cell. 3. Buffer used is non-isotonic or pH is out of physiological range. 4. Cells are fragile and stressed by preparation procedure. 	<ol style="list-style-type: none"> 1. Ensure sample mixing option was selected in WorkEdit Software. 2. Run Quick Clean. Restain fresh cell sample. 3. Check osmolarity and pH. pH should be 7.2–7.4. 4. Resuspend cells in medium containing 10–20% FBS for 30 min with frequent, gentle agitation. Restain.

Problem	Possible Cause	Solutions
Cell count is significantly different than expected.	<ol style="list-style-type: none"> 1. Dilution factor or original volume entered incorrectly. 2. Sample was not mixed properly before acquisition. 3. Adherent cells were not adequately removed from culture surface. 4. Sample was not stained completely. 5. Sample dilution factor is too low. 6. Clogged flow cell. 7. Cells are aggregated. 8. Instrument settings not optimal. 9. Incorrect sample tubes or plates used. 	<ol style="list-style-type: none"> 1. Ensure dilution factor and original volume are correct. 2. Ensure sample mixing option was selected in WorkEdit Software. 3. Check culture vessel to ensure cells were removed. Repeat procedure to release cells if necessary. 4. Ensure reagent is not expired or was not exposed to excessive light. Ensure sufficient amount of ViaCount Flex Reagent was used. 5. Use at least a 1:10 of sample to Viacount reagent, or use Viacount Flex CS Reagent (Catalog No. 4500-0110). 6. Perform a Backflush. Follow with Quick Clean. 7. Prepare uniform suspension. Use ViaCount CDR to disaggregate cells. 8. Adjust settings so positive populations appear on scale and debris is below threshold. 9. See Order Information for list of supported tubes and plates.
guava Excel Utility will not start Microsoft Excel when you try to open a CSV file	<ol style="list-style-type: none"> 1. File is not a ViaCount file or it was created by a version of CytoSoft Software prior to version 2.0. 2. Templates folder is not located in same directory as utility program. 	<ol style="list-style-type: none"> 1. Excel Utility can be used only for ViaCount files created in CytoSoft Software, version 2.0 or later. 2. Be sure Templates folder and executable are in same folder.

Guava Excel Utility

The Guava Excel Utility Program can be used to extract pertinent analysis information (cell concentration, percent viability, dilution factor, and original volume) from the CSV file and create an Excel spreadsheet file. The information is organized in the following four worksheets of the Excel workbook:

- manual analysis summary sheet
- EasyFit Analysis summary sheet (If EasyFit was not enabled, "--" appears in the fields under the EasyFit columns.)
- manual analysis sheet (a subset of relevant information found in the CSV)
- data sheet (contains the same information found in the CSV file)

The utility program can be used by all guavaSoft Software users including administrators, supervisors, and operators. You may use the program to open CSV files created in CytoSoft Software, version 2.0 and later.

■ **NOTE:** The utility cannot be used if the apoptosis gate was enabled.

To open a file with the utility program, you can either use the drag-and-drop method or start the program.

■ **NOTE:** If a dialog box appears indicating that the document contains macros, select the Enable Macros option.

Drag and Drop

- 1 Locate GuavaExcelUtil.exe icon.
It is located in the C:\Program Files\Millipore\guavaSoft\GuavaExcelUtil directory along with a Templates folder that the program uses.
- 2 Drag and drop a CSV file onto the utility icon.
The utility program starts Microsoft Excel and creates a workbook with the appropriate information.
The Excel file is saved automatically. If you make changes to the file, you must save the changes.
The file name assigned to the Excel file is the same name as the CSV file but with the extension .xls. The original CSV file is unchanged.
- 3 Excel automatically write protects the summary sheet. To disable the protection, select Protection from the Tools menu in the Excel menu bar. Then choose Unprotect Sheet from the list of options.

■ **NOTE:** If you wish to place a copy of the Guava Excel Utility Program on the desktop, you must also place a copy of the Templates folder on the desktop with the executable.



Open the Utility Program

- 1 Locate GuavaExcelUtil.exe icon (or shortcut) and double-click to start the program.
- 2 Choose a CSV file from the file dialog box.
The utility program starts Microsoft Excel and creates a workbook with the appropriate information.
The file name assigned to the Excel file is the same name as the CSV file but with the extension .xls. The original CSV file is unchanged.
- 3 Excel automatically write protects the summary sheet. To disable the protection, select Protection from the Tools menu in the Excel menu bar. Then choose Unprotect Sheet from the list of options.

CHAPTER 5

guava ExpressPlus Assay

Introduction

The guava ExpressPlus Assay allows you to acquire and analyze up to three fluorescence parameters in combination with forward scatter (FSC) and side scatter (SSC), provided as an optional parameter, to identify cells with specific phenotypic markers or specific properties. The software provides absolute cell counts and allows you the flexibility to stain samples with the guava Express[®] Reagents, your own fluorochrome-conjugated antibody reagents, DNA intercalating dyes, or other green, yellow, and/or red fluorescent reagents.

The guava ExpressPlus Assay can be used for any one-, two-, or three-color assay including:

- protein expression or other cell-surface marker experiments
- screening and analyzing cells expressing Green Fluorescent Protein (GFP)

The Green parameter can be used for FITC, GFP, or Alexa Fluor 488. The Yellow parameter has been optimally configured for use for phycoerythrin (PE)-based reagents, although fluorochromes with comparable emission spectra, such as propidium iodide (PI), TRITC, DS Red, sulforhodamine, Cy[™]3, and Alexa Fluor[®] 532 can also be used. The Red parameter can be used to detect PE-Cy5, PerCP, 7-AAD, and PI. Refer to Specifications for the guava easyCyte HT System fluorescence detection range.

To run the guava ExpressPlus Assay, stain samples using an appropriate fluorochrome combination for up to three fluorescence parameters. Acquire the samples on the guava easyCyte HT System using guavaSoft Software. The data are displayed in three plots, which can be set up to show histograms or dot plots with any parameter combination (FSC, SSC, if available, GRN, YLW, and RED). You may set up to two gates to select a specific subpopulation(s) for analysis, then choose the gate(s) for which you want to display statistics for the remaining two plots.

Statistics (histogram and dot plot) include the cell count, cells/mL, mean signal intensity, and %CV (for histogram data) for the data within the entire plot and the subset of data within the markers/gate. Additionally, the data are expressed as a percentage of the total data within the plot (whether gated from the dot plot or histogram), and as a percentage of data within the gate.

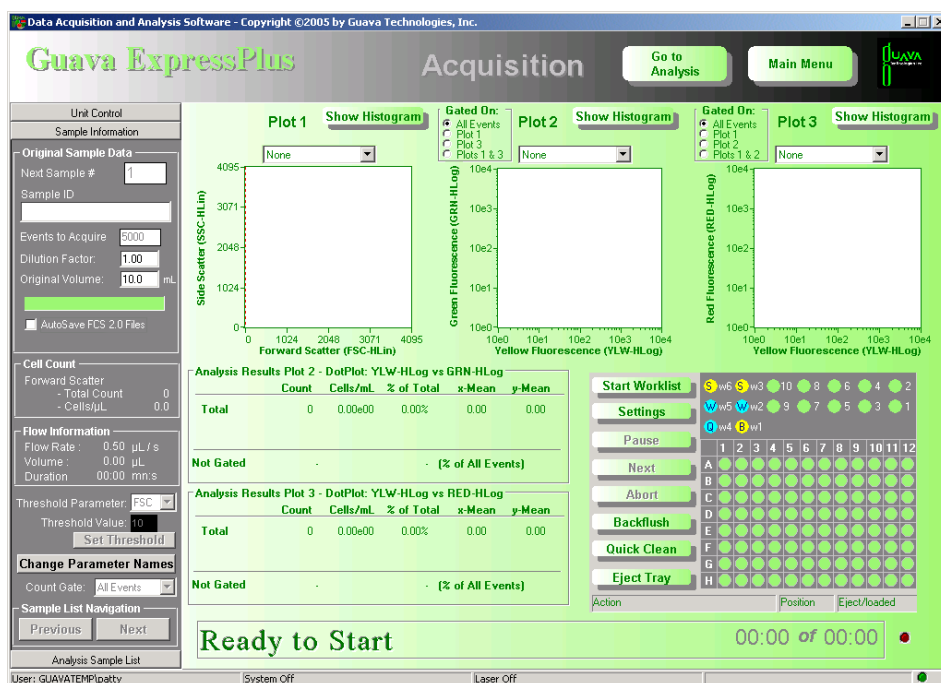
The guava ExpressPlus data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you

selected to save FCS 2.0 files. In addition to the saved data file, all results, instrument settings, and the acquisition summary information are exported to a spreadsheet file.

Running the guava ExpressPlus Assay

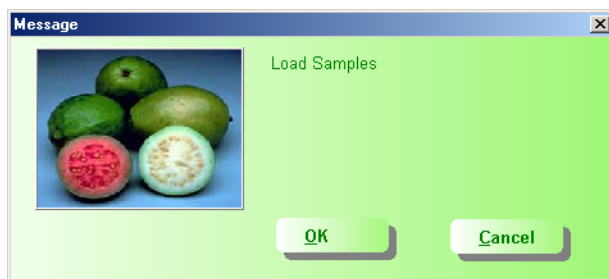
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “guava ExpressPlus Software Module Control Panels” on page 5-19.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.
- 2 Open guavaSoft Software by double-clicking the guavaSoft 2.7 icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.
- 3 Click **ExpressPlus** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 5 minutes before acquiring samples.

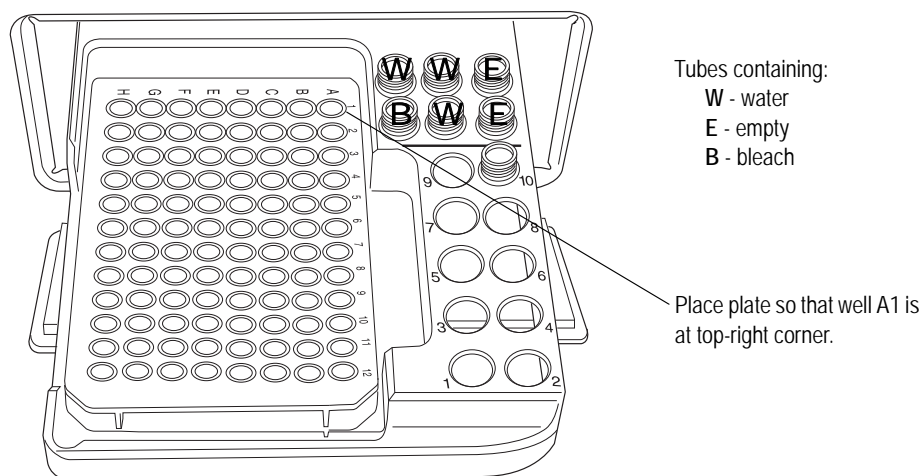


- 4 Prepare samples for analysis in a microplate or 0.5-mL tubes.
- 5 Click **Start Worklist** on the Guava ExpressPlus screen.
The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

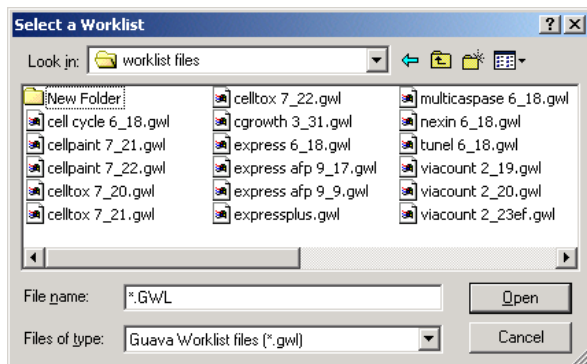


- 6 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

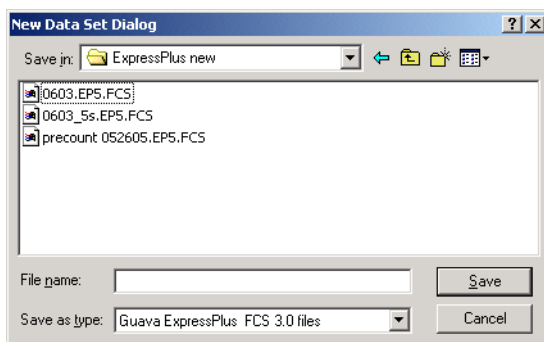
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select worklist file and click Open.

- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

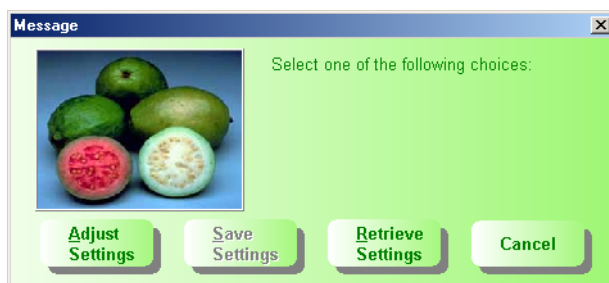
- **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



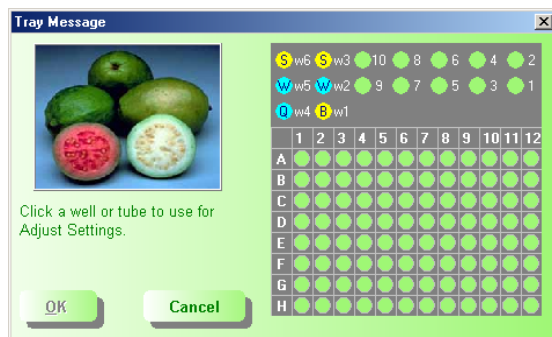
Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.

- To adjust instrument settings, click **Adjust Settings**.
- To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



- 11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends using a stained negative or isotype control sample for the initial adjustments. Click to select the well/tube used to adjust settings, then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

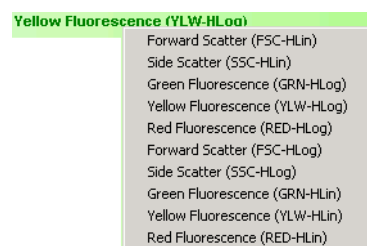
- 12 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is less than or equal to 500 (for medium flow), less than or equal to 800 (for low flow), or less than or equal to 1200 (for very low flow).

If your sample is heterogeneous in cell size or contains no cells that would stain with more than one fluorochrome, consider using cell concentrations below these default limits to minimize the number of coincident events. EMD Millipore recommends cell concentrations of 200 cells/ μ L or lower.

- **NOTE:** If the value is greater than the high limit for the corresponding flow rate, click **Abort**, then click **Eject Tray**. Dilute the sample with staining buffer or fixative solution to lower the concentration. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

- 13 If you need to fine tune the settings, you can make the following adjustments once events start to appear on the screen:

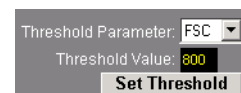
- **NOTE:** Click **Show Histogram/Show DotPlot** above each plot to display the type of plot you want. Then, right-click the axis label(s) and select the appropriate label from the pop-up menu. You can choose linear and log scale for each parameter. If your easyCyte HT System is not equipped with the SSC parameter, SSC will not be available.



- Set the **Refresh Rate** to the maximum number of events you want to display.
- Set the **Flow Rate** to Very Low (0.12), Low (0.24 μ L/s), or Medium (0.59 μ L/s). The recommended flow rate is Medium.
- **NOTE:** If you change the flow rate during the adjust settings step, EMD Millipore recommends that you repeat the adjust settings step at the new flow rate to ensure that the markers and threshold are still set correctly.
- Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the cells are visible and on scale. Use the drop-down menu to change the FSC signal by a factor of 2. Use the FSC slider to change the FSC signal by steps of 1% between two

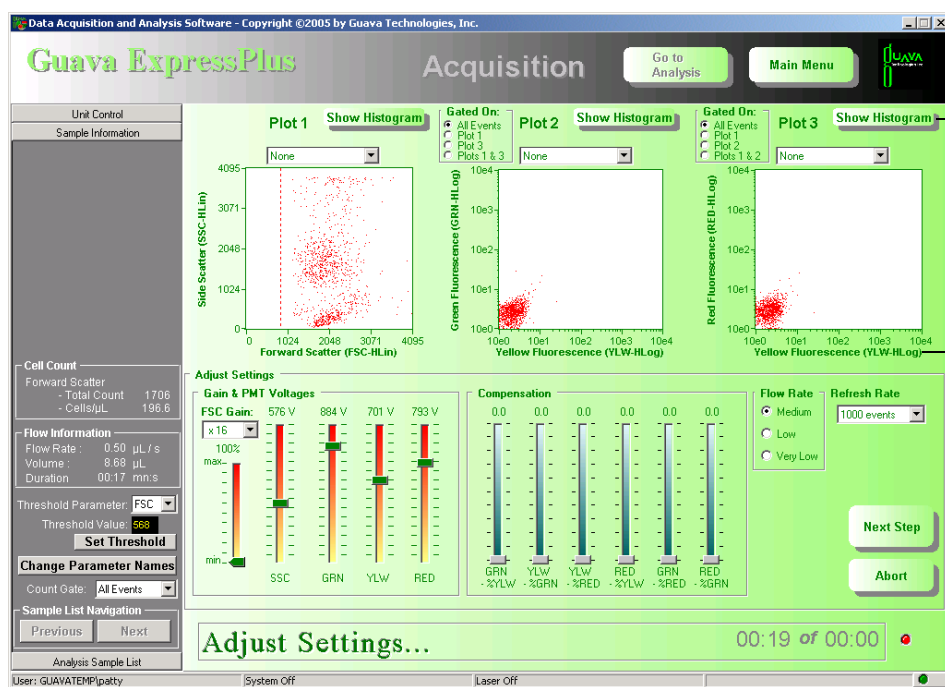
coarse gain levels. If the slider is set to 100% (min), the gain is equal to the value appearing in the drop-down box. If the slider is set to 200% (max), the gain is 2 times the value appearing in the drop-down box.

- Adjust the voltages (using the SSC, GRN, YLW, and/or RED sliders, or the arrow keys on the keyboard) so that the negative population is positioned in the lower-left corner of the fluorescence plot and the cells are evenly distributed between 10e0 and 10e1. Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.
- Select the threshold parameter (FSC, SSC, GRN, YLW, or RED) from the drop-down menu in the Sample Information control panel.



- To adjust the threshold, click and drag the threshold marker (dotted red line) up or down the axis of the dot plot displaying the threshold parameter until the desired amount of debris or other unwanted events are eliminated below the threshold. You can also enter a numerical value in the Threshold Value text box in the Sample Information control panel, then click **Set Threshold**.

■ **NOTE:** When setting the threshold, place it at least 2 to 3 mm to the left of the cells of interest to ensure that you are not excluding cells. Any debris that gets acquired can be excluded during analysis with the optional dot plot gate.



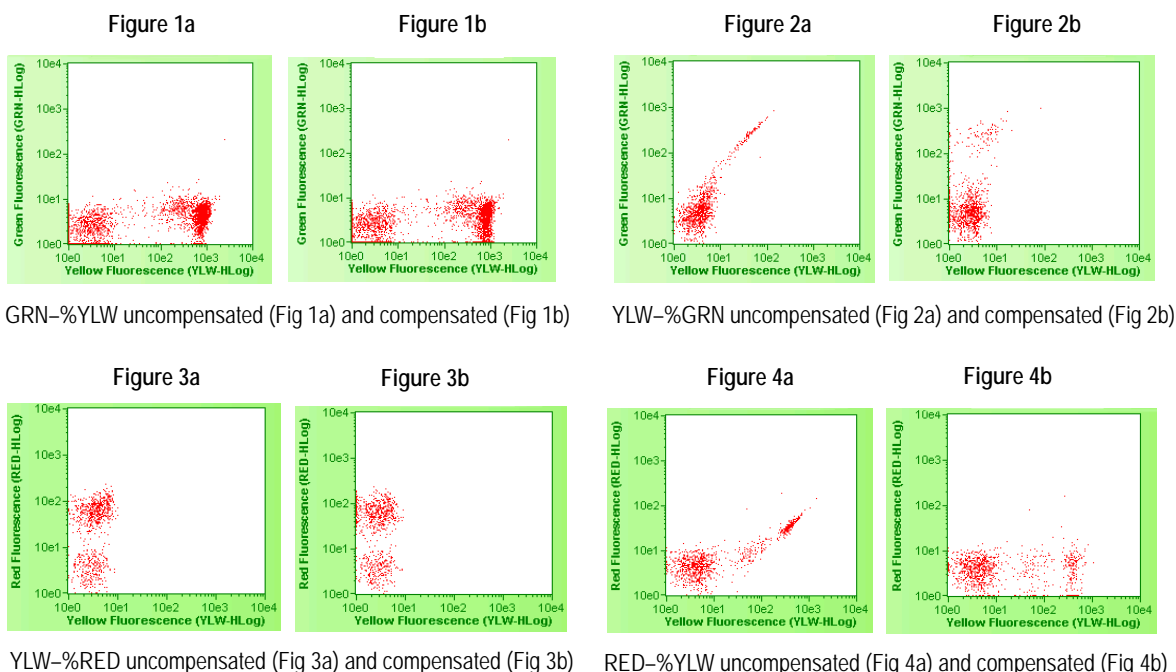
Click to change plot type.

Right-click to change axis label.

- To adjust compensation, click **Next Step**, **Settings**, **Adjust Settings**, then select the location of a positive control sample. You may want to adjust each compensation setting individually using single-stained controls.

■ **NOTE:** You must check the compensation for each fluorochrome combination you are using. For example, if using FITC and PE fluorochromes, which correspond to the GRN and YLW parameters, respectively, ensure that YLW-%GRN and GRN-%YLW compensation settings are correctly adjusted (see Figures 1 and 2 below for examples). If using PE-Cy5 as well, which corresponds to the RED

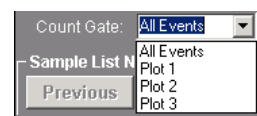
parameter, ensure that the YLW-%RED and RED-%YLW compensation settings are correctly adjusted (see Figures 3 and 4). You may also need to change the plot parameters to display RED vs GRN and adjust RED-%GRN and GRN-%RED. Some fluorochromes require very little compensation because they have little overlap, such as PE into the GRN parameter (GRN-%YLW) [see Figures 1a and 1b]; whereas others require much more compensation because they have more overlap, such as FITC into the YLW parameter (YLW-%GRN) [see Figures 2a and 2b]. Compensation settings are correct when the edge of the stained population touches the axis, as shown below. Avoid having too many cells touching the axis (overcompensated) or the population too far from the axis (undercompensated).



- **NOTE:** If you adjust the compensation with single-color samples, check the compensation settings again with a sample stained with all fluorochromes, and fine-tune, if necessary.
- **NOTE:** If you acquire samples with a new fluorochrome combination or the same fluorochrome combination conjugated to different antibodies, you must ensure that the compensation is set correctly for the new samples.

14 You may set gates and markers prior to acquiring the samples. Refer to “Dot Plot Gate” on page 5-11, “Histogram Gate” on page 5-12, and “Setting Markers” on page 5-13 for information.

If you wish to apply a count gate, select the gate from Count Gate drop-down menu in the Sample Information control panel, then set the region in the appropriate plot. You can select the count gate from the Adjust Settings screen only.



- **NOTE:** Gates can only be the rectangular region, elliptical region, or histogram marker in Plot 1, or the rectangular region, elliptical region, or histogram marker 1 in Plots 2 and 3. You cannot use quadrant markers or the second or third histogram marker in Plots 2 or 3. If you select a count gate on a plot that does not have a valid gate

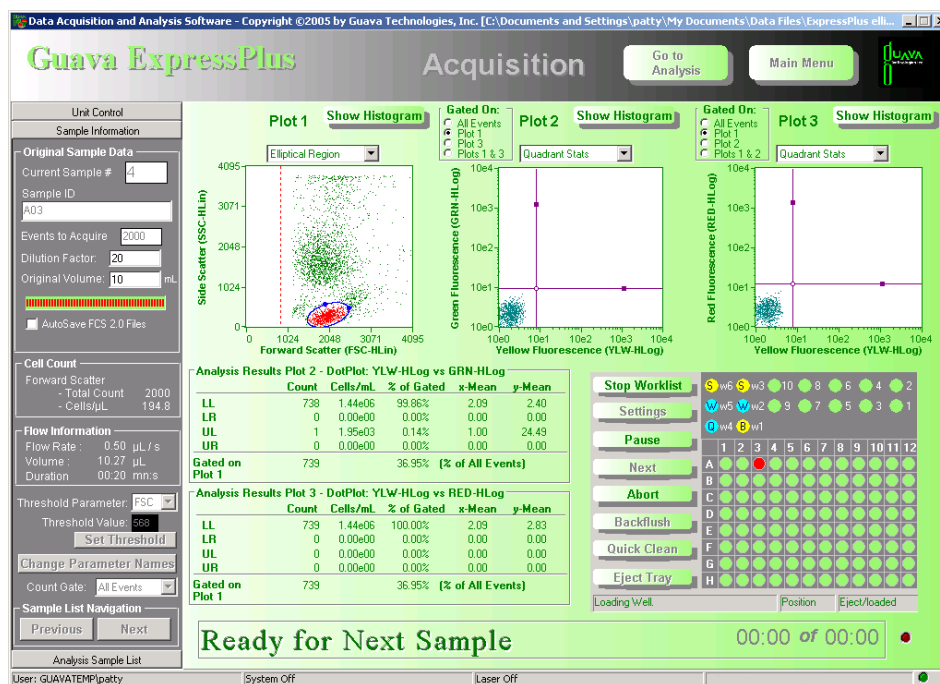
enabled, when you exit the Adjust Settings screen, the count gate will automatically revert to All Events.

- 15 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.

If necessary, you can repeat the adjust settings step to ensure that other samples (such as another positive control) are on scale, appropriately positioned, and compensated, by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

- 16 Click **Resume**.

The system acquires the first sample and automatically displays the results. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 3.5 minutes (medium flow rate), 7 minutes (low flow rate), or 10 minutes (very low flow rate).

The system automatically performs a Quick Clean at the end of the assay.

guava ExpressPlus Analysis

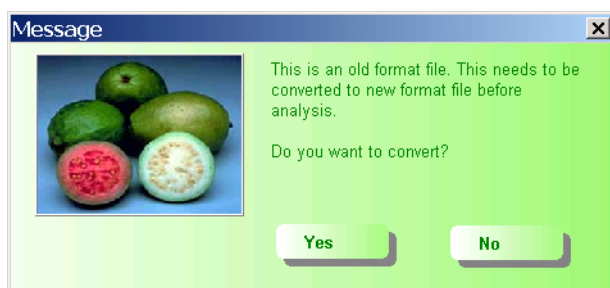
Use the Analysis screen to analyze samples, print results, log comments, or view the event log from a data set that was saved previously. You can also export data to FCS 2.0 files or a spreadsheet file.

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

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

This version of the ExpressPlus Software Module within guavaSoft 2.7 Software allows you to open and analyze the old format of guava ExpressPlus files (*.GXP.FCS). Remember to select All FCS 3.0 files from the *Files of type* text box. A dialog box appears prompting you to convert the file.

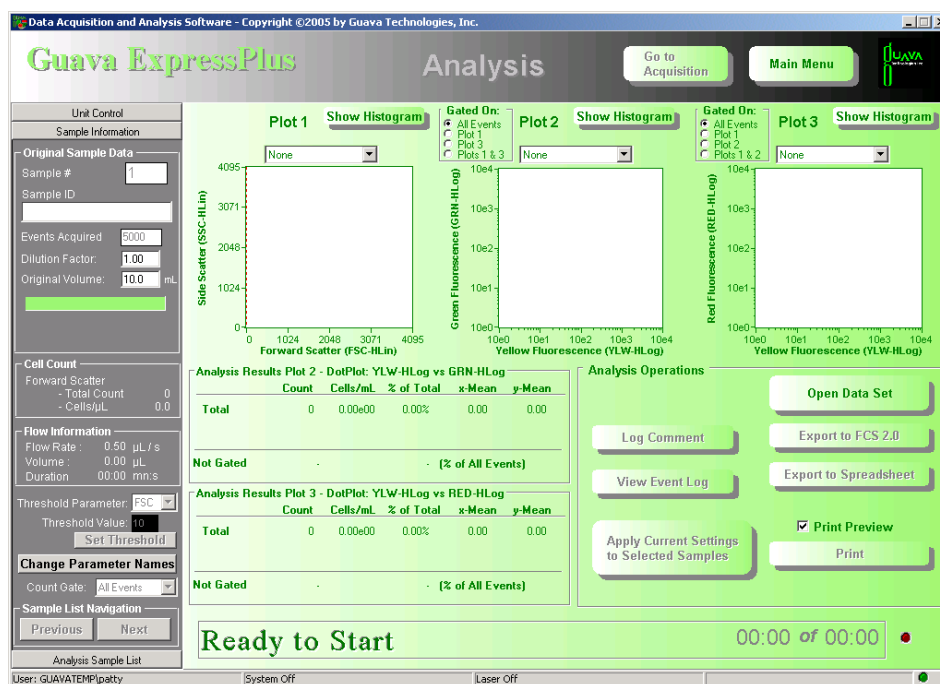


When you click **Yes** to convert the file, a copy of the converted data is saved as an .EP5 file with the same file name as the original .GXP file.

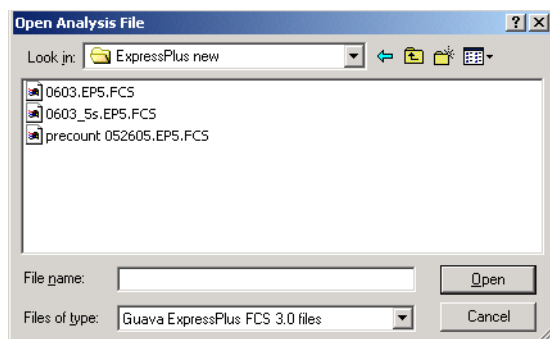
- **NOTE:** A spreadsheet file (.CSV) corresponding to the converted file does not get saved automatically. If you do not make changes to the converted file, you must click **Export to Spreadsheet** if you want to generate a spreadsheet file.

- 1 Click **ExpressPlus** from the main menu.

2 Click **Go to Analysis** from the Acquisition screen.



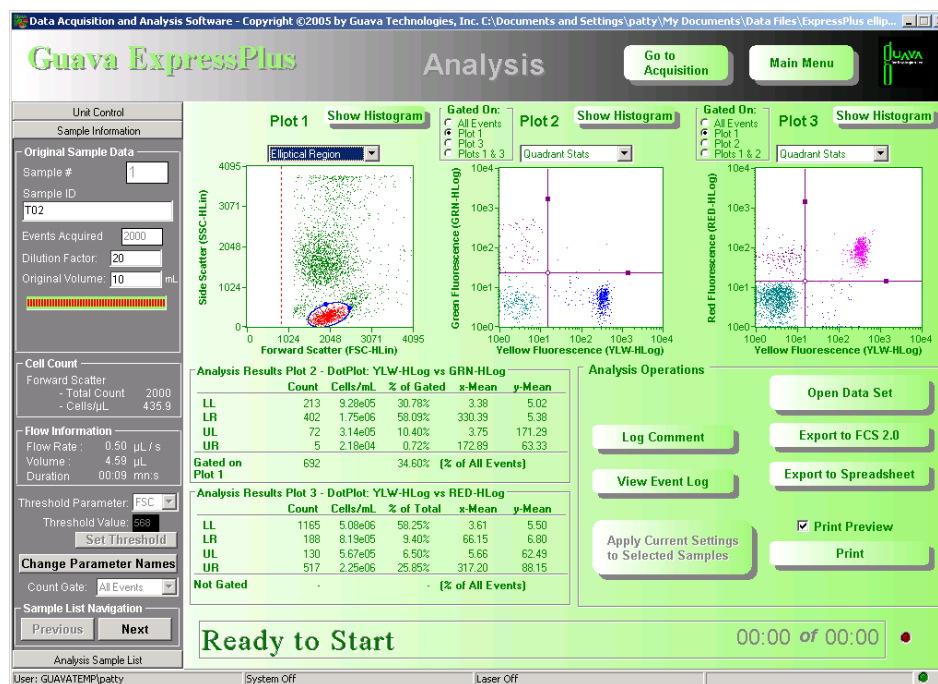
3 Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker settings appear as they were when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.

■ **NOTE:** If the number of events acquired per sample is greater than 15,000, it will take several seconds to see the results appear on the analysis screen.

Ensure that the gates and markers are set appropriately for negative and positive controls. Adjust if necessary.



Three-color data—sample stained with CD8-FITC, CD4-PE, and CD3-PE-Cy5

Gates

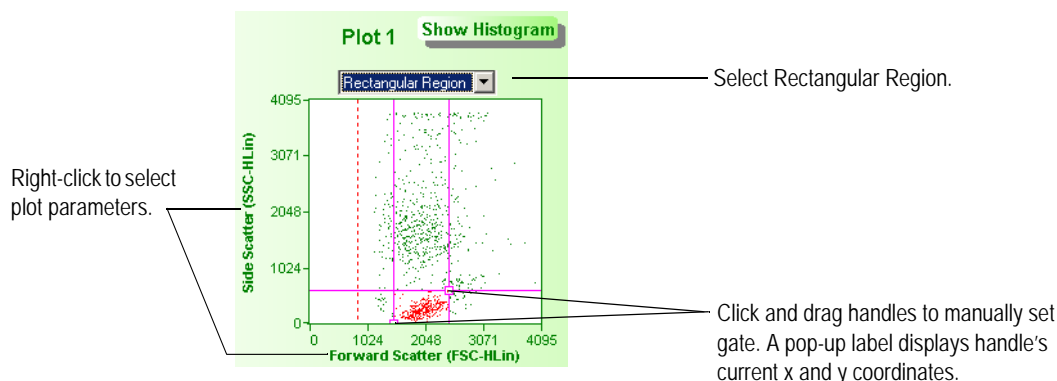
You can set up to two analysis gates—one gate in Plot 1 and a second gate in either Plot 2 or Plot 3. Then, while generating statistics for certain subpopulations, you can apply one or both gates to the data displayed in Plots 2 and 3. You can also choose to analyze without using gates.

Dot Plot Gate

The dot plot gate allows you to select a specific population for analysis, while viewing two parameters simultaneously. You can set a dot plot gate using the rectangular or elliptical region in any of the plots. If necessary, use the **Show DotPlot** button above the plot to display the dot plot.

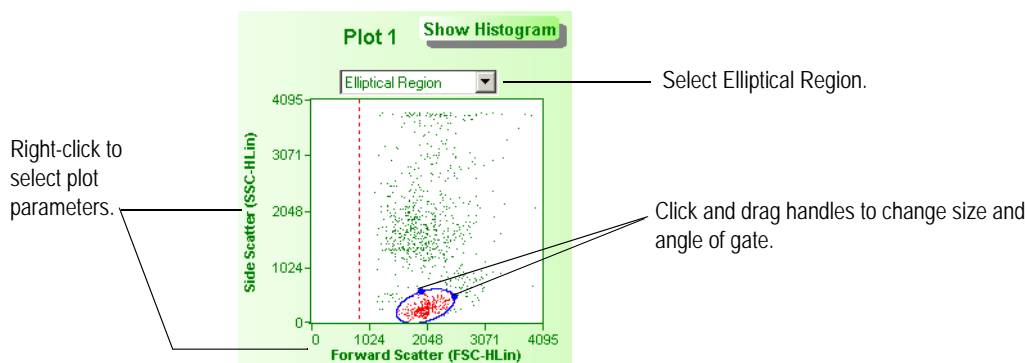
- 4 Select the plot parameters by right-clicking the axis labels and choosing the parameter from the pop-up menu.
- 5 To select the gate in Plot 1, 2 or 3, choose Rectangular Region or Elliptical Region from the drop-down menu above the plot.
- 6 Set the gate.

- To set a rectangular gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle and appear in red are included in the gate.
- **NOTE:** When using the negative control to set the gate, if one of the axes displays a fluorescence parameter, be sure to extend the gate high enough up that axis so that positive events in subsequent samples are included in the gate.



Rectangular region gate set on lymphocytes in a lysed whole blood sample.

- To set an elliptical gate, click anywhere on the edge of the ellipse, except on a handle, and drag it to a new location. Click on a handle to change the size and angle of the ellipse.



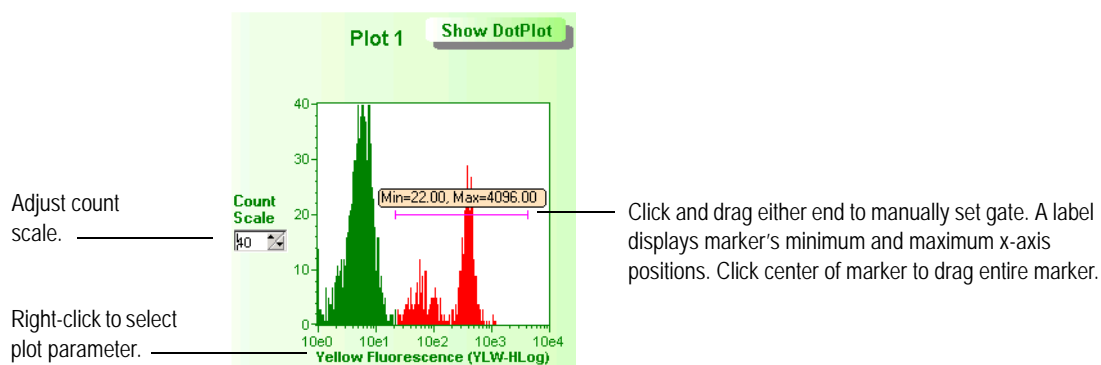
Elliptical region set on lymphocytes in a lysed whole blood sample.

Histogram Gate

The histogram gate allows you to select a specific population for analysis within a single parameter. You can set a histogram gate using the marker in Plot 1 or the first marker (M1) in Plots 2 and 3. If necessary, use the **Show Histogram** button above the plot to display the histogram.

- 4 Select the plot parameter by right-clicking the axis label and choosing the parameter from the pop-up menu.
- 5 Use the Count Scale to adjust the histogram y-axis scale.

- 6 To set the histogram gate, click and drag either side of the marker to size it. Click the horizontal line to drag the entire marker. If you gate the histogram in Plot 1, events that fall within the marker appear in red and are included in the gate. If you gate the histogram in Plot 2 or 3, events that fall within marker 1 (the gating marker) appear in pink. You may overlap the markers by clicking Allow Overlapping Markers, but only events within marker 1 are gated.



YLW histogram gate set on CD4-positive lymphocytes and monocytes.

Markers for Statistics

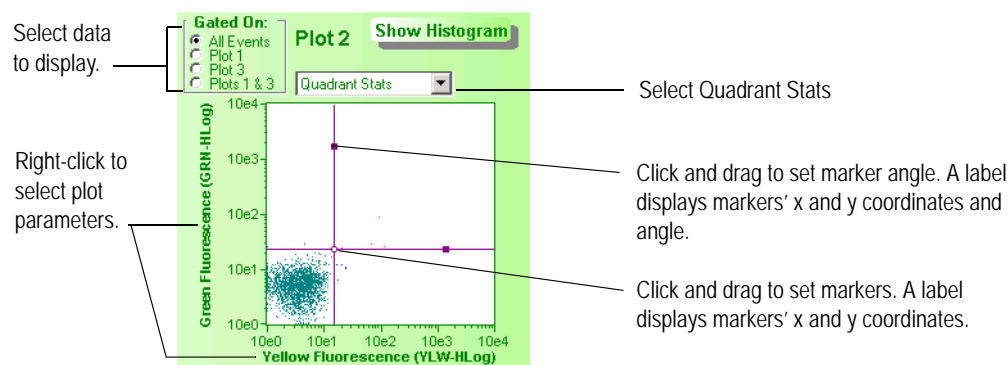
Use Plot 2 and Plot 3 to set quadrant markers, a rectangular region, an elliptical region, or histogram markers to generate statistics on all the data acquired or the data within the analysis gate(s).

- If necessary, click **Show Histogram/Dot Plot** above the plot to display the appropriate plot.
- Select the plot parameters by right-clicking the axis labels and choosing the parameter from the pop-up menu.

Setting Markers

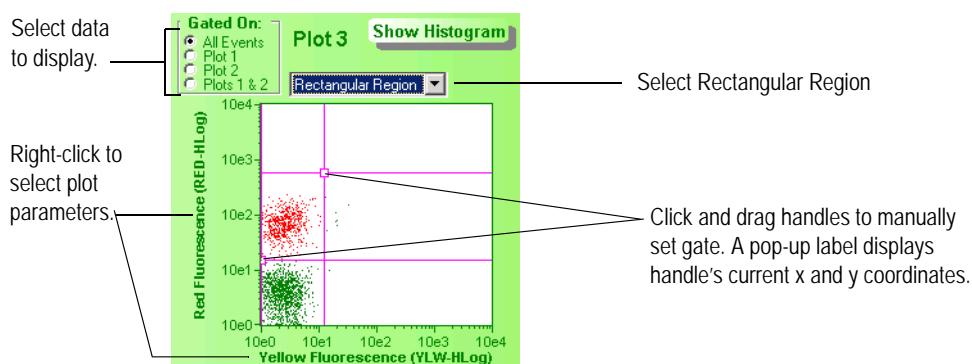
- 7 For quadrants, a rectangular region, or an elliptical region, select **Quadrant Stats**, **Rectangular Region**, or **Elliptical Region** from the drop-down menu above the plot.
- 8 Select the data you wish to display by clicking the appropriate button under Gated On, in the top-left corner of the plot.
You may choose to view and analyze all the events acquired, or only the events that are included in the gate. For Plot 2, gated data may include the gate in Plot 1, Plot 3, or both. For Plot 3, gated data may include the gate in Plot 1, Plot 2, or both.
 - To set *quadrant markers*, position the cursor over the handle at the intersection, then click and drag to the desired location

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



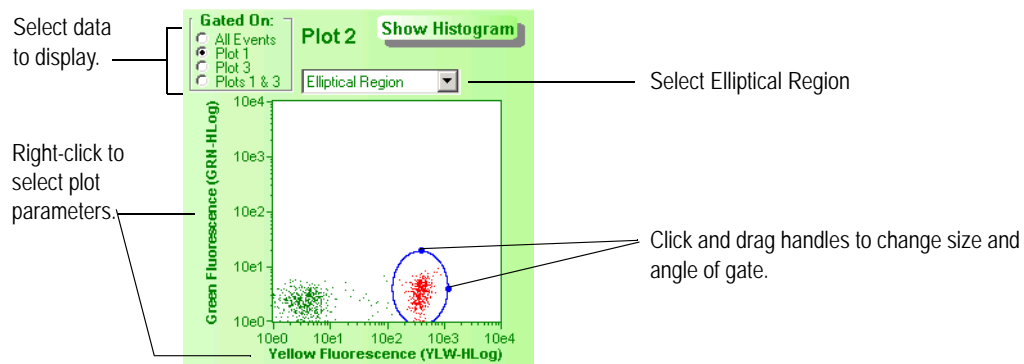
Quadrant markers set on negative control.

- To set a *rectangular region*, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle and appear in red are included in the region.



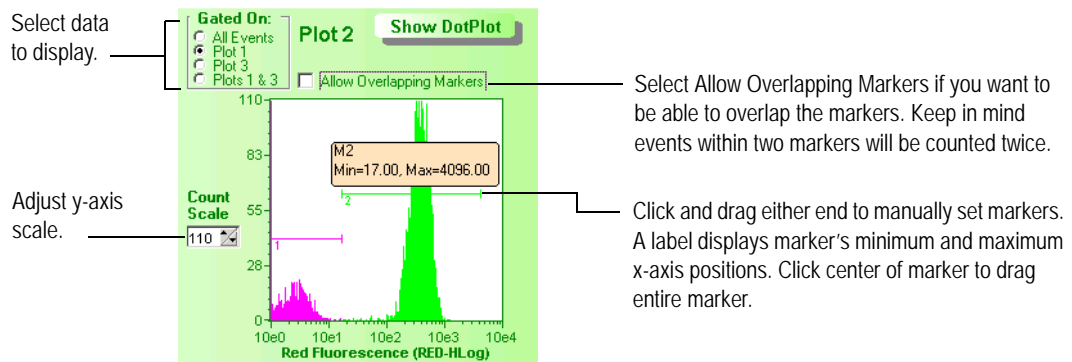
Rectangular region set on CD3-positive lymphocytes.

- To set an elliptical region, click anywhere on the edge of the ellipse, except on a handle, and drag it to a new location. Click on a handle to change the size and angle of the ellipse.



Elliptical region set on CD4-positive lymphocytes.

- To set the *histogram markers*, click and drag either side of the marker to size it. Click the horizontal line to drag the entire marker. Events that fall within marker 1 appear in pink, events that fall within marker 2 appear in light green, events that fall within marker 3 appear blue. You may overlap the markers by clicking Allow Overlapping Markers. Be aware that events falling within two markers will be counted twice in the statistics.



Histogram regions set on CD3-positive lymphocytes.

- Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- You can apply gate and/or markers settings, as well as changes to parameter names from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**. Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.

- 11 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.

Results are automatically exported to a CSV file that is given the same name as the FCS file.

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

- 12 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

guava ExpressPlus Results

You can view subsets of the data and generate individual statistics for Plots 2 and 3 by selecting an option under Gated On (All Events, Plot 1, then depending on which plot you are viewing—Plot 2 or 3, Plots 1 & 3, and Plots 1 & 2).

- Click All Events to view all data in the plot. Click P1, P3, or P1 and P3 in Plot 2 to view the gated data from Plot 1, Plot 3, or data that is common to both gates. Click P1, P2, or P1 and P2 in Plot 3 to view the gated data from Plot 1, Plot 2, or data that is common to both gates.

The statistics provide you with the count, cell concentration, mean signal intensities, and %CV (for histogram data) for all data in the plot (if ungated) or for data within the gate.

Histogram Statistics

Analysis Results Plot 2 - Histogram: GRN-HLog					
	Count	Cells/mL	% of Gated	x-Mean	% CV
Marker 1	1363	5.76e04	32.21%	2.15	61.94%
Marker 2	2868	1.21e05	67.79%	269.80	41.81%
Marker 3	0	0.00e00	0.00%	0.00	0.00%
Gated on Plot 1	4231		50.72% [% of All Events]		

guava ExpressPlus GRN histogram statistics.

The histogram statistics provide the count, cells/mL, mean signal intensity, and %CV for each population within a marker. Additionally, the % of Total or Gated shows the percentage of the data displayed in that plot or the subset of data within the gate.

The *Gated on* row shows the count (number of events in the gate) and the gated events as a percent of all events. If a gate was not used, *Not Gated* appears.

Quadrant Statistics

Analysis Results Plot 2 - DotPlot: GRN-HLog vs YLW-HLog					
	Count	Cells/mL	% of Gated	x-Mean	y-Mean
LL	1387	5.86e04	32.78%	2.28	1.69
LR	2844	1.20e05	67.22%	272.00	3.22
UL	0	0.00e00	0.00%	0.00	0.00
UR	0	0.00e00	0.00%	0.00	0.00
Gated on Plot 1	4231		50.72% [% of All Events]		

guava ExpressPlus quadrant statistics.

The quadrant statistics provide the count, cells/mL, and x and y mean signal intensity for each population within each quadrant. Additionally, the % of Total or Gated shows the percentage of the data displayed in that plot or the subset of data within the gate.

The *Gated on* row shows the count (number of events in the gate) and the gated events as a percent of all events. If a gate was not used, *Not Gated* appears.

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

Quadrant	Staining	Color
lower left	x negative, y negative	teal
lower right	x positive, y negative	blue
upper right	x positive, y positive	pink
upper left	x negative, y positive	purple

Rectangle Statistics

Analysis Results Plot 2 - DotPlot: GRN-HLog vs YLW-HLog					
	Count	Cells/mL	% of Gated	x-Mean	y-Mean
Rect	2819	1.19e05	66.63%	274.21	3.22
Gated on Plot 1	4231		50.72% (% of All Events)		

guava ExpressPlus rectangular region statistics.

The rectangle statistics provide the count, cells/mL, and x and y mean signal intensity for the population within the region. Additionally, the % of Total or Gated shows the percentage of the data displayed in that plot or the subset of data within the gate.

The *Gated on* row shows the count (number of events in the gate) and the gated events as a percent of all events. If a gate was not used, *Not Gated* appears.

Ellipse Statistics

Analysis Results Plot 2 - DotPlot: GRN-HLog vs YLW-HLog					
	Count	Cells/mL	% of Gated	x-Mean	y-Mean
Ellipse	2065	8.79e04	50.10%	3.50	901.87
Gated on Plot 1	4122		67.20% (% of All Events)		

guava ExpressPlus elliptical region statistics.

The ellipse statistics provide the count, cells/mL, and x and y mean signal intensity for the population within the ellipse. Additionally, the % of Total or Gated shows the percentage of the data displayed in that plot or the subset of data within the gate.

The *Gated on* row shows the count (number of events in the gate) and the gated events as a percent of all events. If a gate was not used, *Not Gated* appears.

Exporting guava ExpressPlus Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting guava ExpressPlus Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**. The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

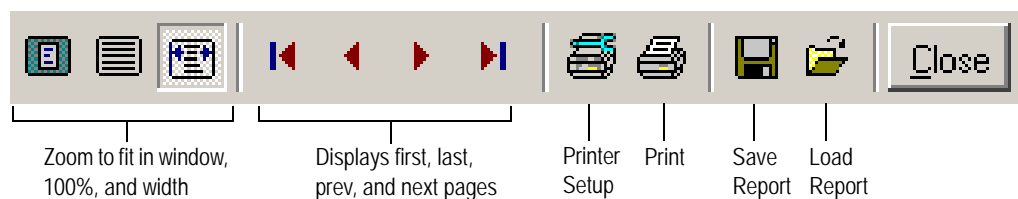
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

Move the cursor over the icon to display the description.



guava ExpressPlus Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

Sample Information

Original Sample Data

Current Sample #: 1

Sample ID: A01

Events to Acquire: 5000

Dilution Factor: 1.00

Original Volume: 10.0 mL

AutoSave FCS 2.0 Files

Cell Count

Forward Scatter

- Total Count: 5149
- Cells/μL: 139.2

Flow Information

Flow Rate: 0.59 μL/s

Volume: 37.00 μL

Duration: 01:02 mn:s

Threshold Parameter: FSC

Threshold Value: 819

Set Threshold

Change Parameter Names

Count Gate: Plot 1

Sample List Navigation

Previous Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 5000.
- Displays the dilution factor and the original sample volume. The default values are 1 and 10, respectively.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells and cells/μL that have exceeded the FSC threshold.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Threshold Parameter and *Threshold Value* allow you to select the threshold parameter and enter its value. Click **Set Threshold** after entering the Threshold Value.

Click **Change Parameter Names** to enter your own parameter names (for example, the name of the monoclonal Ab you use). See “Changing Parameter Names” on page 5-20.

Count Gate allows you to select a gate used as a counting gate. All events above the threshold are saved to the file whether they are in the gate or not. However, the number of Events to Acquire is applied to events that fall within the gate.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Changing Parameter Names

You can change the default long (or stain) name of any parameter. The short name, for example, GRN-HLog, will still appear in parentheses after the new long name.

- 1 Click **Change Parameter Names** in the Sample Information control panel.
- 2 Highlight the existing name in the *Long or Stain Name* text field and type in the new name.
- 3 Click **Update**.



Param No.	Short Name	Long or Stain Name
1	FSC-HLin	Forward Scatter
2	SSC-HLin	Side Scatter
3	GRN-HLog	Green Fluorescence
4	YLW-HLog	Yellow Fluorescence
5	RED-HLog	Red Fluorescence
6	FSC-HLog	Forward Scatter
7	SSC-HLog	Side Scatter
8	GRN-HLin	Green Fluorescence
9	YLW-HLin	Yellow Fluorescence
10	RED-HLin	Red Fluorescence

Update Cancel

- You can change the long or stain names for individual samples.
- If you change the names before starting acquisition, the new names will apply to all samples in the run.
- If you use the *Apply Current Settings to Selected Samples* button during analysis, any changes made to long names will be applied to the selected samples.

Unit Control

The Unit Control panel is divided into several sections:

- Detection:** Includes a Laser status indicator (red dot), FSC Gain: x 32 (100%), SSC: 750 V, and voltage settings for GRN (750 V), YLW (630 V), and RED (750 V).
- Pump Status:** Includes indicators for End of Stroke (red dot), Waste (red dot), and Pump Reset (red dot). It also shows Position: 10 Steps and Actual Speed: 0 Steps/s.
- Pump Action:** Includes indicators for Pump on (red dot), Waste (yellow dot), Pump Reset (yellow dot), Purge (yellow dot), and Sample (green dot). It also shows Speed: 0 Steps/s.
- Threshold parameters:** Includes Offset and Threshold settings for FSC (0.00 V and 800 units).
- Sample List Navigation:** Includes Previous and Next buttons.

Detection

Displays the laser status, the SSC (if installed) GRN, YLW, and RED voltage settings, and the FSC gain.

■ **NOTE:** Do not change the voltages from this panel. Use the sliders in Adjust Settings to adjust the SSC, GRN, YLW, and RED voltages.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold settings for the threshold parameter.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava ExpressPlus Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava ExpressPlus spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava ExpressPlus FCS file to another directory or give it a new name.
guava ExpressPlus Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Cell Count section of Sample Information control panel.	1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled.	1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate).	1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying GRN, YLW, or RED.	1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris.	1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but the events appear in the wrong places in plots displaying GRN, YLW, and/or RED.	1. Sample was not stained. 2. Cell lysis.	1. Check sample. If necessary, restain sample from original suspension. 2. Check buffers used to process cells.
Events appear in Plot 1 but not in Plot 2 or Plot 3.	Plot 1 gate excludes events, and gate is applied to Plot 2 and/or Plot 3.	Uncheck Plot 1 (under Gated On) for Plots 2 and 3 to see if events appear. Or, set Plot 1 gate to include population of interest.

Problem	Possible Cause	Solutions
Events appear in Plot 2 but not in Plot 3.	Plot 2 gate excludes events, and gate is applied to Plot 3.	Uncheck Plot 2 (under Gated On) for Plot 3 to see if events appear. Or, set Plot 2 gate to include population of interest.
Events appear off scale in dot plots or histograms.	FSC gain or SSC, GRN, YLW, and/or RED voltages set incorrectly, or samples staining brightly.	Adjust gain setting or voltage settings so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Voltages too low to detect fluorescent signals. 2. Incomplete staining with fluorescent probe, or fluorescent probe inappropriate for cell type. 3. Fluorescent probes over-exposed to light, stored improperly, or expired. 4. Non-specific binding of fluorescent probes. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Ensure positive control is staining adequately and with correct reagent. 3. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 4. If using antibody-based probes, try Fc blocking reagent during staining to minimize non-specific binding. Otherwise, titer the fluorescent probes down to reduce the nonspecific staining. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.

Problem	Possible Cause	Solutions
Poor resolution between positive populations in plots displaying GRN, YLW, and/or RED.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s). 2. Too much reagent in staining tube. 3. Fluorescence background too high. 4. Voltage too high causing signal to bleed into other parameters. 5. Voltage too low to optimally detect positive signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. 2. Washing cells may remove residual reagent. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce voltage. Adjust compensation settings. 5. Adjust settings to increase voltage. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris. Or, select one of the fluorescence parameters as the threshold.

CHAPTER 6

guava ExpressPro Assay

Introduction

The guava ExpressPro Assay allows you to acquire and analyze up to six fluorescence parameters in combination with forward scatter (FSC) and side scatter (SSC), provided as an optional parameter, as well as area, width, and time, to identify cells with specific phenotypic markers or specific properties. The software provides absolute cell counts and allows you the flexibility to stain samples with the guava Express Reagents, your own fluorochrome-conjugated antibody reagents, DNA intercalating dyes, or other green, yellow, red, and/or near infrared (NIR) fluorescent reagents that are excited by a 488-nm laser, as well as red (RED2) or near infrared (NIR2) fluorescent reagents that are excited by a 640-nm laser.

The guava ExpressPro Assay can be used for any one-, two-, three-, four-, five-, or six-color assay including:

- protein expression or other cell-surface marker experiments
- intracellular staining
- screening and analyzing cells expressing Green Fluorescent Protein (GFP)

The Green parameter can be used for FITC, GFP, or Alexa Fluor 488. The Yellow parameter has been optimally configured for use for phycoerythrin (PE)-based reagents, although fluorochromes with comparable emission spectra, such as propidium iodide (PI), TRITC, DS Red, sulforhodamine, Cy3, and Alexa Fluor 532 can also be used. The Red parameter can be used to detect PE-Cy5, PerCP, 7-AAD, PE-Cy5.5, and PI. The NIR parameter can be used to detect PE-Cy7 or PE-AlexaFluor 750. The RED2 parameter can be used to detect Allophycocyanin (APC) or fluorochromes with comparable emission spectrum. The NIR2 parameter can be used to detect Cyanine 5 (Cy5), APC-Cy7, APC-AlexaFluor 750, or fluorochromes with comparable emission spectrum. Refer to Specifications for the guava easyCyte HT System fluorescence detection range. In addition to the height value for each pulse measured, you can select to save width and area of each pulse for a single parameter. These measurements are especially useful in cell cycle analysis to discriminate doublets from singlets.

To run the assay, stain samples using an appropriate fluorochrome combination for up to six fluorescence parameters. Acquire the samples on the guava easyCyte HT System using guavaSoft Software. The data are displayed in eight plots, which can be set up to show histograms or dot plots with any parameter combination (FSC, SSC, GRN, YLW, RED, near infrared [NIR], RED2, and NIR2, as well as area, width, and time). You may set up to 16 global regions and 32 global gates to select a specific subpopulation(s) for

analysis, then choose the gate(s) for which you want to display statistics for the remaining plots.

Histogram and dot plot statistics include the cell count, cells/mL, mean signal intensity, geometric mean signal intensity, median signal intensity, and %CV for the data within the entire plot and the subset of data within the markers/gate. Additionally, the data are expressed as a percentage of the total data within the plot (whether gated from the dot plot or histogram) and as a percentage of data within the gate.

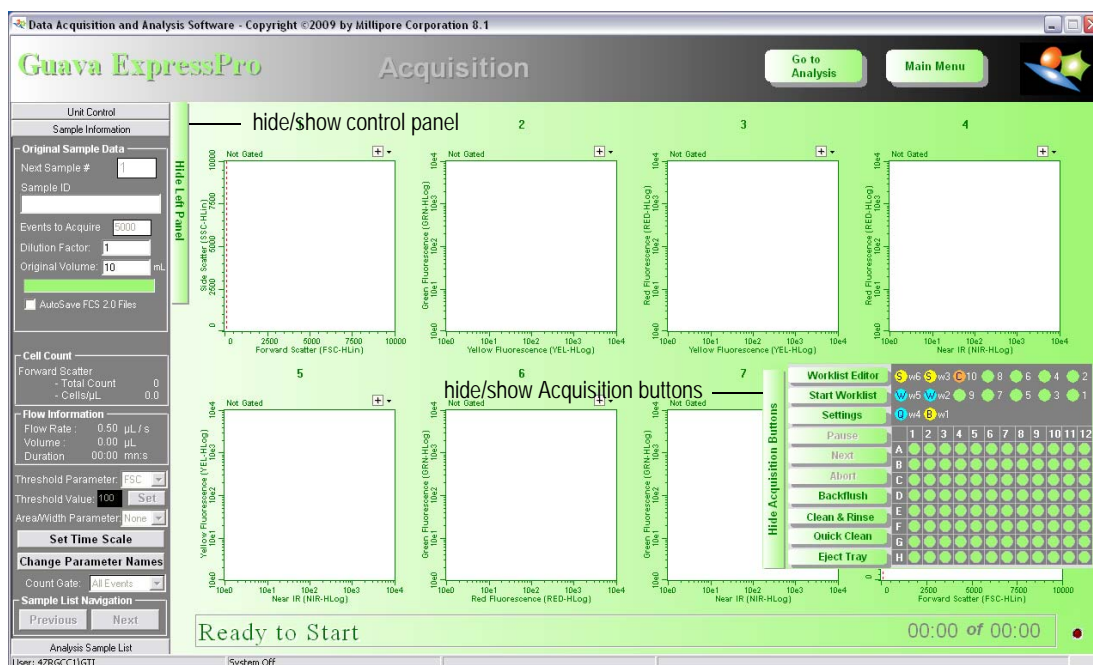
The guava ExpressPro data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you selected to save FCS 2.0 files. In addition to the saved data file, user-selectable statistics, instrument settings, and the acquisition summary information are exported to a spreadsheet file.

Running the guava ExpressPro Assay

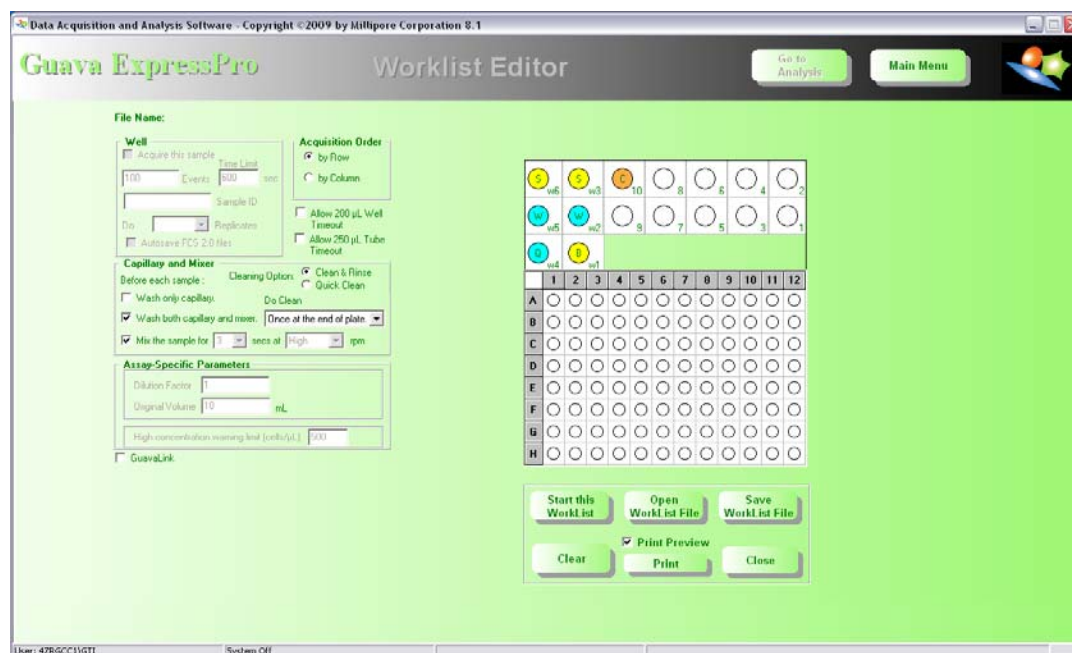
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “guava ExpressPro Software Module Control Panels” on page 6-25.

- 1 Open guavaSoft Software by double-clicking the guavaSoft 2.7 icon on the desktop.
- 2 Click **ExpressPro** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples. The guava ExpressPro application window opens displaying eight plots. The control panel and acquisition buttons are hidden. To display the control panels, click **Show**

Left Panel at the top-left area of the window. To display the acquisition buttons, click **Show Acquisition Buttons** at the bottom-right area of the window.

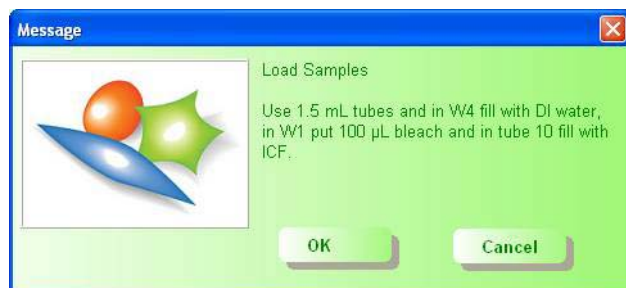


- 3 Prepare samples for analysis in a microplate or 0.5-mL tubes.
- 4 Click **Worklist Editor** to open the Worklist Editor application and define the worklist parameters.
 - **NOTE:** If you already created a worklist, you can click **Start Worklist**. The tray ejects and dialog boxes appear prompting you to select the worklist file, save the data set, and select the sample to adjust instrument settings.

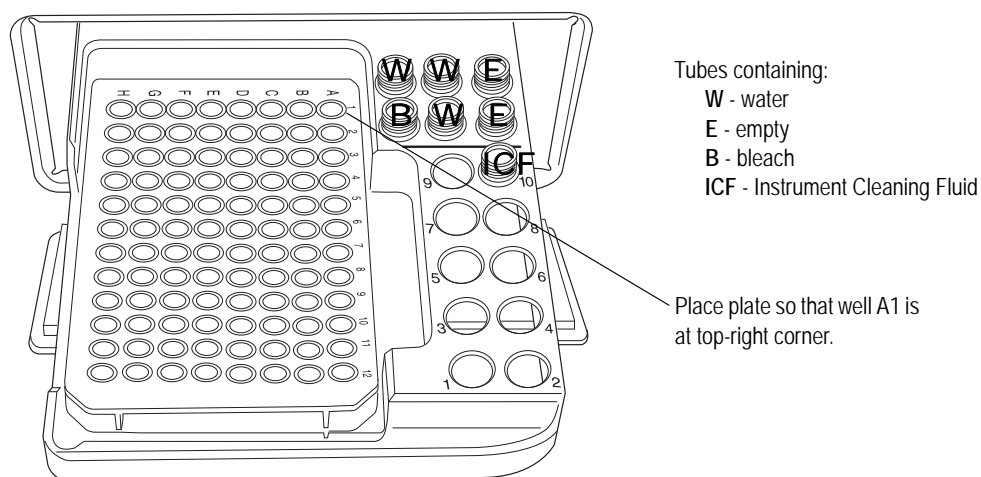


- 5 Define the worklist parameters for the run and click **Start this Worklist**. See “Creating a Worklist” on page 1-29 for more information on setting up a worklist.
When you start a worklist, the sample tray ejects. A dialog box appears prompting you to load samples.

◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.



- 6 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 µL of bleach in position w1 (for performing a backflush).
 - Load a tube containing 1.5 mL of ICF in position 10 for Clean & Rinse.



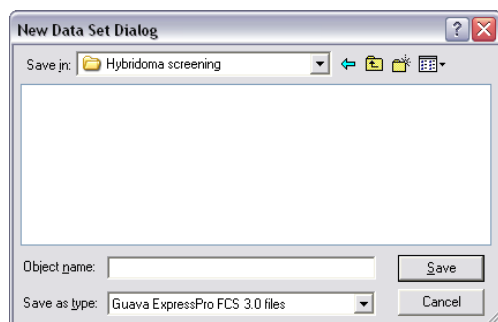
- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.

◆ **WARNING:** Keep the area clear as the tray loads.

◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

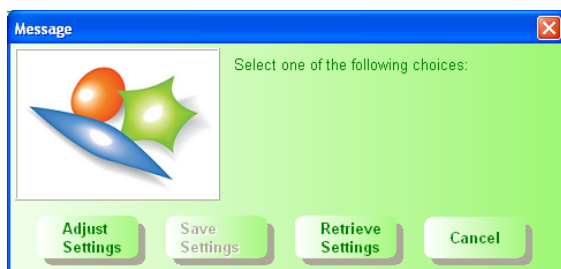
- 8 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



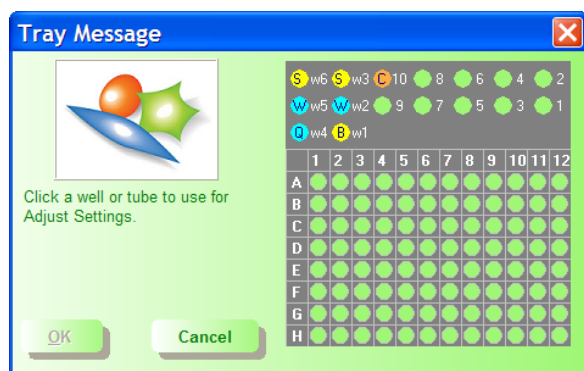
Enter a file name for the data set and click Save.

- 9 Select an instrument settings option.
- To adjust instrument settings, click **Adjust Settings**.
 - To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



- 10 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends using a stained negative or isotype control sample for the initial

adjustments. Click to select the well or tube (1–9) used to adjust settings, then click **OK**.



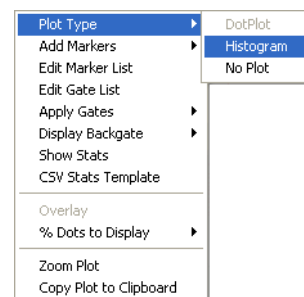
The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

- 11 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is less than or equal to 500.

■ **NOTE:** If the value is greater than the high limit for the corresponding flow rate, click the **Abort**, then **Eject Tray**. Dilute the sample with the appropriate buffer to lower the concentration and minimize the risk of coincident events. For optimal performance, EMD Millipore recommends a concentration of 50–500 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

- 12 To adjust or fine tune the settings, you can make the following adjustments once events start to appear on the screen:

■ **NOTE:** Click the plus sign (+) at the upper-right corner of a plot or right-click the plot to open the plot menu. Point to **Plot Type** and select the type of plot you wish to display. Select **No Plot** to hide the plot. To replace it, double-click the blue rectangle that appears in place of the plot. Click the axis label(s) and select the appropriate label from the pop-up menu. You can choose linear and log scale for each parameter except time and width.



- Set the **Refresh Rate** to the maximum number of events you want to display.
- Set the **Flow Rate** to Very Low (0.12), Low (0.24 μ L/s), Medium (0.59 μ L/s) or High (1.2 μ L/s). The recommended flow rate is Medium.
- **NOTE:** If you change the flow rate during the adjust settings step, EMD Millipore recommends that you repeat the adjust settings step at the new flow rate to ensure that the markers and threshold are still set correctly.
- Use the **FSC Gain** slider to reduce or amplify the FSC signal so that the cells are visible and on scale.
- Adjust the gains (using the SSC, GRN, YLW, RED, NIR, RED2, or NIR2 sliders or the arrow keys on the keyboard) to a value from 1 to 1024. Adjust so that the negative population is positioned in the lower-left corner of the fluorescence plot and the cells are evenly distributed in the lower-left quadrant. Start from a lower

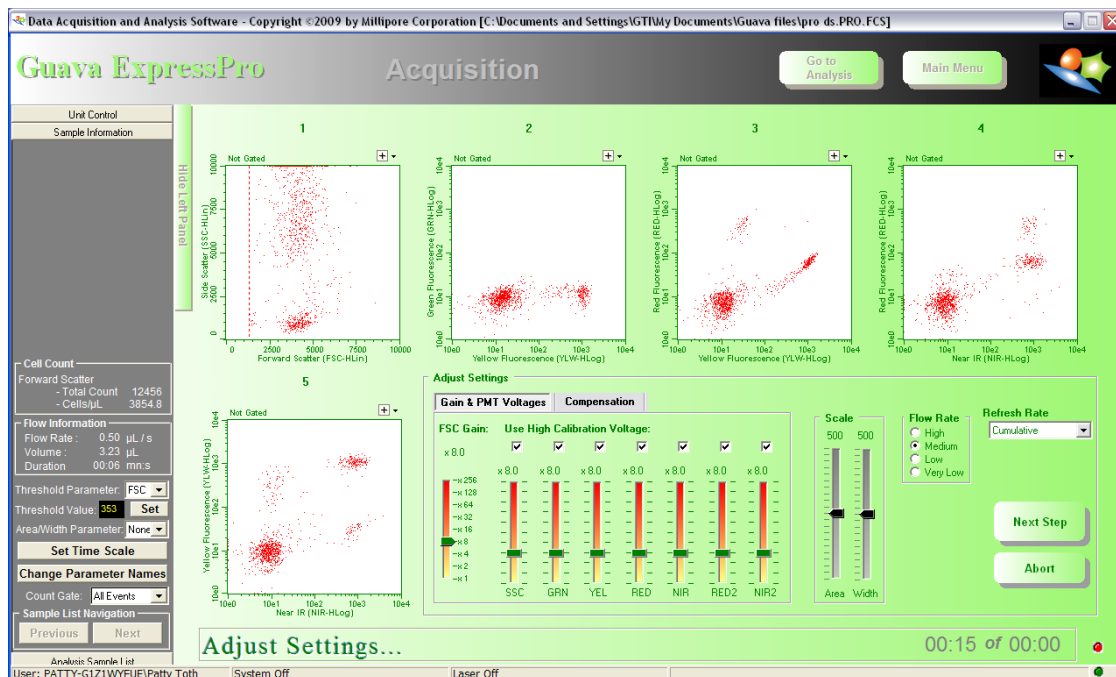
gain setting and gradually increasing the value. For greater detection and sensitivity across a broad range of fluorescence, the overall gain range can be adjusted using the High check box. By default, all boxes are checked. If further adjustment is needed, remove the checks from the High Calibration Voltage check boxes and allow the voltage to stabilize for 2–5 seconds.

- Select the threshold parameter (FSC, SSC, GRN, YLW, RED, NIR, RED2, or NIR2) from the drop-down menu in the Sample Information control panel.



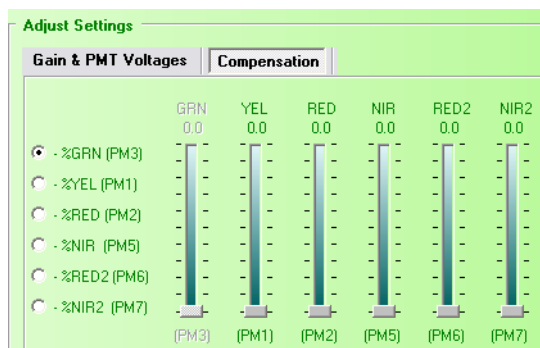
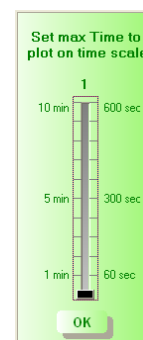
- To adjust the threshold, click and drag the threshold marker (dotted red line) up or down the axis of the dot plot displaying the threshold parameter until the desired amount of debris or other unwanted events are eliminated below the threshold. You can also enter a numerical value in the Threshold Value text box in the Sample Information control panel, then click **Set**.

■ **NOTE:** When setting the threshold, place it at least 2 to 3 mm to the left of the cells of interest to ensure that you are not excluding cells. Any debris that gets acquired can be excluded during analysis with an optional dot plot gate.



- If you wish to save area and width measurements for a specific parameter, select the parameter from the Area/Width Parameter menu in the Sample Information control panel. Use the Area and/or Width Scale sliders to reduce or amplify the signal so that the cells are visible and on scale.

- If you wish to save a time parameter, select Time from the parameter list. Click **Set Time Scale** in the Sample Information control panel and use the slider to set the maximum time to plot, then click **OK**. Adjust the y-axis (count) scale, if necessary.
- To adjust compensation, click **Next Step, Settings, Adjust Settings**, then select the location of a positive control sample. You may want to adjust each compensation setting individually using single-stained controls. Select the parameter radio button, then use the slider to remove the overlapping signal.



- **NOTE:** You must check the compensation for each fluorochrome combination you are using. For example, if using FITC and PE fluorochromes, which correspond to the GRN and YLW parameters, respectively, ensure that YLW-%GRN and GRN-%YLW compensation settings are correctly adjusted (see Figures 1 and 2 on page 6-8 for examples). If using PE-Cy5 as well, which corresponds to the RED parameter, ensure that the YLW-%RED and RED-%YLW compensation settings are correctly adjusted (see Figures 3 and 4).

Figure 1a

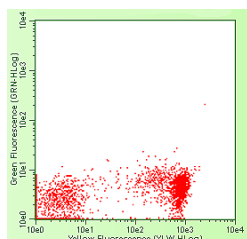


Figure 1b

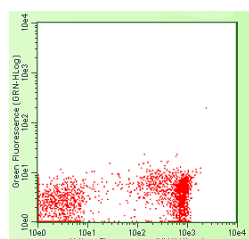


Figure 2a

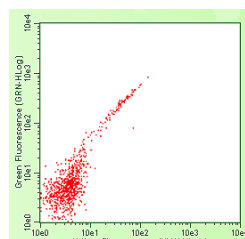
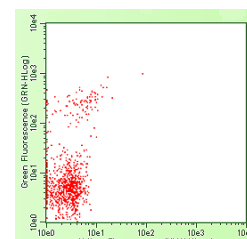


Figure 2b



GRN-%YLW uncompensated (Fig 1a) and compensated (Fig 1b)

YLW-%GRN uncompensated (Fig 2a) and compensated (Fig 2b)

Figure 3a

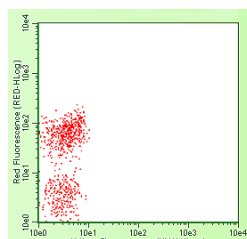


Figure 3b

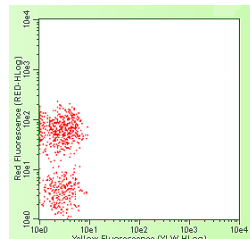


Figure 4a

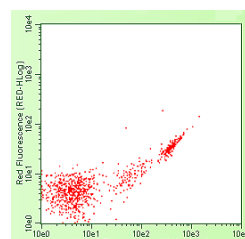
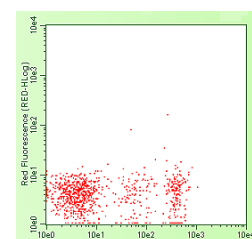


Figure 4b



YLW-%RED uncompensated (Fig 3a) and compensated (Fig 3b)

RED-%YLW uncompensated (Fig 4a) and compensated (Fig 4b)

Adjust YLW-%NIR and NIR-%YLW (see Figures 5 and 6). If necessary, adjust RED-%NIR (see Figure 7). Typically NIR-%RED compensation does not need to be adjusted.

Figure 5a

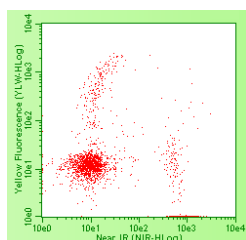


Figure 5b

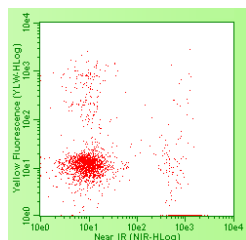


Figure 6a

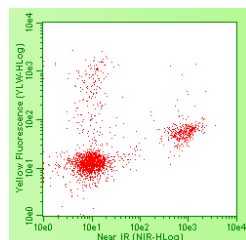
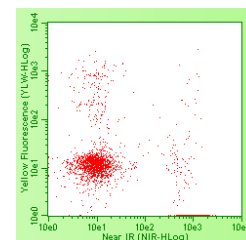


Figure 6b



NIR-%YLW uncompensated (Fig 5a) and compensated (Fig 5b)

YLW-%NIR uncompensated (Fig 6a) and compensated (Fig 6b)

Figure 7a

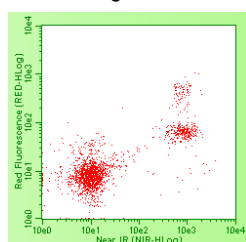
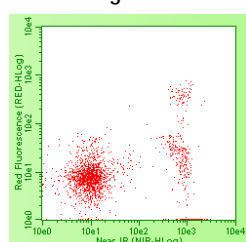


Figure 7b



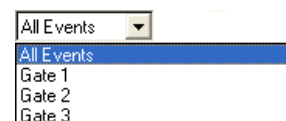
RED-%NIR uncompensated (Fig 7a) and compensated (Fig 7b)

If necessary, select GRN vs RED and/or GRN vs NIR to adjust these compensation settings. Some fluorochromes require very little compensation because they have little overlap, such as PE into the GRN parameter (GRN-%YLW) [see Figures 1a and 1b]; whereas others require much more compensation because they have more overlap, such as FITC into the YLW parameter (YLW-%GRN) [see Figures 2a and 2b]. Compensation settings are correct when the edge of the stained population touches the axis. Avoid having too many cells touching the axis (overcompensated) or the population too far from the axis (undercompensated).

- **NOTE:** If you adjust the compensation with single-color samples, check the compensation settings again with a sample stained with all fluorochromes and fine-tune, if necessary.
- **NOTE:** If you acquire samples with a new fluorochrome combination, you must ensure that the compensation is set correctly for the new samples.

- 13** You may set gates and markers prior to acquiring the samples. Refer to “Setting Markers” on page 6-13 and “Defining Gates” on page 6-18 for information.

If you wish to apply a count gate, define the gate, then select the appropriate gate from Count Gate drop-down menu in the Sample Information control panel. You can select the count gate from the Adjust Settings screen only.



- **NOTE:** Gates can only be a rectangular, elliptical, or octagonal region, or a histogram marker. You cannot use quadrant markers as gates.

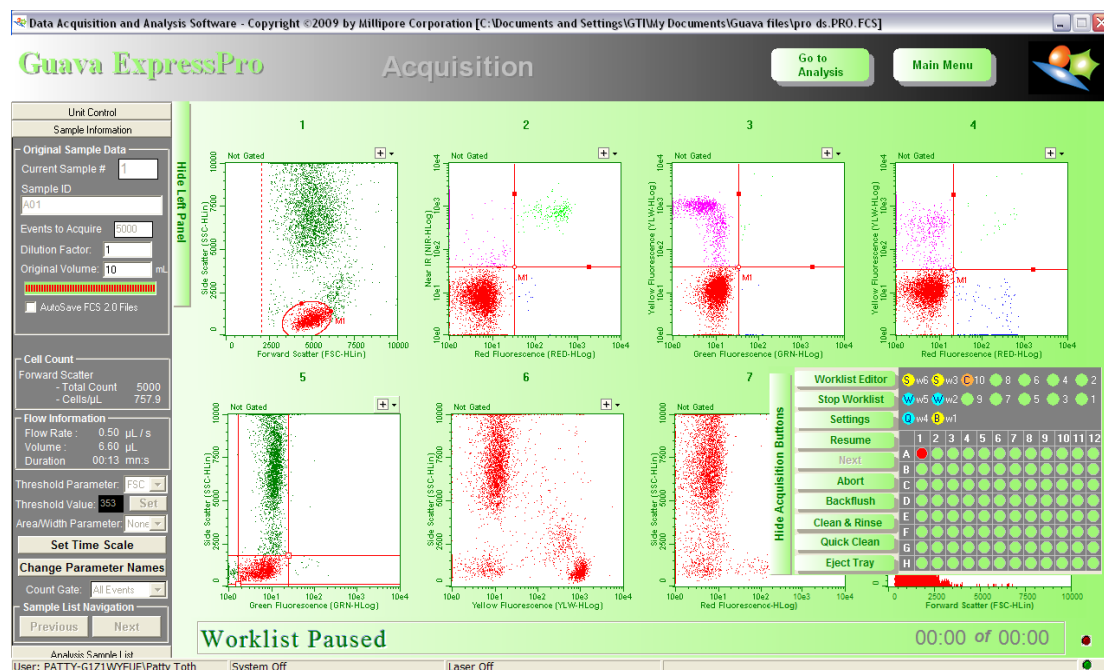
14 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.

If necessary, you can repeat the adjust settings step to ensure that other samples (such as another positive control) are on scale, appropriately positioned, and compensated, by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

If you made changes to an existing settings file, when you click **Next Step** you will be prompted to save it as a new file or overwrite the existing file.

15 Click **Resume**.

The system acquires the first sample and automatically displays the results. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Clean & Rinse**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 1.75 minutes (high flow rate), 3.5 minutes (medium flow rate), 7 minutes (low flow rate), or 10 minutes (very low flow rate).

The system automatically performs a Quick Clean or a Clean & Rinse at the end of the assay.

guava ExpressPro Analysis

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

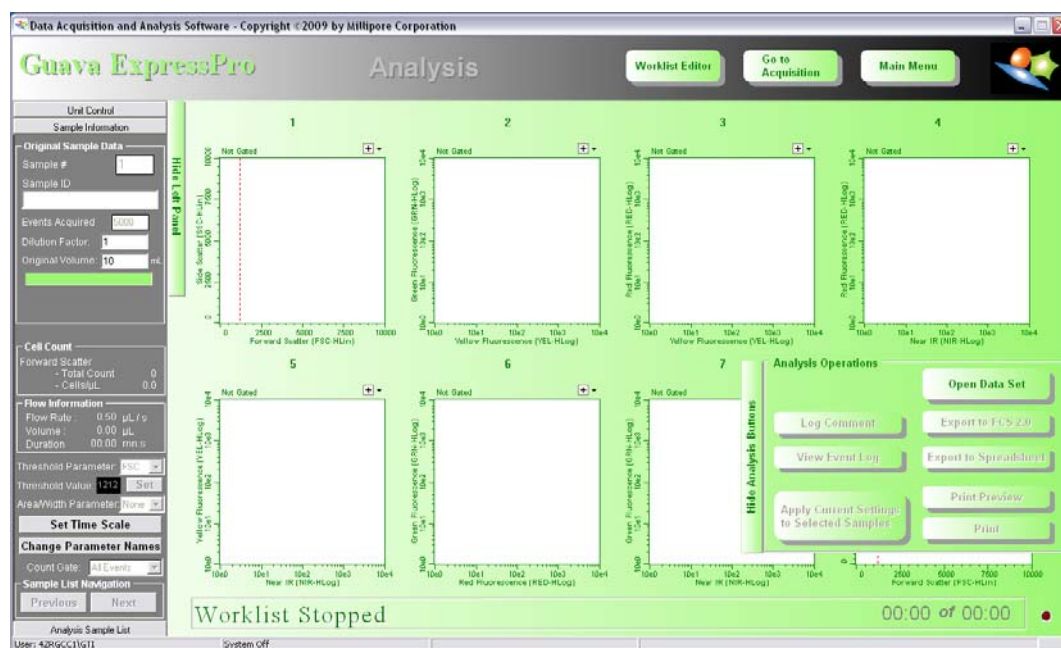
You can save any changes made within Analysis by overwriting the existing file or saving a new file.

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

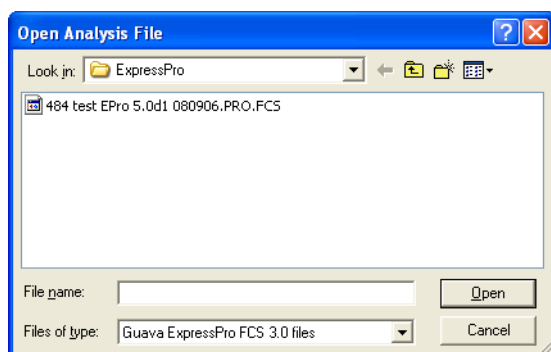
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis settings (plots, axes parameters, gates, markers, etc) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

This version of the ExpressPro Software Module within guavaSoft Software, version 2.7 allows you to open and analyze files from guava ExpressPro 5.2.7, as well as guava ExpressPlus files (*.EP5.FCS). Remember to select All FCS 3.0 files from the *Files of type* text box.

- 1 Click **ExpressPro** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.
The Analysis window opens displaying eight plots. The control panel and analysis buttons are hidden. To display the control panel, click **Show Left Panel** at the left of the window. To display the analysis buttons, click **Show Analysis Buttons** at the right of the window. You can hide the analysis buttons once the data are displayed.

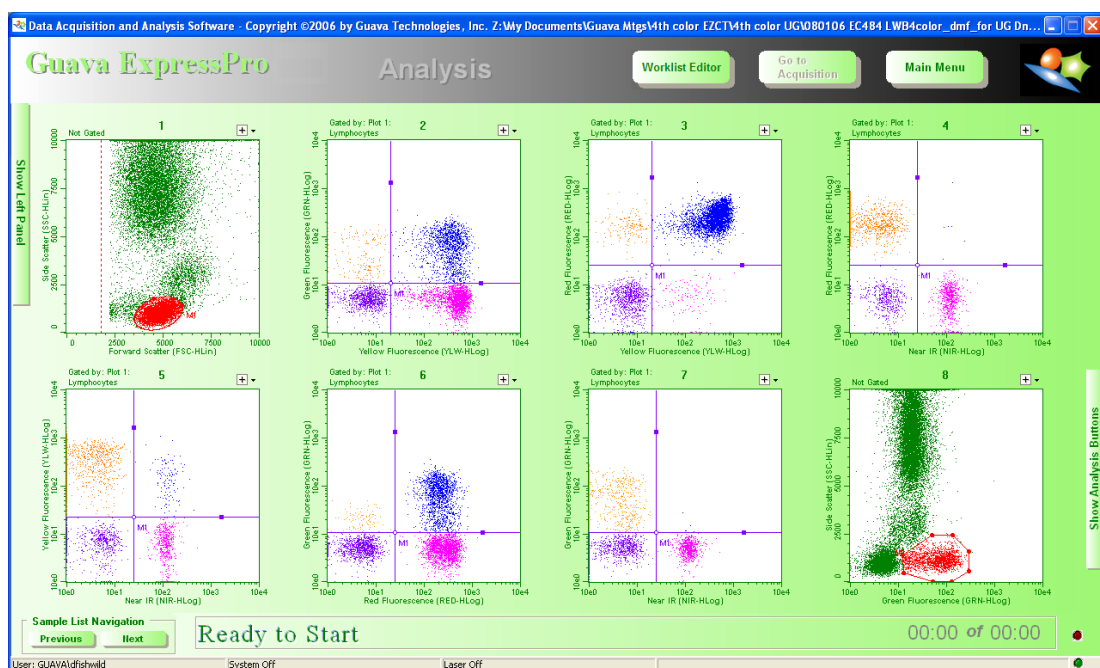


- Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker settings appear as they were when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.

- **NOTE:** If the number of events acquired per sample is greater than 15,000, it will take several seconds to see the results appear on the analysis screen. Ensure that the markers and/or gates are set appropriately for negative and positive controls. Adjust, if necessary.



Four-color data—sample stained with CD45RO-FITC, CD27-PE, CD3-PE-Cy5, and CD19-PE-Cy7

Plot Menu

Most of the analysis features are available from the plot menu. Click the plus sign (+) in the upper-right corner above the plot or right-click the plot to open the plot menu. You can copy a plot to the Microsoft Windows clipboard, by selecting **Copy Plot to Clipboard**. If you need a large copy of a plot, zoom the plot before you copy it. For more information on the options in the plot menu refer to “Editing Markers” on page 6-16, “Creating Global Regions” on page 6-18, and “Changing the Plot Appearance” on page 6-19.



Markers

Select any plot to set markers. If the plot is a histogram, you can set histogram markers. If the plot is a dot plot, you can set rectangular, quadrant, octagonal, or elliptical markers. Markers allow you to generate statistics on all the data acquired or the data within the analysis gates. You can set up to 10 markers on the data within a single plot, but only one set of quadrant markers per plot. Each marker is assigned a number that increments by one for each additional marker created. Once markers are set, they can be added to a global region list. You can then use the regions within the global region list to define gates.

Markers will stay in the same locations on the plot if different parameters are displayed. If scaling is switched between log and linear, the elliptical, octagonal, and quadrant markers will remain in the same location; while the histogram and rectangular markers will adjust to the selected scale.

- Click the plus sign (+) in the upper-right corner above the plot to open the plot menu. Point to **Plot Type** and select the type of plot you wish to display.
- Select the plot parameters by clicking the axis labels and choosing the parameter from the pop-up menu.

Setting Markers

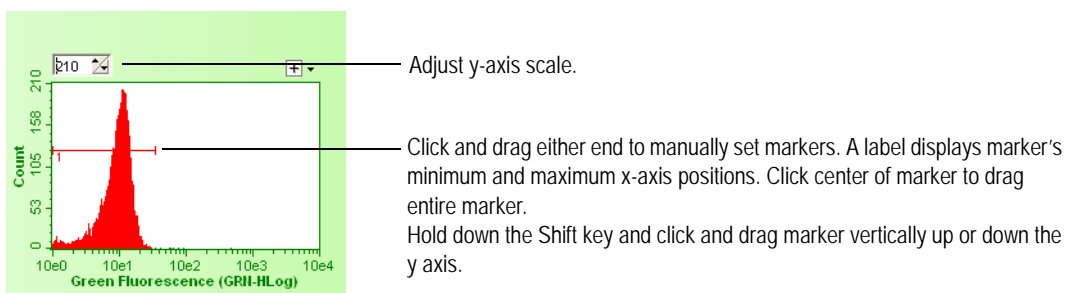
- 4 Open the plot menu, point to **Add Markers**, then select the type of marker you wish to set. Select **Histogram Marker** for a histogram plot, or **Rectangular Marker**, **QuadStat Marker**, **Octagonal Marker**, or **Elliptical Marker** for a dot plot.

- **NOTE:** If you already have a gate defined, you can apply it to the data in the plot by opening the plot menu, pointing to **Apply Gates**, then selecting the gate.

After you set the appropriate markers for analysis, you can define and apply gate (see “Defining Gates” on page 6-18).

Histogram Markers

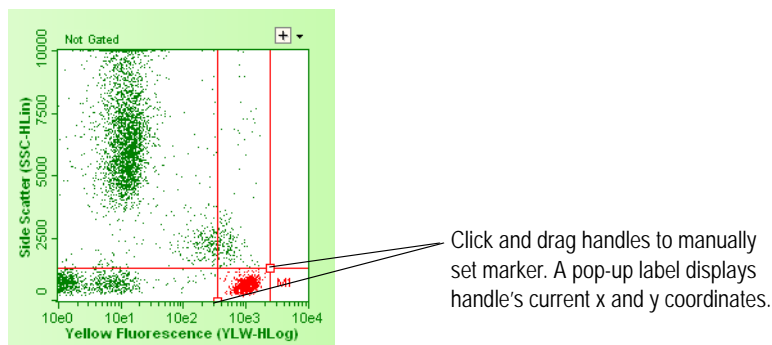
- To set the *histogram marker*, click and drag either side of the marker to size it. Click the horizontal line to drag the entire marker. Events that fall within marker 1 appear in red, events that fall within marker 2 appear in blue, and so on. There are 10 default colors for the 10 markers. However, you can change the colors of events falling within any marker set (see “Editing Markers” on page 6-16). You can move the histogram marker vertically along the y axis of the plot by holding down the Shift key and clicking and dragging the marker up or down. Use the Count Scale in the left corner above the plot to adjust the histogram y-axis scale.



Histogram marker set on negative control.

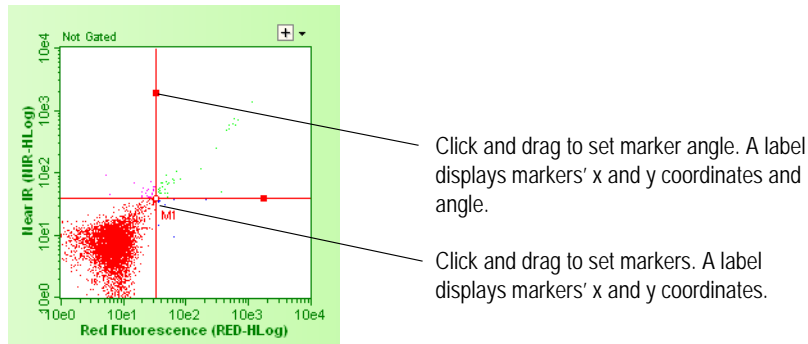
Rectangular, Quadrant, Octagonal, and Elliptical Markers

- To set a *rectangular marker*, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle are included in the marker.



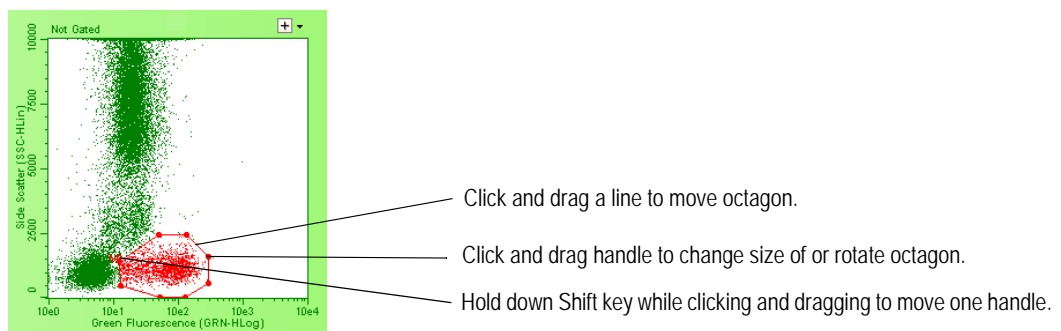
Rectangular marker set on CD3+ lymphocytes.

- To set *quadrant markers*, position the cursor over the handle at the intersection, then click and drag to the desired location
If necessary, you can adjust the angle of the markers $\pm 44^\circ$ from their original locations. Click and drag the handle (solid square) towards the end of the marker and tilt it to the desired location.



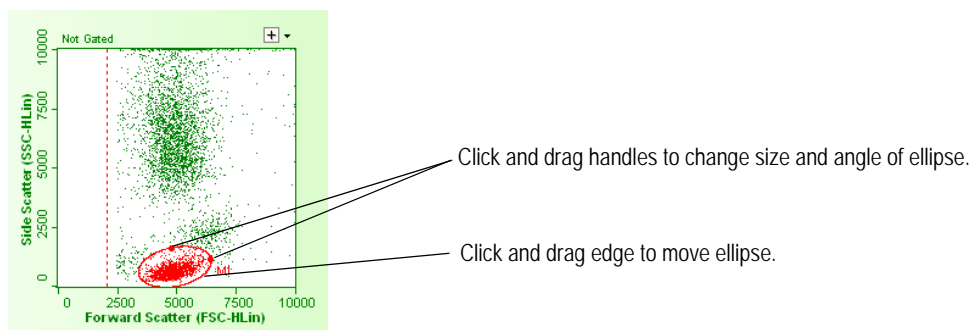
Quadrant markers set on negative control.

- To set an *octagonal marker*, click on a line of the octagon to drag it to a new location. Click on a handle to change the size of or rotate the octagon. To move one of the handles, press and hold the shift key while clicking and dragging a handle.



Octagon marker set on CD3+, CD4+ lymphocytes.

- To set an *elliptical marker*, click anywhere on the edge of the ellipse, except on a handle, and drag it to a new location. Click on a handle to change the size and angle of the ellipse.

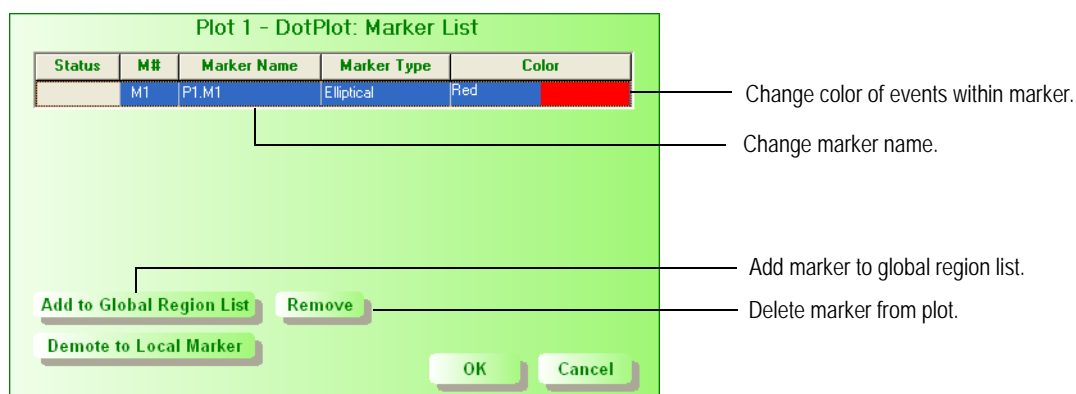


Elliptical marker set on lymphocytes.

- 5 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 6 You can apply gate and/or markers settings, as well as changes to parameter names from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
 - **NOTE:** Apply Current Settings to Selected Samples applies to parameter names as well, so make name changes last and apply only to the samples with the same custom name.
- 7 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
 - **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 8 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

Editing Markers

Use **Edit Marker List** in the plot menu to add markers to the global region list, delete markers from the plot, and change the color of events that fall within the markers. Click and drag the window to move it to a new location on the screen.



- To add a marker to the global region list, click to select the marker from the list and then click **Add to Global Region List**. *Global* will appear in the Status column. Click

OK. You cannot add quadrant markers to the global region list. To return the settings in this dialog to their original values, right-click a marker and select **Reset**.

- To change the default name, click in the Marker Name field and type a new name. You can use any alphanumeric characters and symbols, except spaces and parentheses.
- To remove a marker from the global region list, click **Demote to Local Marker**. *Demote* will appear in the Status column.
- To delete a marker from the plot, click the marker in the list, and click **Remove**. Click **OK**. You can also right-click the marker and select **Remove**. If the marker was in the global region list, it will be removed from the list. See the note on page 6-19 for more information on removing a marker from the global region list.
- To change the color of the events within a marker, click on the color in the Color column, then click the palette icon and select the desired color from the standard Windows pop-up color palette. Click **OK**.

Plot 2 - DotPlot: Marker List

Status	M#	Marker Name	Marker Type	Color
	M1	P2.M1	QuadStat	Red Blue Magenta Green

QuadStat Colors

LowerLeft	Red	Palette Icon
LowerRight	Blue	Palette Icon
UpperLeft	Magenta	Palette Icon
UpperRight	Green	Palette Icon

Buttons: Add to Global Region List, Remove, Demote to Local Marker, OK, Cancel

Callouts:

- Click any color to display the palette icons.
- Click any color to display the palette icons.
- Then, click the palette icon for the color you want to change and select a color from the standard Windows color palette. Click OK. Click the close box in the Quad Stat Colors pop-up window.

- To specify the height of a histogram marker as a percentage of the height of the histogram, click in the Y Pos (%) and enter a number from 10 to 100 or use the arrows to increase or decrease the value.
- To overlap histogram markers, select **Edit Marker List** from the plots menu, then click **Allow Overlapping Markers**. Be aware that events falling within two markers will be counted twice in the statistics. However, if the right side of one marker and left side of the second marker are at the exact same plot location, typically events will not be counted twice.

Plot 6 - Histogram: Marker List

Status	M#	Marker Name	Y Pos (%)	Color
	M1	P6.M1	30	Red
	M2	P6.M2	36	Blue
	M3	P6.M3	42	Fuchsia

Buttons: Add to Global Region List, Remove, Demote to Local Marker, OK, Cancel

☒ Allow Overlapping Markers

Callouts:

- Click to overlap histogram markers.

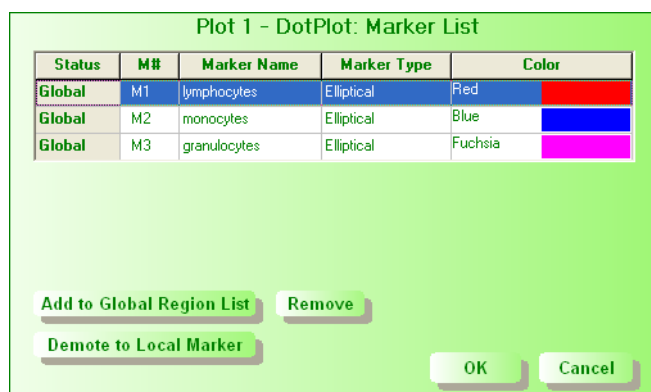
Defining Gates

Once you create markers to identify various subpopulations, you can add the markers to a global region list. These regions can then be used to define gates. You can set up to 32 analysis gates on the data within the plots. You can also choose to analyze without using gates.

Creating Global Regions

To create global regions, start by setting markers on the populations of interest.

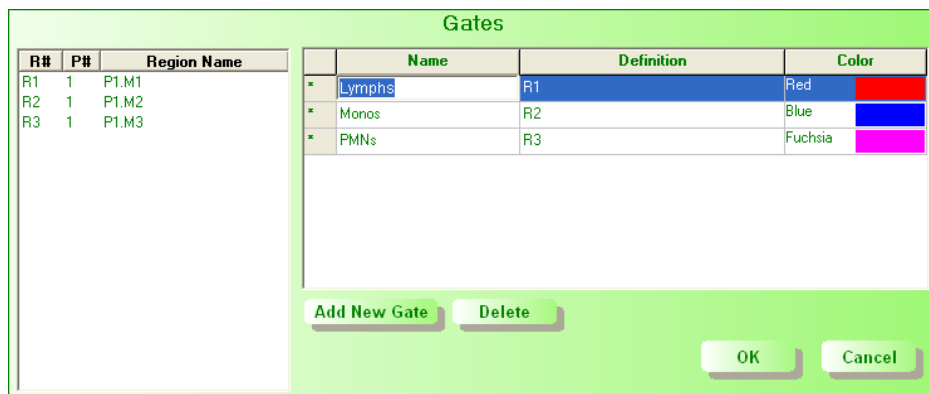
- Select **Edit Marker List** from the plot menu to display the Marker List dialog box with a list of markers in the selected plot. Select a marker and click **Add to Global Region List**. *Global* will appear in the left column. Click **OK**.



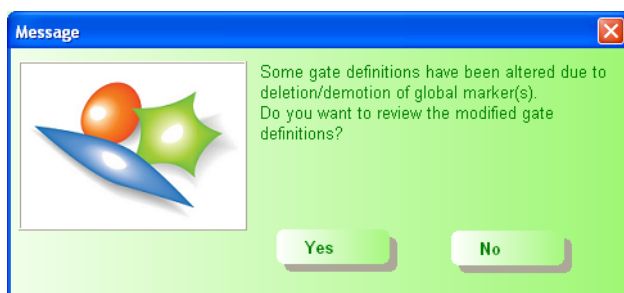
The Marker List dialog box allows you to add a marker to the global region list, delete a marker from the plot, and change the color of events that fall within the marker.

A marker can be demoted from a global marker back to a local marker.

- Select **Edit Gate List** from the plot menu to display the Gates dialog box. The left side of the box displays a list of markers that were added to the global region list. The list on the right displays a list of gates. To define a gate, click **Add New Gate**. Enter the definition. The definition may include the following operators—AND, OR, NOT. For example:
 - R1 AND R2 means the event must be in both the R1 and R2 regions to be included in the gate.
 - R1 OR R2 means the event must be in either the R1 or R2 region to be included in the gate.
 - R1 AND (NOT R2) can be used if R1 and R2 overlap and you want to include events in R1 but not in R2.



- To add a gate, click **Add New Gate**, or right-click a gate and select **Add Gate**. Click in the Definition field, type the gate definition, and click **OK**.
 - To change the default name, click in the Name field and type a new name to describe the gate. You can use any alphanumeric characters and symbols, except spaces and parentheses.
 - Once you have defined a gate, you can apply that gate to data appearing in any of the plots. Open the plot menu, point to **Apply Gates**, then select the gate.
 - To change the color of events within a gate, click the color box under the Color column.
 - To delete a gate, select the gate from the list and click **Delete**, or right-click a gate and select **Delete**. Right-click and select **Reset** to restore a deleted gate.
- **NOTE:** If you select Edit Marker List and use the Marker List dialog box to remove a marker that was a global region and that was used to define a gate, a dialog box will appear warning you that some gate definitions have been altered. Click **Yes** to review those changes which appear in red in the Gates dialog box.



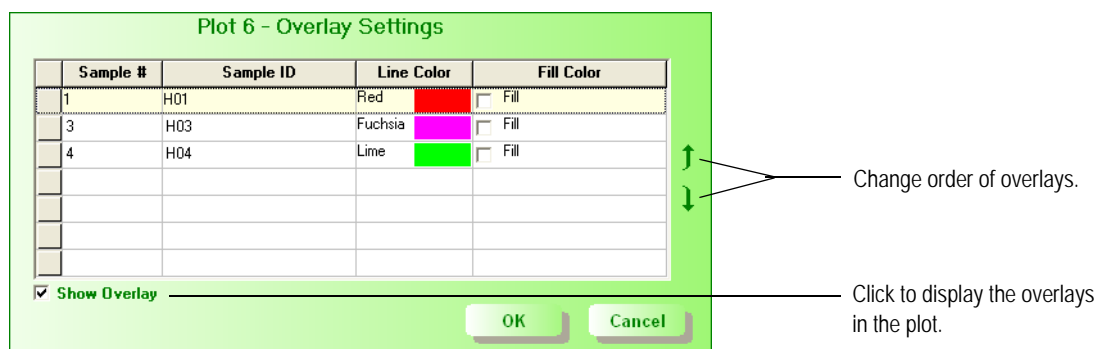
When a global region is removed from a local plot, that global region is removed from the regions list in the Gates dialog box, and the gate definition now shows a "1" in place of the global region number, indicating all events are used in that gate definition.

Changing the Plot Appearance

Overlaying Plots

You can overlay the data of up to six samples in a dot plot and seven samples in a histogram plot. By selecting a different color for each sample, you can see the different sample data overlaid within the same plot.

- 1 Select **Overlay** from the plot menu.
The Overlay Settings dialog box appears (histogram overlay dialog box shown).



- 2 Click the **Sample #** column and select the sample from the drop-down list. You may select up to six samples for dot plots and seven samples for histograms.
 - 3 To change the line or fill color, click the color in the **Line and/or Fill Color** column for a sample, then click the palette icon and select the desired color from the color palette. Click **OK**.
 - 4 Click **Show Overlay** and then click **OK**.
The plot will display the selected sample data in the selected colors. The first sample's dot plot/histogram is drawn, then the other plots are layered over it, with the last plot appearing on top. When you overlay plots, any markers you had previously set as well as the stats, apply to the base plot only.
To change the overlay order, click a sample in the list then click the up or down arrow at the right of the list to reorder the sample. To remove an overlaid plot, right-click on the overlay and select **Remove**. The overlay plots are gated from the same gate as the initial plot.
- **NOTE:** You cannot overlay sample data with different plot parameters. A message will appear informing you that some samples do not have the required parameters.

Displaying a Backgate

A backgate allows you to display the gated events in the color for the selected gate. This enables you to see the gated events within the plot.

- 1 Open the plot menu, point to **Display Backgate**, then select the gate from the list.
The events within the plot that are contained in the selected gate will appear in the color for that gate.

Changing the Number of Dots to Display

You can change the number of dots displayed in the plot.

- 1 Open the plot menu, point to **% Dots to Display**, then select a percentage from the menu. If you choose **Custom**, you can enter the number of events you wish to display.

Zooming a Plot

- 1 Select **Zoom Plot** from the plot menu. To unzoom the plot, right-click the zoomed plot and select **Normal Size Plot**.

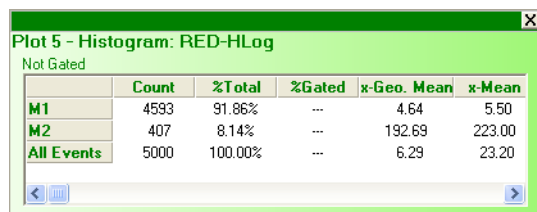
guava ExpressPro Results

You can view subsets of data and generate individual statistics for the data in any of the plots. You must manually select any statistics that you wish to export. See "Selecting Statistics for the Spreadsheet File" on page 6-22.

You can resize the stats windows by clicking and dragging the bottom or right edge of the window. You can resize the columns by clicking and dragging the right edge of the column header. If the column becomes too small to adjust, close, then reopen the window to restore the column width.

- Open the plot menu and click Show Stats.
The statistics provide you with the count, % total, % gated, cell concentration, signal intensities, and %CV (for histogram data) for all data in the plot (if ungated) or for data within the gate.

Histogram Statistics



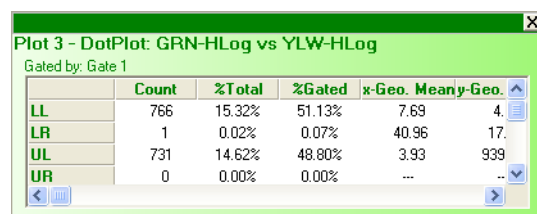
	Count	%Total	%Gated	x-Geo. Mean	x-Mean
M1	4593	91.86%	---	4.64	5.50
M2	407	8.14%	---	192.69	223.00
All Events	5000	100.00%	---	6.29	23.20

The histogram statistics provide the count, % total, % gated, mean signal intensity, geometric mean, median, %CV, and cells/mL for each population within a marker.

The *All Events* row shows the statistics for the events in the gate. If a gate was not applied, *All Events* shows the stats for all events.

guava ExpressPro histogram statistics.

Quadrant Statistics



	Count	%Total	%Gated	x-Geo. Mean	y-Geo. Mean
LL	766	15.32%	51.13%	7.69	4.17
LR	1	0.02%	0.07%	40.96	17.00
UL	731	14.62%	48.80%	3.93	939.00
UR	0	0.00%	0.00%	---	---

The quadrant statistics provide the count, % total, % gated, x and y mean signal intensity for each population within each quadrant, x and y geometric Mean, x and y median, x and y %CV, and cells/mL.

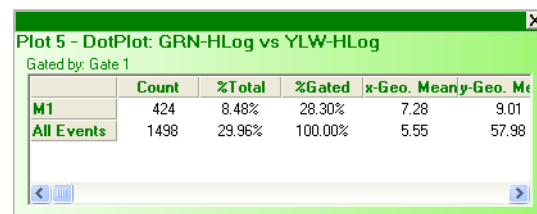
The *All Events* row shows the statistics for the events in the gate. If a gate was not applied, *All Events* shows the stats for all events.

guava ExpressPro quadrant statistics.

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

Quadrant	Staining	Default Color
lower left	x negative, y negative	red
lower right	x positive, y negative	blue
upper right	x positive, y positive	green
upper left	x negative, y positive	fuchsia

Rectangle Statistics



	Count	%Total	%Gated	x-Geo. Mean	y-Geo. Mean
M1	424	8.48%	28.30%	7.28	9.01
All Events	1498	29.96%	100.00%	5.55	57.98

The rectangle statistics provide the count, % total, % gated, x and y mean signal intensity for the population within the rectangle, x and y geometric mean, x and y median, x and y %CV, and cells/mL.

The *All Events* row shows the statistics for the events in the gate. If a gate was not applied, *All Events* shows the stats for all events.

guava ExpressPro rectangular region statistics.

Octagonal Statistics



Plot 5 - DotPlot: GRN-HLog vs YLW-HLog

Not Gated

	Count	%Total	%Gated	x-Geo.	Meany-Geo.	Me
M1	3502	70.04%	---	10.38	13.60	
All Events	5000	100.00%	---	8.87	28.05	

guava ExpressPro octagonal region statistics.

The octagon statistics provide the count, % total, % gated, x and y mean signal intensity for the population within the octagon, x and y geometric mean, x and y median, x and y %CV, and cells/mL.

The *All Events* row shows the statistics for the events in the gate. If a gate was not applied, *All Events* shows the stats for all events.

Ellipse Statistics



Plot 6 - DotPlot: YLW-HLog vs SSC-HLin

Not Gated

	Count	%Total	%Gated	x-Geo.	Meany-Geo.	Me
M1	2929	58.58%	---	14.48	6479.16	
All Events	5000	100.00%	---	28.05	2905.98	

guava ExpressPro elliptical region statistics.

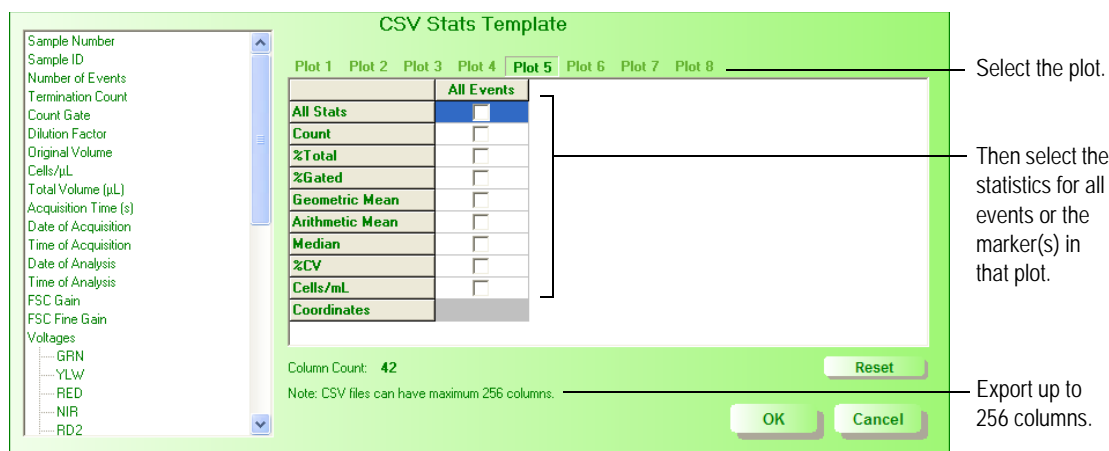
The ellipse statistics provide the count, % total, % gated, x and y mean signal intensity for the population within the ellipse, x and y geometric mean, x and y median, x and y %CV, and cells/mL.

The *All Events* row shows the statistics for the events in the gate. If a gate was not applied, *All Events* shows the stats for all events.

Selecting Statistics for the Spreadsheet File

Although you can view any statistics on screen, you must manually select the statistics that you wish to export to the spreadsheet file.

- 1 Open the plot menu from any plot and select CSV Stats Template. The CSV Stats Template dialog box appears.



CSV Stats Template

Plot 1 Plot 2 Plot 3 Plot 4 **Plot 5** Plot 6 Plot 7 Plot 8

	All Events
All Stats	<input checked="" type="checkbox"/>
Count	<input type="checkbox"/>
%Total	<input type="checkbox"/>
%Gated	<input type="checkbox"/>
Geometric Mean	<input type="checkbox"/>
Arithmetic Mean	<input type="checkbox"/>
Median	<input type="checkbox"/>
%CV	<input type="checkbox"/>
Cells/mL	<input type="checkbox"/>
Coordinates	<input type="checkbox"/>

Column Count: 42

Note: CSV files can have maximum 256 columns.

Reset OK Cancel

Select the plot.

Then select the statistics for all events or the marker(s) in that plot.

Export up to 256 columns.

The left side of the screen displays a list of instrument settings and sample information that is always exported to the spreadsheet file. The right side of the screen allows you to select the specific statistics that will be exported for each plot. If a plot has more than one marker, you can select the stats for each marker. When you click **OK**, the statistic you selected will be remembered the next time you open the CSV Stats Template.

- Click to select the statistic for export. Stats are shown for each marker in the plot or total events in the plot if no markers are set.

- Click **Reset** to ignore any changes made and revert to the stats that were selected when you opened the CSV Stats Template.
- **NOTE:** If you exceed 256 columns, a warning message appears requesting that you review and remove stats until there are no more than 256 columns of stats for export.

Exporting guava ExpressPro Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel. The CSV file will contain only the sample information and instrument settings unless you select the stats you wish to export.

- **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting guava ExpressPro Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.
The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample(s) from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**. If you selected multiple samples or all samples in the run, the results will print in batch mode.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Batch Printing

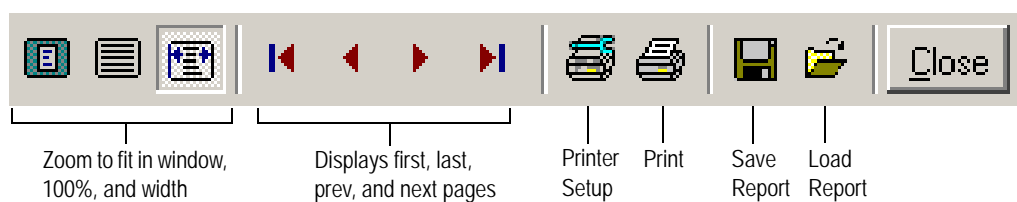
If you wish to print data from multiple samples, highlight the samples in the Sample Analysis List control panel, then click Print.

Note that when printing in batch mode:

- Print preview is not available.
- The number of pages to print shown in the print dialog box refers to the number of pages each sample will print on (typically 2 per sample) and not to the total number of pages that will be printed.
- You can select to print only the 1st page or 2nd page for each sample.

Print Preview Icons

Move the cursor over the icon to display the description.



guava ExpressPro Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

Sample Information

Original Sample Data

Current Sample # 1

Sample ID A01

Events to Acquire 5000

Dilution Factor: 1

Original Volume: 10 mL

AutoSave FCS 2.0 Files

Cell Count

Forward Scatter

- Total Count 5000

- Cells/μL 757.9

Flow Information

Flow Rate : 0.50 μL / s

Volume : 6.60 μL

Duration 00:13 mn.s

Threshold Parameter: FSC

Threshold Value: 353 Set

Area/Width Parameter: None

Set Time Scale

Change Parameter Names

Count Gate: All Events

Sample List Navigation

Previous Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 5000.
- Displays the dilution factor and the original sample volume. The default values are 1 and 10, respectively.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells and cells/μL that have exceeded the threshold.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Threshold Parameter and *Threshold Value* allow you to select the threshold parameter and enter its value. Click **Set** after entering the Threshold Value.

Area/Width Parameter allows you to save area and width measurements for a selected parameter.

Set Time Scale allows you to adjust the time parameter axis scale.

Click **Change Parameter Names** to enter your own parameter names (for example, the name of the monoclonal Ab you use). See “Changing Parameter Names” on page 6-26.

Count Gate allows you to select a gate used as a counting gate. All events above the threshold are saved to the file whether they are in the gate or not. However, the number of Events to Acquire is applied to events that fall within the gate.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Changing Parameter Names

You can change the default long (or stain) name of any parameter. The short name, for example, GRN-HLog, will still appear in parentheses after the new long name.

- 1 Click **Change Parameter Names** in the Sample Information control panel.
The Customize Parameter Names dialog box appears. Area and width appear only if they were selected during the adjust settings step.
- 2 Highlight the existing name in the *Long or Stain Name* text field and type in the new name.
- 3 Click **Update**.

Param No.	Short Name	Long or Stain Name
1	FSC-HLin	Forward Scatter
2	SSC-HLin	Side Scatter
3	GRN-HLog	Green Fluorescence
4	YLW-HLog	Annexin V-PE
5	RED-HLog	7AAD
6	NIR-HLog	Near IR
7	FSC-HLog	Forward Scatter
8	SSC-HLog	Side Scatter
9	GRN-HLin	Green Fluorescence
10	YLW-HLin	Yellow Fluorescence
11	RED-HLin	Red Fluorescence
12	NIR-HLin	Near IR
13	TIME	Time

- You can change the long or stain names for individual samples.
- If you change the names before starting acquisition, the new names will apply to all samples in the run.
- If you use the *Apply Current Settings to Selected Samples* button during analysis, any changes made to long names will be applied to the selected samples.

Unit Control

The screenshot shows the 'Unit Control' software interface with the following sections:

- Detection:** A table of gain settings for FSC, SSC, GRN, YEL, RED, NIR, RED2, and NIR2, all set to x 8.0. It also shows 'Use High Calibration Volt' as 'Yes' for all. Below this, 'Scaling Factor' is set to 'Area 500' and 'Width 500'.
- Pump Status:** Displays 'End of Stroke', 'Waste', and 'Pump Reset' with red indicator lights. It also shows 'Position' at 0 Steps and 'Actual Speed' at 0 Steps/s.
- Pump Action:** Displays 'Pump on', 'Waste', 'Pump Reset', 'Purge', and 'Sample' with green indicator lights. It also shows 'Speed' at 0 Steps/s.
- Threshold parameters:** Shows 'Offset' at 0.24 V and 'Threshold' at 100 units. Below this, 'Baseline Values' are shown for NIR2, RED2, NIR, SSC, GRN, RED, YEL, and FSC, all set to 0.

Detection

Displays the laser status, the FSC, SSC, GRN, YLW, RED, NIR, RED2, and NIR2 gain settings. Displays the area and width scaling factor.

■ **NOTE:** Do not change the gains from this panel. Use the sliders in Adjust Settings to adjust the SSC, GRN, YLW, RED, NIR, RED2, and NIR2 gains.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold settings for the threshold parameter.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava ExpressPro Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava ExpressPro spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava ExpressPro FCS file to another directory or give it a new name.
ExpressPro Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Cell Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying GRN, YLW, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but the events appear in the wrong places in plots displaying GRN, YLW, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Sample was not stained. 2. Cell lysis. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, restain sample from original suspension. 2. Check buffers used to process cells.

Problem	Possible Cause	Solutions
Events appear in some plots but not in others.	Ensure correct gate is selected for plot in question.	<ol style="list-style-type: none"> 1. Open plot menu, point to Apply Gates and select gate. 2. Check gate definition to ensure it includes the correct regions and operators.
Events appear off scale in dot plots or histograms.	FSC, SSC, GRN, YLW, RED, NIR, RED2, and/or NIR2 gains set incorrectly, or samples staining brightly.	Adjust gain setting so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Gains too low to detect fluorescent signals. 2. Incomplete staining with fluorescent probe, or fluorescent probe inappropriate for cell type. 3. Fluorescent probes over-exposed to light, stored improperly, or expired. 4. Non-specific binding of fluorescent probes. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Ensure positive control is staining adequately and with correct reagent. 3. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 4. If using antibody-based probes, try Fc blocking reagent during staining to minimize non-specific binding. Otherwise, titer the fluorescent probes down to reduce the nonspecific staining. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.
Cannot resolve dim positive staining from background signal.	Dirty capillary.	<p>Perform at least one cycle of Guava Clean.</p> <p>While acquiring samples, select Clean & Rinse instead of Quick Clean, and if necessary, run it frequently.</p>

Problem	Possible Cause	Solutions
Poor resolution between positive populations in plots displaying GRN, YLW, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s). 2. Too much reagent in staining tube. 3. Fluorescence background too high. 4. Gains too high causing signal to bleed into other parameters. 5. Gain too low to optimally detect positive signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. 2. Washing cells may remove residual reagent. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce gain. Adjust compensation settings. 5. Adjust settings to increase gain. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris. Or, select one of the fluorescence parameters as the threshold.

CHAPTER 7

guava InCyte Assay

7.1 Introduction

guava InCyte was developed to be an open assay module providing all the basic tools for sample acquisition and data analysis. The guava InCyte module allows you to acquire and analyze up to six fluorescence parameters in combination with forward scatter (FSC) and side scatter (SSC), as well as area, width, and time. Unlike other flow cytometry-based software, InCyte allows you to focus analysis at either the sample or the experiment level. An experiment can be described as a single acquisition of samples, acquisition across multiple days, or multiple conditions acquired within one day.

Experimental analysis within InCyte allows you to perform both simple data set analyses, as well as more complex analyses such as; comparative analyses both within data sets (between samples) and between data sets (control vs stimulated, healthy vs disease, replicates - day 1 vs 2 vs 3) or different parameters (expression of multiple proteins or functional assays such as apoptosis).

InCyte offers the following features:

- simple gating and display of samples, including drag and drop gating capabilities
- easy export of statistics
- the simultaneous application of Analysis Methods (analysis/gating strategies) across entire data sets or subsets of a data set
- visualization of statistical results in the form of a plate map
- analysis of FCS 3.0 data files acquired using any guava software module
- “grouping” which allows you to analyze subsets of samples from guava FCS files, as well as combine samples from different guava FCS files
- sample pooling for rare event analysis
- post-acquisition compensation
- semi-automated compensation
- IC-50 and EC-50 curves

A single Method (or analysis strategy) can be applied to multiple data sets in parallel, or multiple Methods can be applied to a single data set to obtain statistics to be displayed in the heat map. The process of creating equation-based gating schemes has been simplified through use of drag-and-drop regions; “draggable” features are used throughout InCyte.

The HeatMap allows you to visually compare results using a plate map that displays varying shades of blue to represent relative statistical values. Results from up to six parameters or six data sets can be displayed simultaneously.

The instrument is configured to detect the following fluorochromes:

- The Green parameter has been optimally configured for FITC, GFP, or Alexa Fluor® CF-488.
- The Yellow parameter has been optimally configured for phycoerythrin (PE)-based, Cy3, Alexa Fluor® 532, propidium iodide (PI), TRITC, and DS Red reagents.
- The Red parameter can be used to detect PE-Cy5, PerCP, 7-AAD, PE-Cy5.5, and PI.
- The NIR parameter can be used to detect PE-Cy7.
- The Red2 parameter can be used to detect Allophycocyanin (APC), Cy5, or Alexa Fluor® CF-647.
- The NIR2 parameter can be used to detect APC-Cy7, or APC-Alexa Fluor® 750.

Additionally, all channels can be used with reagents that fluoresce in appropriate channels. Refer to "Specifications" for filter information.

Components of InCyte Files

InCyte files can be thought of as having two components—the raw FCS data and the analysis components (regions, gates, markers, statistics, etc). Both of these components form the Analysed Group. When you open an InCyte FCS file during analysis, these three components (data, Method, and Analysed Group) appear in their respective pane of the Analyse control panel. This allows you to easily apply different Methods to different data files or subsets of a single data file, and to analyze and save FCS files from guavaSoft programs other than InCyte, using different analysis strategies.

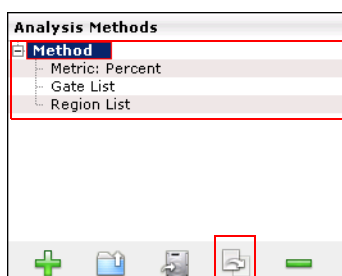
Methods

Analysis Methods are the tools used to analyze data. They can also be used during acquisition to define subsets of data or to set count gates. In InCyte, gating layout—plots and parameters, statistical setup, and all gates, regions, and markers are saved within the Method.

Every data set acquired in InCyte already has an associated Analysis Method, which was created or selected during acquisition. This is the case even if you do not perform any analysis during acquisition. This Method becomes part of the FCS file. Data acquired using any guavaSoft module other than InCyte, for example ExpressPro, will not have an Analysis Method associated with it. However, when you open the file in InCyte, a default Method will automatically be created.

While Methods are part of an InCyte-acquired FCS file, they can also be saved as separate files. To save an individual Method file, use the **Save Method** button in the Analysis Methods pane (see below). You can access a Method during analysis by opening the Method file (.gsy) or an FCS file and thus its associated Method. The saved Method file will contain only the regions, gates, metrics, and stats (if stats were derived). It will not contain instrument settings or data. Instrument settings are contained in the FCS file (data file and/or Analysed Group).

You can use the Duplicate Method icon to quickly create new Methods that are the same as the original Method. Then, modify the duplicated Method by a region or metric to create a similar analysis strategy.



Analysis Method containing a metric, gate list, and region list.

Duplicate a Method to modify the analysis by a metric, region, or gate.

Instrument Settings

Instrument settings are saved as part of the FCS file. You can also save instrument settings as a separate file (.gst). Instrument settings files contain all gains, thresholds, and compensation values.

Analysed Group

An Analysed Group is an FCS file that contains the sample data and the Method. It is created by pairing an FCS file with a Analysis Method. When data is acquired in InCyte, the software will automatically pair the data set and Method for analysis. If data were acquired in any other guavaSoft module, you will need to pair the data with a new or existing Method.

7.2 InCyte Application Window

The application window opens displaying three plots. A tool bar at the left edge of the window allows you to access most of the application controls (see Figure 7-1). You can find these same options in the Tools menu in the menu bar.

The control panel displays the controls for acquiring and analyzing data. Click **Analyse** or **Acquire** to display the desired control panel. Click in the top bar (above the Analyse and Acquire buttons) to drag the floating control panel to any area of the screen or dock it on the right side of the window. To redock it on the left, double-click the top bar. To hide the control panel, click the X in the top-right corner. To redisplay the panel, choose **Tools > Show/Hide Acquisition Controls** from the menu bar, or click the *Acquisition Controls* icon in the tool bar on the left edge of the application window. Place your cursor over any button in the Analyse or Acquire button panel to see text describing the button.

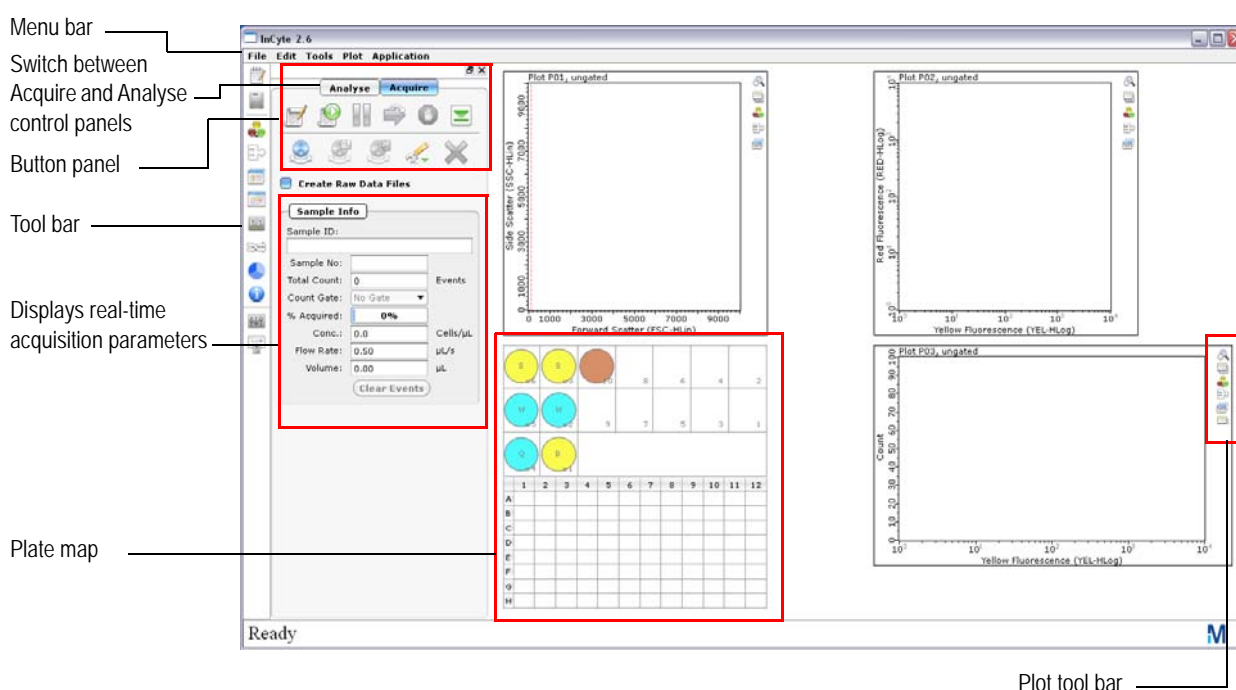
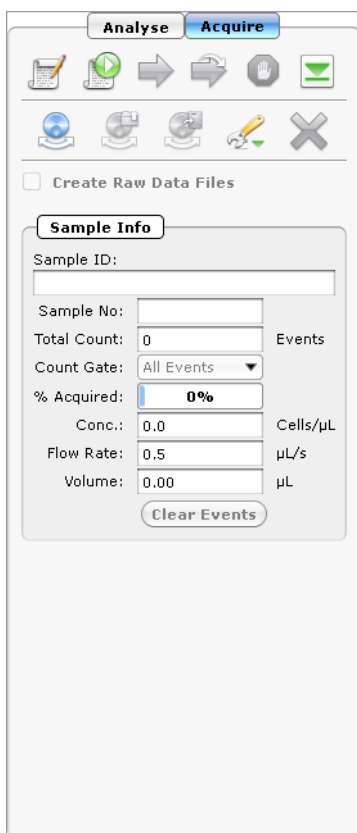


Figure 7-1 InCyte application window – Acquisition screen

Control Panels

To move between acquisition and analysis, click the Acquire and Analyse buttons at the top of the control panel.

Acquire Control Panel



Acquisition Buttons

Place the cursor over the icon to display text describing the button.

Sample Info

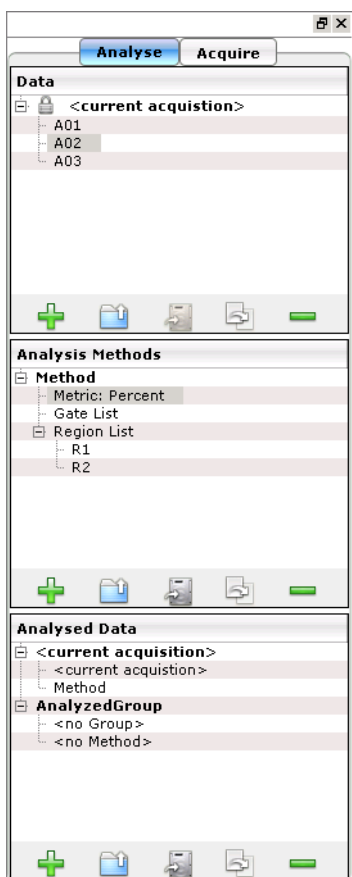
- **Sample ID:** Displays the Sample ID for the individual sample. Defaults to the plate well number.
- **Sample No:** Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- **Total count:** Displays the number of events to acquire. The default is 5000. Total count changes to Gated count if you apply a count gate.
- **Count Gate** allows you to select a gate used as a counting gate. All events above the threshold are saved to the file whether they are in the gate or not.
- **% Acquired:** A progress bar provides an estimate of the target event count during acquisition.
- **Conc.:** Displays the cells/μL that have exceeded the threshold.
- **Flow Rate:** Displays the flow rate selected during the adjust settings step (μL/s).
- **Volume:** Displays the sample volume that is acquired.
- **Clear Events:** Click to clear the display and restart acquisition during adjust settings.

Acquisition Buttons

Place the cursor over the icon to display text describing the button



Analyse Control Panel



Analysis Buttons

Place the cursor over the icon to display text describing the button. The buttons have the same function for each pane but apply specifically to that pane.



Data

Displays the open data files, as well as any user-created subsets of these files or groups and allows you to select a data set or group for analysis.

Analysis Methods

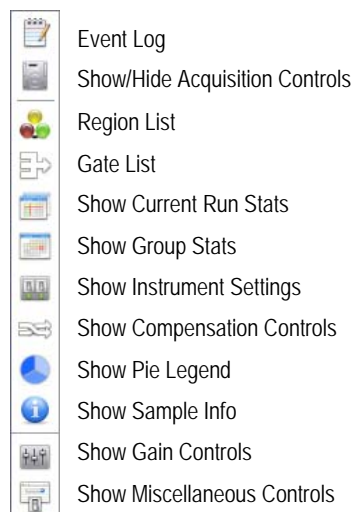
Displays the Analysis Methods for the current experiment. Each Analysis Method contains a gate list, a region list, and a metric (statistical parameter). InCyte-acquired files contain Methods. For data files acquired using a program other than InCyte, you must create a new Method or open an existing Method before you can perform analysis.

Analysed Data

Displays the FCS file and the associated Method. Created by pairing a non-InCyte-acquired data file with a new or existing Method during analysis, or created automatically during acquisition using InCyte.

Tool Bar

The tool bar appearing on the far left side of the window provides additional features for acquisition and analysis. The features are also accessible from the Tools menu. Other than the Pie Legend, which is described on page 7-10, the tools are described in detail at the point in the workflow where they are used.



Plots

Use the Plot menu in the menu bar to set up the plot layout. Select the number of plots to display (2x2 to 5x5 represents the number of plots displayed down vs across). Up to 24 plots can be displayed at one time.

Plot Tool Bar

A tool bar is located to the right of each plot.

- Use the *Undock* icon to detach and enlarge the plot. The plot will detach in its own floating window on top of the application window. Drag the bottom-right corner to increase the size of the plot window. To reattach the plot, click the undock icon again or the X in the top-right corner.
- Use the *Plot type* icon to select Dot Plot, Histogram, or Contour Plot, and to adjust settings such as smoothing histograms and contours and selecting the dots to display in dot plots. Contour plots can be used only during analysis. See “Changing the Display of Data in Plots” on page 7-8 for information.
- Use the *New region* and *Plot gate* icons to create regions and apply gates, respectively. See “7.5 Regions, Gates, and Statistics” on page 7-25.
- *New Stat Marker* is described in “Stat Markers” on page 7-32. *Edit overlay list* is described in “Overlaying Histograms” on page 7-54.
- To change the plot parameters once data is displayed, click the parameter name and select the new parameter from the pop-up menu. To customize a parameter name, see “Changing Parameter Names” on page 7-9.
- To copy a plot to the clipboard, right-click the plot and select **Copy Plot to Clipboard**.
- Use the *Zoom* icon to zoom an area of the plot. Click and drag a rectangle in any direction to encompass the population you wish to zoom in on. To scroll the data in the plot, click the icon, then hold down the Shift key while you click and drag. To unzoom, click the Zoom icon again, then press and hold the Ctrl key while you click anywhere in the plot.



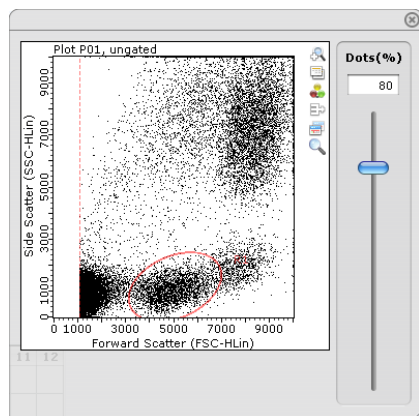
Changing the Display of Data in Plots

You can make the following changes to the display of data in these plots. The settings are saved with the Analysed Group. Click the Plot Type icon in the plot tool bar and select **Plot Settings**.



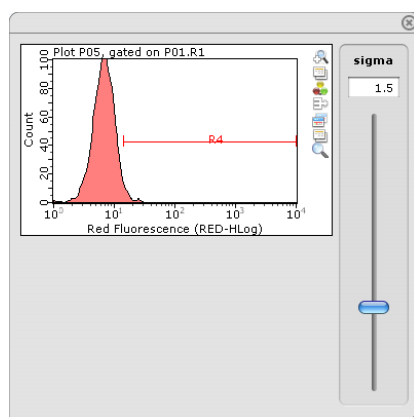
- Set the number of dots displayed in a dot plot

Use the slider to set the number of dots from 1 to 100%. The default is 100%.



- Smooth histograms

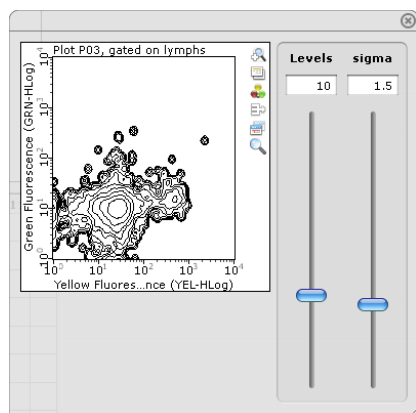
Click the Plot Type icon in the plot tool bar and select **Plot Settings**. Use the slider to set the amount of smoothing (sigma) from 0 to 5.0, where 0 is no smoothing. The default is 1.5.



- Smooth contour plots

Click the Plot Type icon in the plot tool bar and select **Plot Settings**. You can adjust both the number of levels from 5 to 20, and the amount of smoothing (sigma) from 0

to 5.0, where 0 is no smoothing. The defaults are 10 and 1.5, respectively. Contour plots can be created during analysis only.



Changing Parameter Names

You can change the default long (or stain) name of any parameter. The short name, for example, GRN-HLog, is fixed and will still appear in the plots and in parentheses after the new long name.

- 1 Choose **Application > Change Parameter Names** from the menu bar.

The Parameter Names dialog box appears. Area and width are listed only if they were selected during the adjust settings step prior to acquisition.

- 2 Double-click the existing name in the *Long or Stain Name* text field and type in the new name, or use the tab key to highlight the desired field and begin typing the new name.

The short name will appear in parentheses following the long name under the plot.

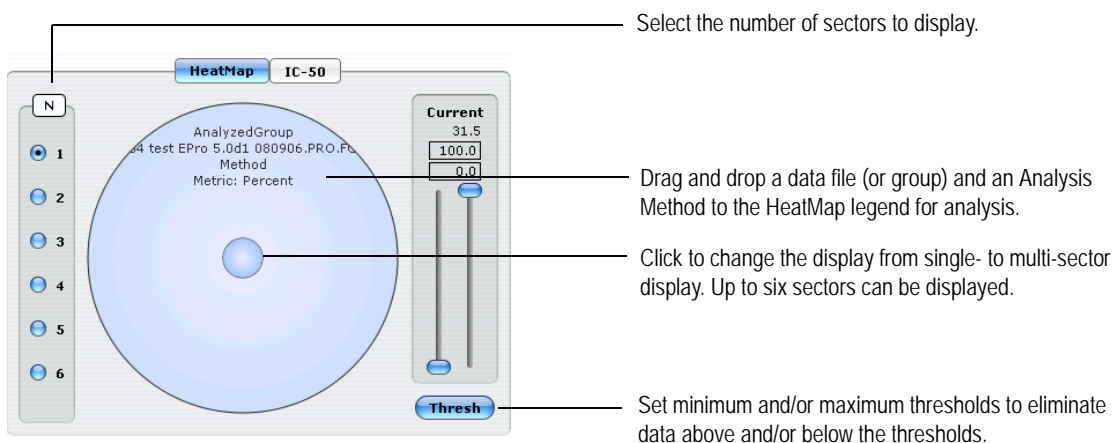
- 3 Click **Update**.

	Short Name	Long or Stain Name
1	FSC-HLin	Forward Scatter
2	SSC-HLin	Side Scatter
3	GRN-HLog	Green Fluorescence
4	YLW-HLog	Yellow Fluorescence
5	RED-HLog	Red Fluorescence
6	NIR-HLog	Near IR
7	RD2-HLog	Red2 Fluorescence
8	NI2-HLog	Near IR2
9	FSC-HLog	Forward Scatter
10	SSC-HLog	Side Scatter
11	GRN-HLin	Green Fluorescence
12	YLW-HLin	Yellow Fluorescence
13	RED-HLin	Red Fluorescence
14	NIR-HLin	Near IR
15	NI2-HLin	Near IR2

Pie Legend

The Pie Legend contains the HeatMap legend and the IC-50 application. The HeatMap legend (circle) is used to display the data results for each Analysed Group (data + Method) in the plate map. Use the **Show Pie Legend** icon in the tool bar to display this workspace.

The HeatMap legend shows one sector by default. You can create up to six sectors for six experiments. Each sector will have a data file (or group) paired with an Analysis Method. To divide the legend into multiple sectors click N=2, 3, etc. To clear a sector, right-click it and select **Clear Sector**. For more information on Heatmapping, see “7.7 HeatMap” on page 7-45.



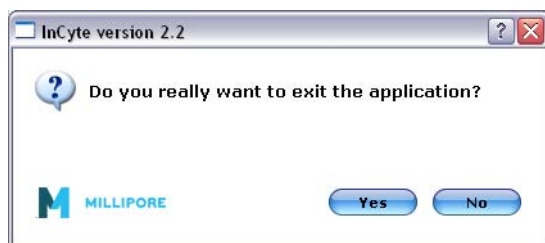
- **N** allows you to select the number of sectors you want to display. Sectors are numbered 1 to 6 with each new sector entering the upper-left portion of the circle.
- **Threshold** allows you to apply upper and/or lower limits on statistical values to eliminate data above or below the thresholds. The Current panel is only functional when one sector is being displayed, or when multiple sectors are used but the HeatMap legend is in single-mode display. See “Thresholds” on page 7-49 for more information.
- The IC-50 application allows you to rapidly convert time course or dose response studies into standard curves for the calculation of compound IC50/EC50 values. See “7.8 IC-50/EC-50” on page 7-50 for more information.

Exiting the Assay

Before quitting the software, be sure to save your current analysis using the Save Analysed Group icon in the Analysed Data pane.

- 1 To quit InCyte and return to the guavaSoft main menu, select **Quit** from the File menu.

- 2 Select **Yes** in the confirmation dialog.



7.3 guava InCyte Sample Acquisition

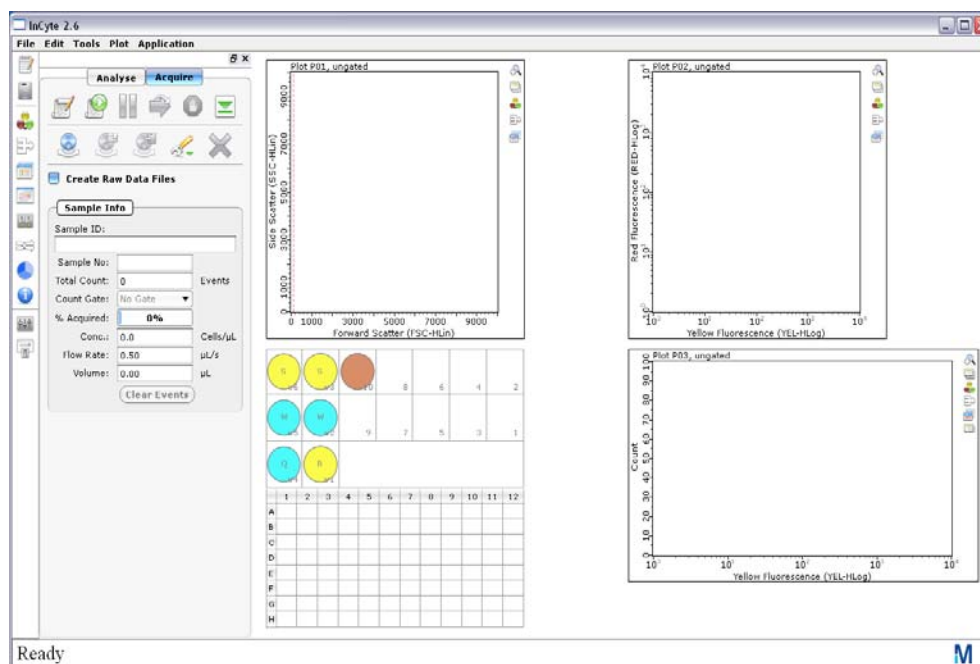
To ensure optimal performance, allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

- 1 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop.
- 2 Click **InCyte** from your list of Favorites, or select **InCyte** from the Program Search list and click **Launch**.

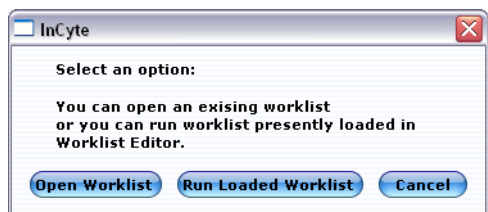
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.

The guava InCyte window opens in Acquisition mode, if the easyCyte instrument is turned on. The top portion of the control panel contains 11 buttons used to control acquisition, obtain and save instrument settings, select cleaning options, and eject the tray. For information on the control panel, refer to “Control Panels” on page 7-5.

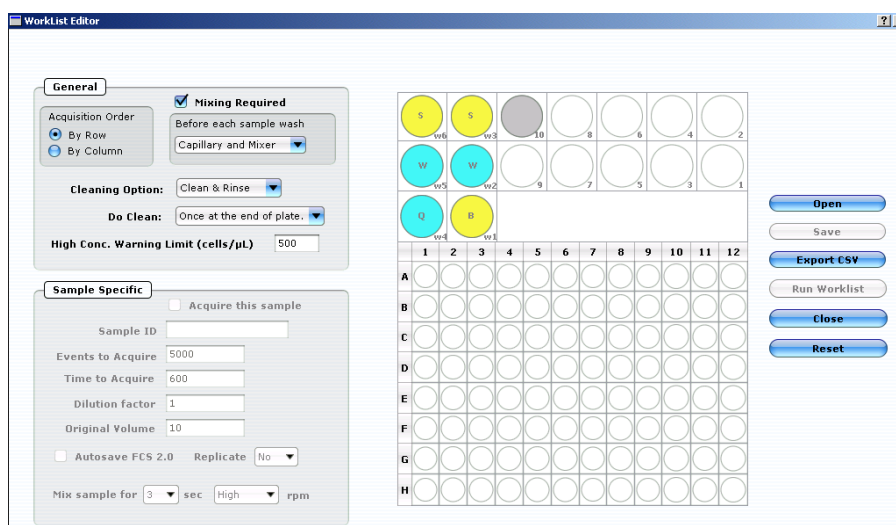
- **NOTE:** If you go to Acquisition following analysis of a previous data set, all the plots, regions, and gates remain from the analysis. The regions and gates will be cleared (or updated) when you select a Method for the acquisition.



- 3 Prepare samples for acquisition in a microplate or tubes.
- 4 Click the **Edit Worklist** button, the first in the button panel, or select **Worklist Editor** from the Application menu to open Worklist Editor and define the worklist parameters. You can also click the **Start Worklist** button, to open an existing worklist. Then choose between opening a saved worklist or running the currently loaded worklist. InCyte worklists are .xml files. If you are opening an existing worklist, proceed to step 6.



- **NOTE:** If you have a saved worklist, you can click **Open** from the Worklist Editor screen, select the worklist file, then click **Run Worklist**. The tray ejects and a dialog box appears prompting you to enter a data set file name. Proceed to step 6.



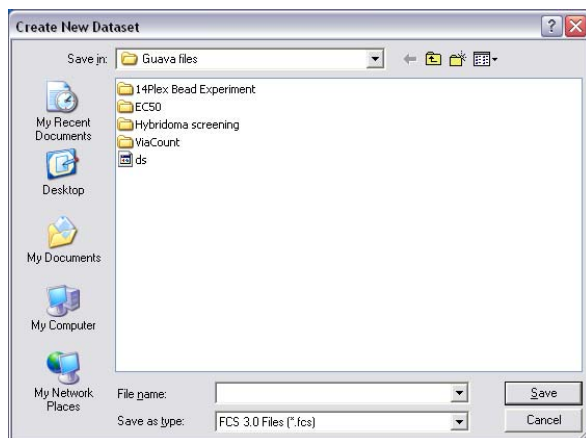
- 5 Define the worklist parameters in WorkList Editor software and click **Run Worklist**. See "Creating a Worklist" on page 1-29 for more information on setting up a worklist. When you start a worklist, the sample tray ejects. A dialog box appears prompting you to enter a data set file name.

◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

- 6 Select the folder where you want to save the data file and enter a file name. Click **Save**.
The FCS data file contains all components of the acquisition—data, Method, and Analysed Group. (InCyte automatically pairs the Method with the data file and creates an Analysed Group at the completion of acquisition.)

- **NOTE:** Always save guavaSoft data files directly to the system's hard drive during acquisition. Saving data to a network or location other than the computer's hard drive

may result in data loss. You may copy the files to another location when acquisition is complete.



Enter a file name for this data set and click Save.

7 A dialog box appears prompting you for an Analysis Method. Every data set acquired in InCyte has an Analysis Method associated with it.

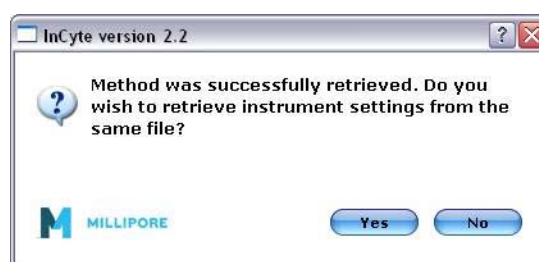
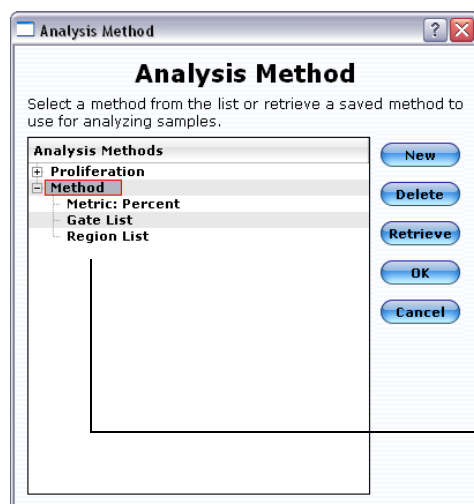
- To open a saved Method from a saved InCyte FCS file (data or Analysed Group) or a separate saved Method (.gsy) file, click **Retrieve**, locate the file, then click **Open**. If you select an FCS file to obtain the Method, a dialog box appears prompting you to also retrieve the instrument settings that were saved with the FCS file.

■ **NOTE:** An FCS file created using a version of InCyte before 2.0 will not contain instrument settings.

- If you are not retrieving a data file or saved Method, click **New** in the Analysis Method window to create a new Method. Click **OK**. You cannot rename a new method at this time. During analysis, you may save the Method with a new name.

■ **NOTE:** If you open a new Method or a saved Method file (.gsy), which will not contain instrument settings, you can retrieve any saved instrument settings when you select the sample for the adjust settings step (see step 10 on page 7-15).

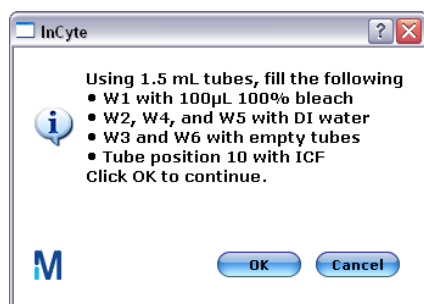
If you cancel out of the Analysis Method dialog box, the worklist is aborted.



Retrieve the instrument settings from the selected FCS file.

Displays Methods used during the current InCyte session.

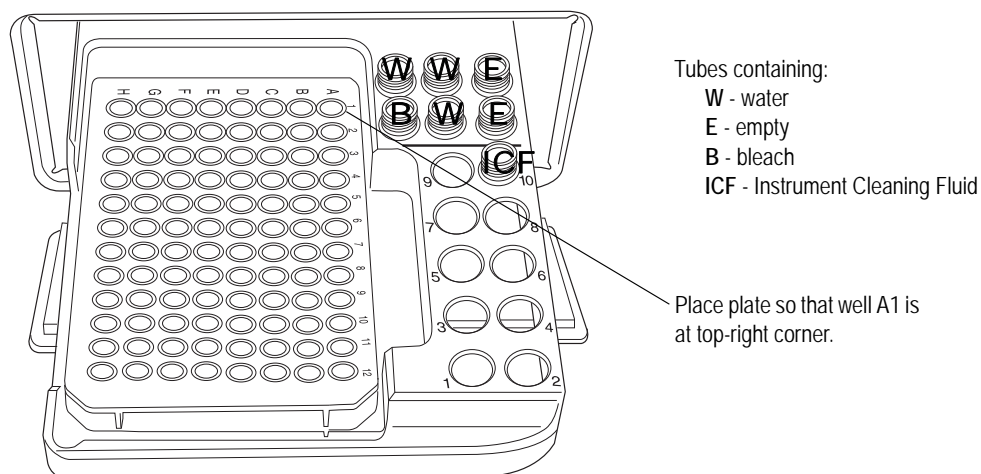
A dialog box appears prompting you to load your samples.



- 8 Place the microplate or tubes, as well as the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner.

Load the following 1.5-mL microcentrifuge tubes in these positions:

- Load tubes containing 1.5 mL of water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
- **IMPORTANT:** Ensure that the tube in position w4 (for Quick Clean) is filled with water.
- Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
- Load a tube containing 100 µL of bleach in position w1 (for a backflush) to disinfect material deposited from a backflush.
- Load a tube containing 1.5 mL of ICF in position 10 for Clean & Rinse.



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

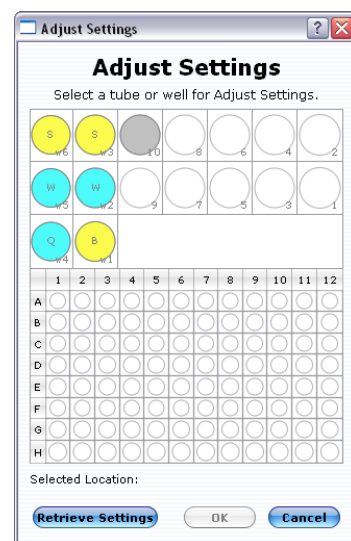
- ◆ **WARNING:** Keep the area clear as the tray loads.
- ◆ **WARNING:** Always use the **Eject Tray** button in the InCyte software module to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

Adjusting Instrument Settings

10 A dialog box appears prompting you to select the sample for adjusting settings.

- Before selecting the sample, if you opened a new Method or a saved Method file (.gsy), you may wish to retrieve instrument settings at this point. Click **Retrieve Settings**, locate the settings file (.gst), and click **Open**. A message appears indicating that settings were successfully retrieved.

- NOTE:** If you wish to save instrument settings later, use the **Save Settings** icon in the acquisition button panel.
- After retrieving the settings, click to select the well or tube (1–9) used to adjust settings, then click **OK**. We recommend using a stained negative or isotype control sample for the initial adjustments.



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

- NOTE:** If the value is greater than the high limit for the corresponding flow rate, click the **Next Step** icon in the button panel, then **Eject Tray**. Dilute the sample with the appropriate buffer to lower the concentration and minimize the risk of coincident events. For optimal performance, we recommend a concentration of 250 cells/ μ L or lower. Click **Load Tray**, then **Adjust Settings**.

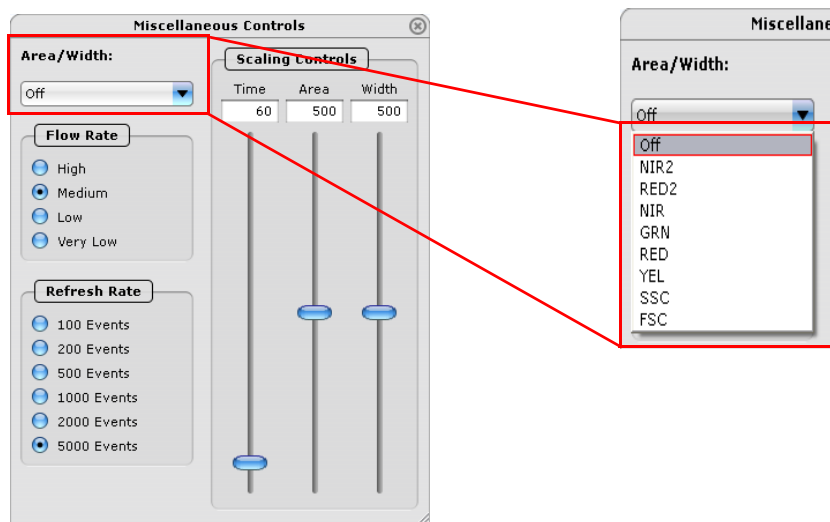
12 Set up the type of plots and parameters you wish to display. If you retrieved a Method, the plots are configured with the regions and gates defined in that Method. Modifications may be necessary to accommodate the new data. For more information on plots, see “Plots” on page 7-7.

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



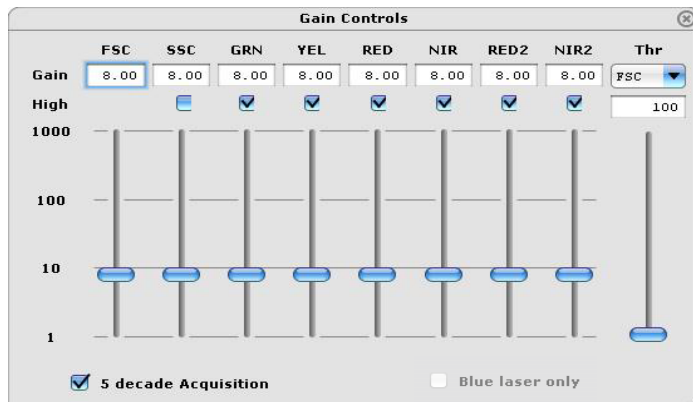
- Use the **Miscellaneous Controls** to set the **Flow Rate** to Very Low (0.12), Low (0.24 μ L/s), Medium (0.59 μ L/s), or High (1.2 μ L/s). The default flow rate is Medium. Set the **Refresh Rate** to the maximum number of events (100–5000) you want to display.
- NOTE:** If you change the flow rate during the adjust settings step, we recommend that you repeat the adjust settings step at the new flow rate to ensure that the markers and threshold are set correctly.
- If you wish to save area and width measurements for a specific parameter, select the parameter from the Area/Width Parameter menu. Use the Area and/or Width Scaling Control sliders to reduce or amplify the signal so that the cells are visible and on scale.

- If you wish to save a time parameter, use the Time Scaling Control slider to set the maximum acquisition time, then click **OK**. Adjust the y-axis (count) scale, if necessary.



- Use the **Gain Controls** to select the Threshold parameter from the drop-down menu.
 - Use the **Gain Controls** to adjust the gains (using the FSC, SSC, GRN, YEL, RED, NIR, RED2, or NIR2 sliders or the arrow keys on the keyboard) to a value from 1 to 1024. You can also type a numerical value in any of the Gain text boxes to adjust the settings. Adjust so that the negative population (or isotype) is positioned in the lower-left corner of the fluorescence plot and the cells are evenly distributed in the lower-left quadrant. Start from a lower gain setting and gradually increase the value. For greater detection and sensitivity across a broad range of fluorescence, the overall gain range can be adjusted using the **High** check box. By default, all boxes are checked. If further adjustment is needed, remove the checks from the High Calibration check boxes and allow the gain to stabilize for 2 to 5 seconds.
- **NOTE:** Use the **Clear Events** button in the Sample Info panel to clear the display.
- To acquire 5 log decades, select **5 decade Acquisition**.
 - Select **Blue laser only** if the red laser is not needed for your application and you wish to turn it off. The checkbox is only available for instruments equipped with a red laser. For information on the supported instruments and the laser required to detect each parameter, see “easyCyte HT Lasers and Parameters” on page 11-2.
 - To adjust the threshold, use the slider or click and drag the threshold marker (dotted red line) up or down the axis of the dot plot displaying the threshold parameter until the desired amount of debris or other unwanted events are eliminated below the

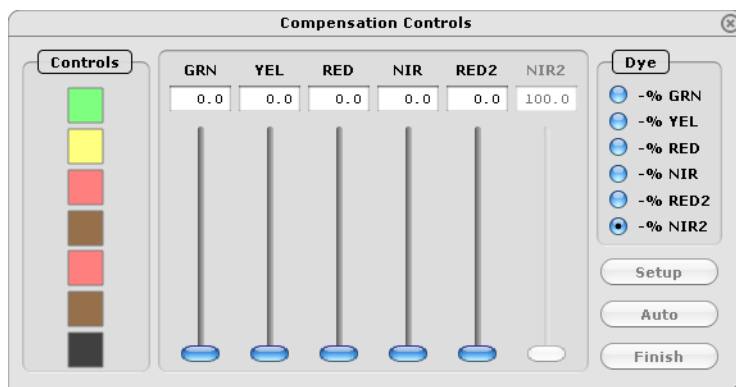
threshold. You can also enter a numerical value in the text box and press **Enter** on the keyboard.



- To adjust compensation, click the **Next Step** button, then **Adjust Settings**, then select the location of a positive control sample, and click **OK**. You may want to adjust each compensation setting individually using single-stained controls.

Use the **Compensation Controls** to select the radio button for the parameter you wish to subtract, then use the slider to define the percentage of the overlapping signal to be removed from the detector.

For more details on specific compensation adjustments and examples, see “Performing Compensation During Acquisition” on page 7-39.



- 14 You may set regions and gates prior to acquiring the samples. Refer to “7.5 Regions, Gates, and Statistics” on page 7-25 for information.

If you wish to apply a count gate, create and define the gate, then select it from the Count Gate drop-down menu under Sample Info. You can select the count gate from the Adjust Settings screen only.

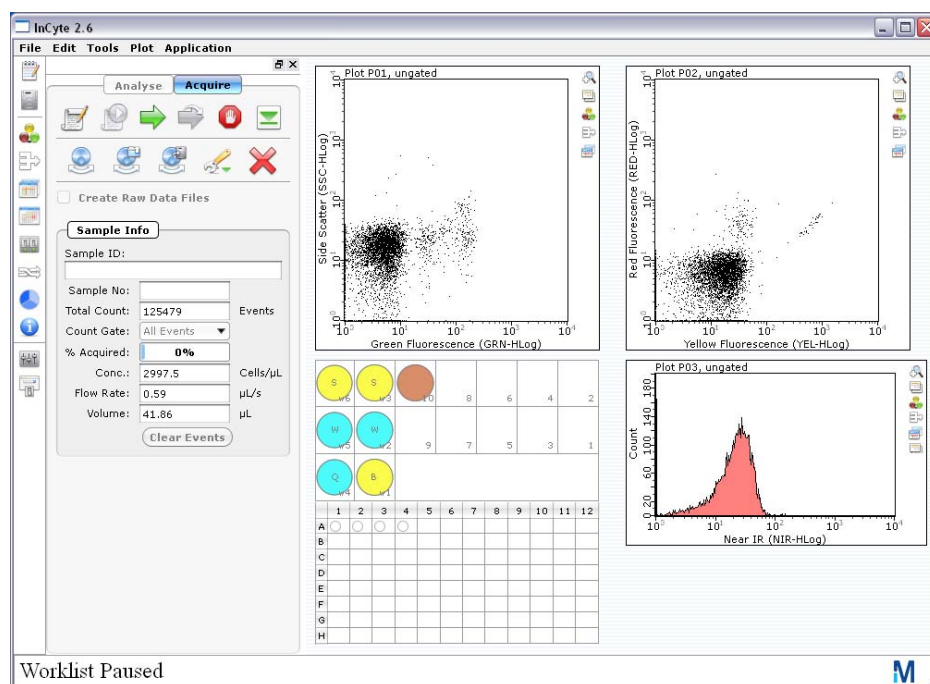
You can also view real-time statistics during the adjust settings step. See “Stat Markers” on page 7-32 for information on creating stat markers and viewing the real-time statistics.

- 15 When you are finished adjusting settings, click the **Next Step** button. If necessary, you can repeat the adjust settings step to ensure that other samples (such as another positive control) are on scale, appropriately positioned, and

compensated, by clicking **Adjust Settings**, then indicating the location of the sample, and clicking **OK**.

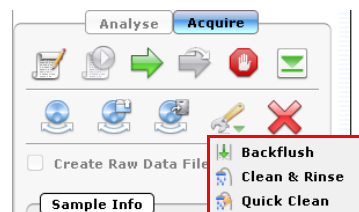
If you wish to save the instrument settings, click the **Save Settings** icon in the control panel. Enter a file name and click **Save** to save a .gst file. Once the worklist is complete you can no longer save instrument settings.

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



■ ACQUISITION NOTES

- You may click the **Pause Worklist** button at any time during the run to select **Eject Tray** or **Capillary Cleaning Tools** then select **Backflush**, **Clean & Rinse**, or **Quick Clean**. The system will complete the current step before pausing. Click **Resume Worklist** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause Worklist**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume Worklist**.
- The % Acquired progress bar provides an estimate of the target event count during the acquisition period, which times out after 1.75 minutes (high flow rate), 3.5 minutes (medium flow rate), 7 minutes (low flow rate), or 10 minutes (very low flow rate).
- The plate map in the control panel provides a visual status of acquisition. The well currently being acquired appears with an open blue circle. Wells acquired appear as a solid blue circle.



The system automatically performs a Quick Clean or a Clean & Rinse at the end of the assay.

At the completion of a worklist, a copy of the Data, Method, and AnalysedGoup are automatically loaded into the Analysis control panel. InCyte saves the data for all sample wells as a single FCS 3.0 file to the specified location. The FCS file contains:

- the acquired data for all tubes and wells in the run
- the Method (plots, regions, gates, and metrics, if applicable)
- an Analysed Group (the data paired with the Method)
- instrument settings (gains, compensation, miscellaneous settings)

7.4 guava InCyte Analysis

InCyte Software allows you to open and analyze any guava FCS 3.0 data file, regardless of the software module used for acquisition. An analysis-only version of InCyte 2.7 is available for MacOS 10.6 and later. See the readme file for more information and details on where to go to download the software.

■ **NOTE:** InCyte does not allow you to analyze data from replicates.

You can proceed to analysis from the acquisition screen.

The Analysis Panes

Click the **Analyse** button at the top of the control panel to access the Analysis screen. If you click Analyse from the Acquisition screen after acquiring a sample, the data from the last sample acquired is displayed.

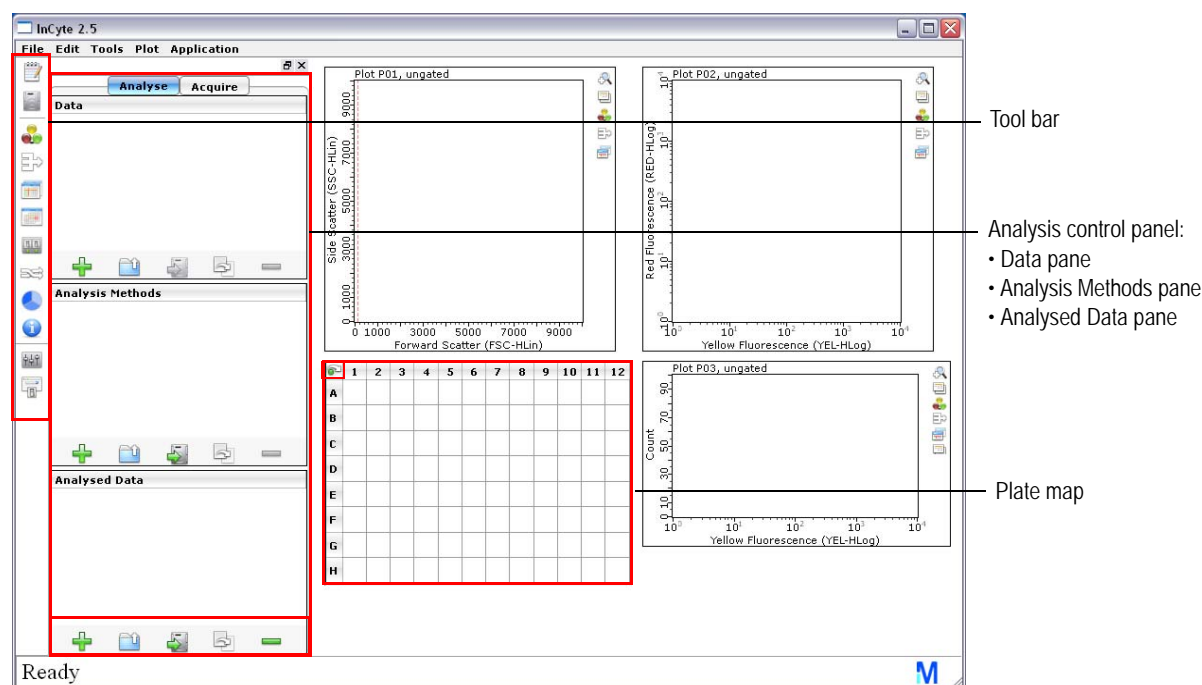



Figure 7-2 InCyte application window – Analysis screen

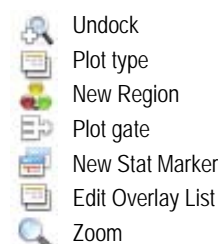
The Analyse control panel provides a working outline for each analysis session. It contains three panes—the Data, Analysis Methods, and Analysed Data. An FCS file generated using InCyte will automatically contain all three elements.

- The **Data** pane lists all open FCS files, as well as any groups (FCS file subsets) created. For more information on groups, see “Creating a Group” on page 7-52.
- The **Analysis Methods** pane contains all Method(s), each comprised of its associated plots, regions, gates, and metrics. Methods can be thought of as gating strategies. Before you can analyze a data file(s) from a guava assay other than InCyte, you must create an Analysis Method. Files acquired with InCyte already have Methods associated with them.
- The **Analysed Data** pane lists all user-defined Analysed Groups. Analysed Groups are created by pairing the data file with Method—each consisting of a data file (or group) with its associated Method. When you close an Analysed Group, the associated FCS file and Method will close automatically.

Place your cursor over an icon in any of the three panes to see text describing the icon . For more information on the control panels, see “Control Panels” on page 7-5.

Plots

The plots area of the window displays three plots by default. Use the Plots menu to change the number of plots displayed. Use the tool bar on the right to change the plot type and set regions and gates. For more information on plots, see “Plots” on page 7-7.



Analyzing Files Acquired Using InCyte

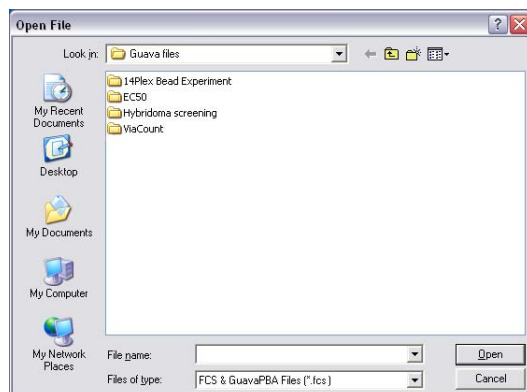
Data files acquired using InCyte will already have the necessary components for each of the three Analysis panes—the data file, the Method, and the Analysed Group. Simply open the FCS file.

During analysis, the original FCS file and any analysis settings (plots, parameters, gates) will not be affected. If you wish to save any changes to the newly analyzed InCyte file, simply resave the file using the Analysed Data pane (Save Analysed Group button).

You can proceed to analysis from the acquisition screen.

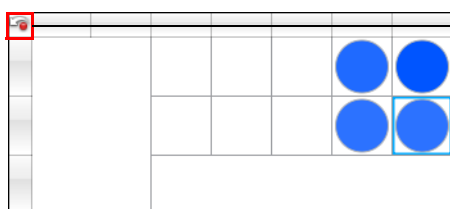
- **NOTE:** If you open and analyze multiple files within a session, all the FCS data files, Methods, and Analysed Groups will remain in the panes from all file that were opened. If they have already been saved, you can remove them from the pane by selecting the item and clicking the **Delete** icon at the bottom of that pane.
- 1 From the Analyse screen, choose **File > Open** from the menu bar. Select an FCS file for analysis and click **Open**. You can also use the **Open Group** icon in the Data pane

or the **Open Analysed Group** icon in the Analysed Data pane. You can also drag and drop an FCS file from a folder to the Data pane.



The data for the first sample well appears. For information on selecting the number and type of plots (dot plot, histogram, or contour plot) you wish to display, and changing the plot parameters, see “Plots” on page 7-7.

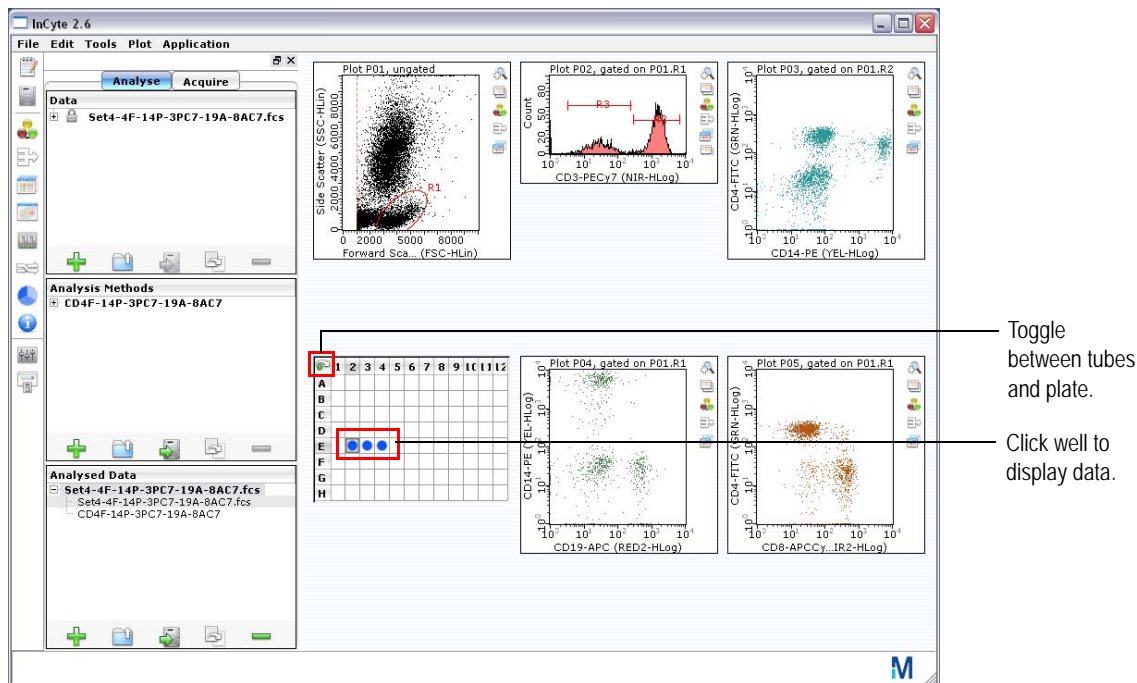
If you acquired tubes, click the icon in the upper-left corner of the plate map to display the tube map.



Click icon to toggle between plate map and tube map.

If you acquired samples from both tubes and plates, use the icon to switch between the tube map and plate map to view the analyzed data for specific samples.

Click the + to the left of the data file name to display a list of all sample wells and tubes contained in the file. All wells with acquired data appear as open circles in the corresponding locations in the plate map.

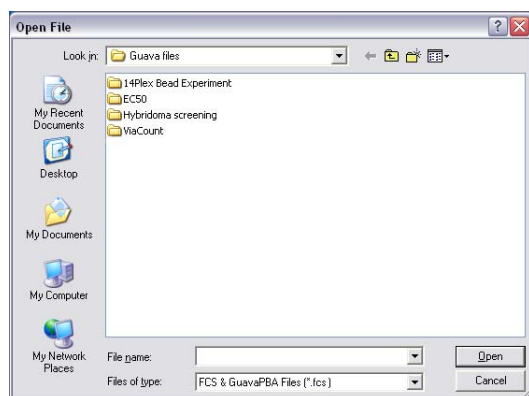


Clicking on a sample in the Data pane will display the unanalyzed data for that sample in the plots, however the information in the plate map will be lost. Click on the Analysed Group to redisplay the information in the plate map. If the data is already analyzed, click on the individual wells in the plate map to display the analyzed data for that well in the plots, or use the arrow keys to quickly scroll through the wells, displaying the data.

- **NOTE:** If you wish to create groups of samples for analysis, see “Creating a Group” on page 7-52.
To rename the Method, double-click it and enter a new name related to this analysis.
- 2** You are now ready to start customizing your Analysis Method using regions and gates. Refer to “7.5 Regions, Gates, and Statistics” on page 7-25.

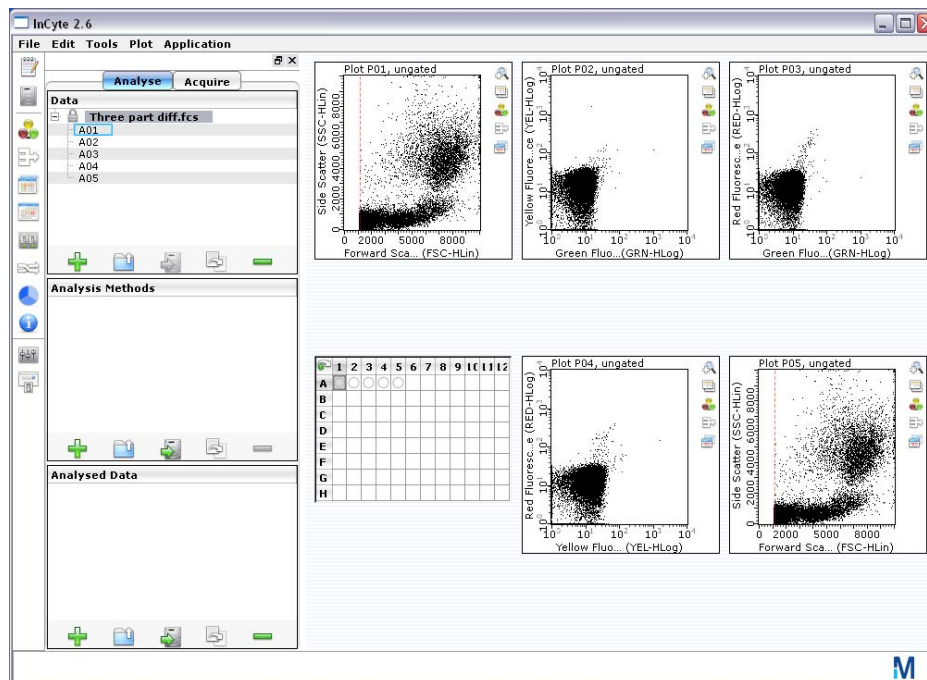
Analyzing Files Acquired Using Other Guava Software Modules

- 1 From the analysis pane, choose **File > Open** from the menu bar. Select an FCS file for analysis and click **Open**. You can also use the **Open Group** icon in the Data pane or the **Open Analysed Group** icon in the Analysed Data pane. You can also drag and drop an FCS file from a folder to the Data pane.



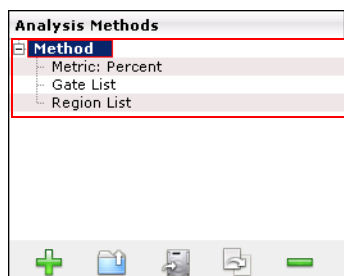
The data for the first sample well appears. For information on selecting the number and type of plots (dot plot, histogram, or contour plot) you wish to display, and changing the plot parameters, see “Plots” on page 7-7.

Click the + to the left of the data file name to display a list of all sample wells and tubes contained in the file. All wells with acquired data appear as open circles in the corresponding locations in the plate map. Data for any well can be displayed in the plots by selecting the sample from the file list or clicking on a well in the plate map.



- 2 When a data file is generated in a Guava software module other than InCyte, InCyte will automatically create a default Analysis Method and Analysed Group for the data file/set. The Method will contain a percent (%) metric, initially. If you wish to open an existing Method use the **Open Method** icon. Method files have the extension .gsy.

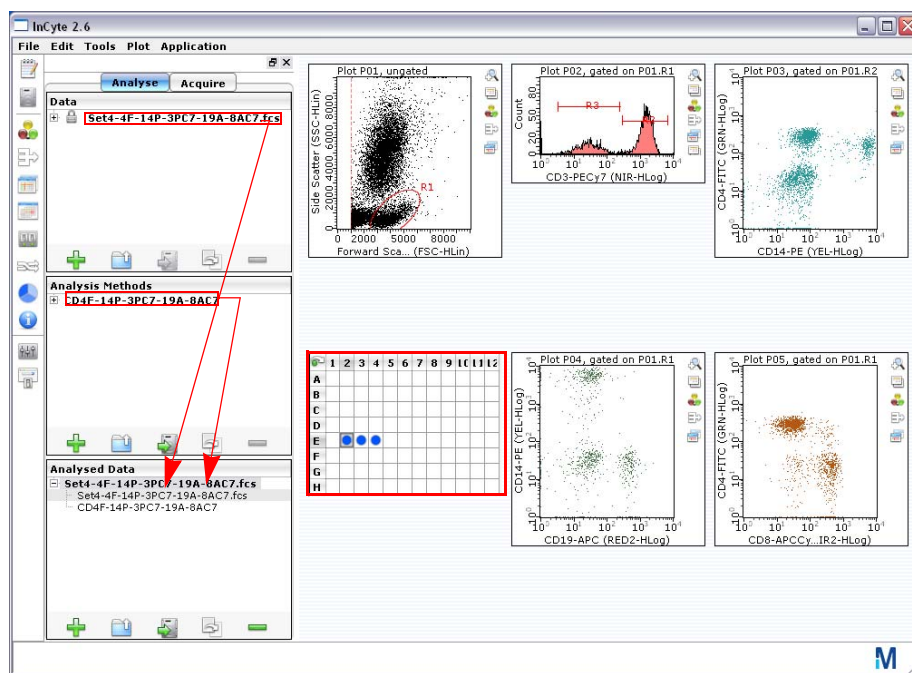
For more information on Analysis Methods, see “Methods” on page 7-2.
To rename the Method, double-click it and enter a new name related to this analysis.



Analysis Method containing a metric, gate list, and region list.

Click New Method to create a new Method.

3 If you opened an existing Method, you will need to drag it to the Analysed Group.

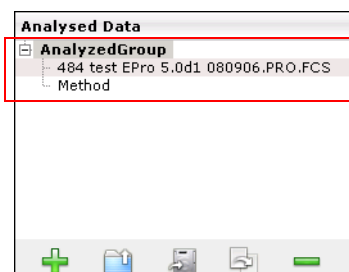


Data and Method are automatically paired to create an Analysed Group. If you open a saved Method, you will need to drag it to the Analysed Group.

Notice the sample wells in the plate map become dark blue (if the Method was newly created) or various shades of blue (if a previously defined Method is used). You can place the cursor over any well in the plate map to view results for that sample.

Clicking on a sample in the Data pane will display the unanalyzed data for that sample in the plots, however the information in the plate map will be lost. Click on the Analysed Group to redisplay the information in the plate map. Click on the individual wells in the plate map to display the analyzed data for that well in the plots, or use the arrow keys to quickly scroll through the wells, displaying the data.

- **NOTE:** Any data file or Method file can be replaced by simply selecting it from the respective pane and dragging to the Analyzed Group. This allows you to quickly interchange data files and Methods.



Analysed Group containing a data file and Analysis Method.

To save an Analysed Group, click on it in the Analysed Data pane to select it, then click the Save Analysed Group icon. Navigate to the location, enter a name, and click **Save**. The Analysed Group will be saved as an FCS file and will contain the Method (regions, gates, metrics, and stats) and the data.

- 4 You are now ready to start customizing your Analysis Method using regions and gates. Refer to "7.5 Regions, Gates, and Statistics" in the following section.

7.5 Regions, Gates, and Statistics

Regions

InCyte offers six types of regions/markers—ellipses, rectangles, octagons, polygons, and quadrant markers for dot plots, and histogram markers for histograms. All of the regions are created using either the New Region icon in the plot tool bar (see “Plots” on page 7-7), except the quadrant marker, or the New Stat Marker icon in the tool bar, which allows you to select statistics for the region/marker. For information on creating quadrants, refer to “Stat Markers” on page 7-32. A single Analysis Method can contain a maximum of 24 regions or stat markers, and you can have multiple stats per marker.

InCyte regions are associated with the one (histogram) or two (dot plot) parameters in which they were created. If you change the x- or y-axis parameter on the plot, the region is no longer displayed. However, it is still contained within the Method.

- 1 Once an Analysed Group is created, by acquiring InCyte data or by dragging a data file and a Method to the Analysed Group, click the **New region** icon in the plot tool bar and select a region from the pop-up menu.

You are prompted to enter a region name. The default for the first region is R1. When creating a polygon, this dialog appears after you create the region.



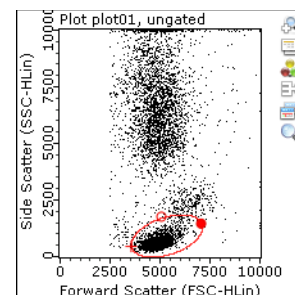
2 Enter a name for the region and click **OK**.

You may leave it R1, but a unique region name can be helpful in differentiating Analysis Methods.

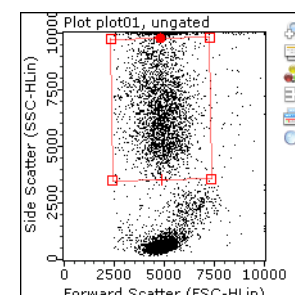
The newly created region appears on the plot with the region name. Click and drag the name to move it. The region is listed in the Region List under the Method and in the Region List table (Region List icon in the main tool bar or **Tools > Show Region List**). To delete a region, see “Region List” on page 7-27.

3 Adjust the region to encompass the data. Or, for a polygon, create the region.

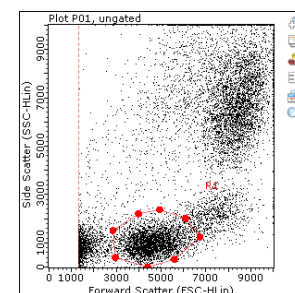
- To adjust an **elliptic region**, click anywhere on the edge of the ellipse, except on a handle, and drag it to a new location. The ellipse has two handles. The open circle allows you to narrow/widen the ellipse. The solid circle allows you to lengthen, as well as rotate the ellipse around a point opposite the solid circle.



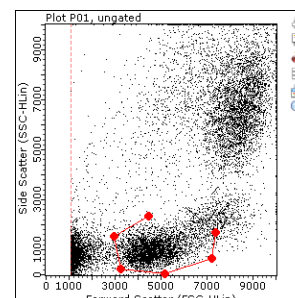
- To adjust a **rectangle region**, click anywhere on the edge of the rectangle, except on a handle, and drag it to a new location. The rectangle has five handles. The open squares allow you to extend at the corresponding corner. The solid square allows you to rotate and resize.



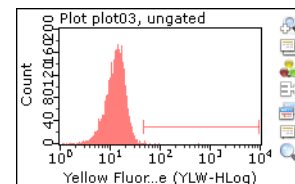
- To adjust an **octagonal region**, click anywhere on the edge of the octagon, except on a handle, and drag it to a new location. The octagon has eight handles. Click and drag the handles to adjust the shape of the octagon. To add or remove segments, right-click on a handle and select **Insert Line Segment** or **Delete Line Segment**.



- To create a **polygon region**, click to add the first handle, then move the cursor to the next point and click. Continue creating up to 32 segments until the shape is complete. Finish by clicking again at the first handle to close it. To move it, click anywhere on the edge, except on a handle, and drag it to a new location. To adjust the shape, click and drag the handles. To resize or rotate, press the Shift key and click and drag a handle.

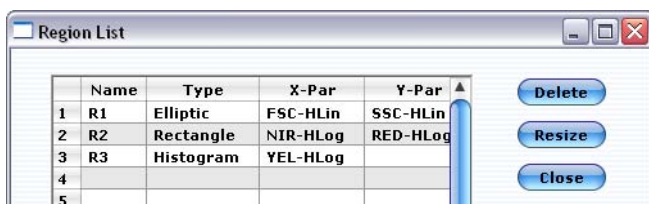


- To adjust a **histogram region**, click either of the two handles. The histogram marker can be moved vertically as well as horizontally.



Region List

When you create a region, it appears in the Region List. To display the list, click the Region List icon in the tool bar or choose **Tools > Show Region List**. The Region List displays a list of all regions and quad stat markers (see page 7-32) in the selected Analysis Method, their region name, type, and the plot axes.



- To display all the columns in the Region List window, click **Resize**.
- To delete a region from the plot, click to select it, then right-click and select **Delete Region**. You can also select the region in the Region List window and click **Delete**. Click **OK** in the confirmation box. The region will be deleted from all associated plots. The region name in the Analysis Method will appear as ???.

Global and Local Region Adjustments

You can make adjustments to regions for specific samples within a data set. For example, you may wish to tweak a region for the data in one or more samples to compensate for a shift in the data.

- To change a region for an individual sample, **press and hold the Ctrl key** while manipulating the region (elliptic, rectangle, octagon, or histogram). To change the region for multiple samples, highlight the desired wells, then hold the Ctrl key while manipulating the region. The change will be applied to all the highlighted samples.

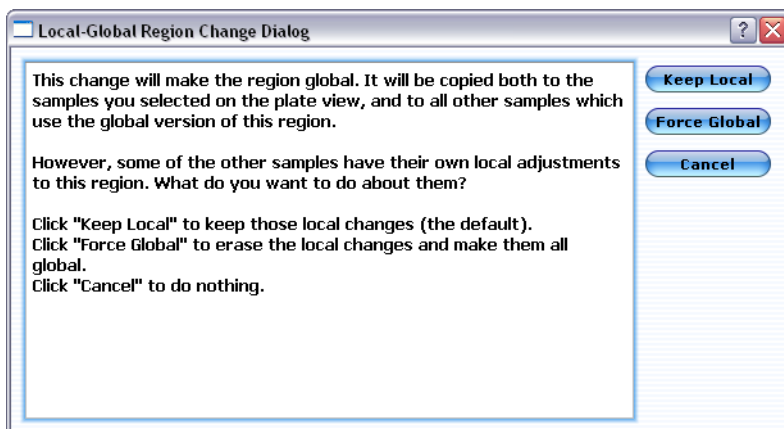
The region will turn from red to black, indicating that it is a local region—it applies only to the sample data displayed in the plots.

- **NOTE:** If you manipulate a global region without holding down the Ctrl key, the change will automatically apply globally (to the data for all samples in the run).

Once a local region is created:

- if you have only one local region in the data set and you attempt to manipulate it without holding down the Ctrl key, the change will become global and apply to all regions in the data set.

- If you have multiple local regions and you attempt to manipulate a single local or global region without holding down the Ctrl key, the following message appears.



- To apply the change to the region you are adjusting and all global regions, but keep any other local regions unchanged, click **Keep Local**.
- To apply the change to all samples in the run, click **Make Global**.
- To cancel the change and not apply it to any regions, click **Cancel**.

If you want to make an adjustment to a local region to manipulate it further, hold down the Ctrl key to keep the change local and not affect any other regions—local or global.

After making a region local, you can still make adjustments to global regions without holding down the Ctrl key. When the dialog box appears, click **Keep Local**. The adjustment will apply to all global regions. The local regions will remain local and unchanged.

Gates

A gate can be as simple as a single region, or a complex combination of regions and multiple parameters. Gates allow you to further characterize and isolate populations based on the regions. A maximum of 32 gates can be created for one Analysis Method.

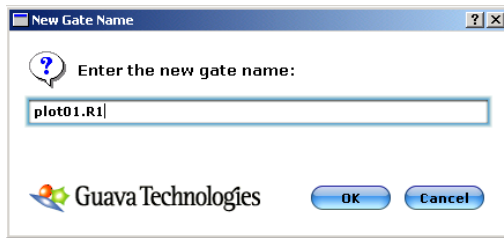
You can create gates for analysis and/or acquisition. Count gates used during acquisition allow you to acquire specific populations of interest. All events above the threshold are saved to the file whether they are in the gate or not. However, the number of Events to Acquire is applied to events that fall within the gate.

Defining a Gate

The gating process has been simplified by dragging and dropping regions. Any region can be dragged from one plot to any other plot. This process allows you to create gates defined with the “AND” operator. You can also create gates using the OR and NOT operators. Refer to “Gate List” on page 7-30.

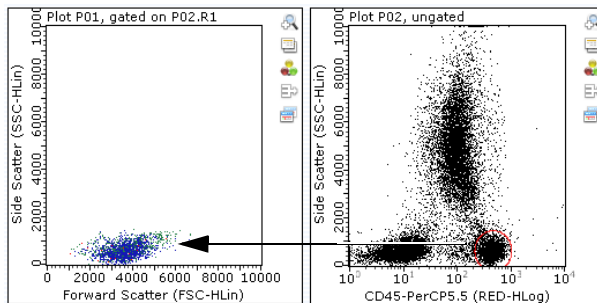
- 1 Click to select the region (handles appear), then click in the center of a region and drag it to another plot.
This “applies” the data within the boundaries of the region to the second plot. For example, if you created a region on live cells in plot 1 and drag the live-cell region to plot 2, only the live cells will be displayed in plot 2. A New Gate Name dialog box

prompts you to rename the newly created gate. You can enter a more meaningful name or use the default.



- **NOTE:** You can also define a gate by typing the regions and the operators AND, OR, and NOT. Refer to “Gate List” on page 7-30.

If the Analysis Method has gates already defined, you can also select a gate from the *Plot gate* menu in the plot tool bar.



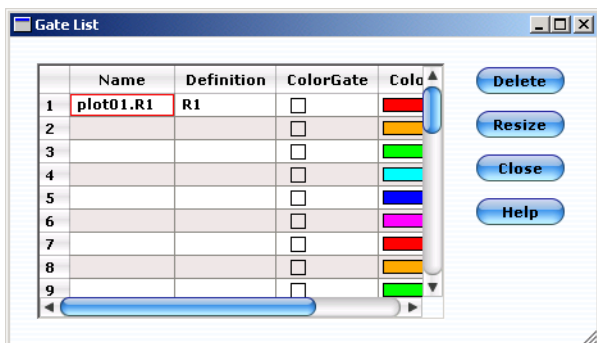
Select the region, then drag it from one plot to another to apply a gate.

When you apply a gate, the following changes occur:

- The events displayed in Plot 2 will automatically change to reflect only those defined by the region that was dragged in. To view the ungated data again, click the **Plot gate** icon to the right of the plot and select *<ungated>*.
 - The color of the newly gated data will reflect the color of the gate defined in the Gate List window. The default color for the first gate is red.
 - The plot heading will reflect the gate applied (for example, *Plot P01, gated on P02.R1*).
 - The newly created gate will appear in the Analysis Methods pane under the Method, in the Gate List (see "Gate List" below), and the Plot gate pop-up menu to the right of the plot.
- **NOTE:** Changes made to the position or size of any region(s) used to define a gate will automatically be reflected in all plots gated by that region.

Gate List

When you create a gate, it appears in the Gate List. To display the list, click the Gate List icon in the tool bar or choose **Tools > Show Gate List**. The Gate List displays a list of all gates in the selected Analysis Method, their definition, and color.



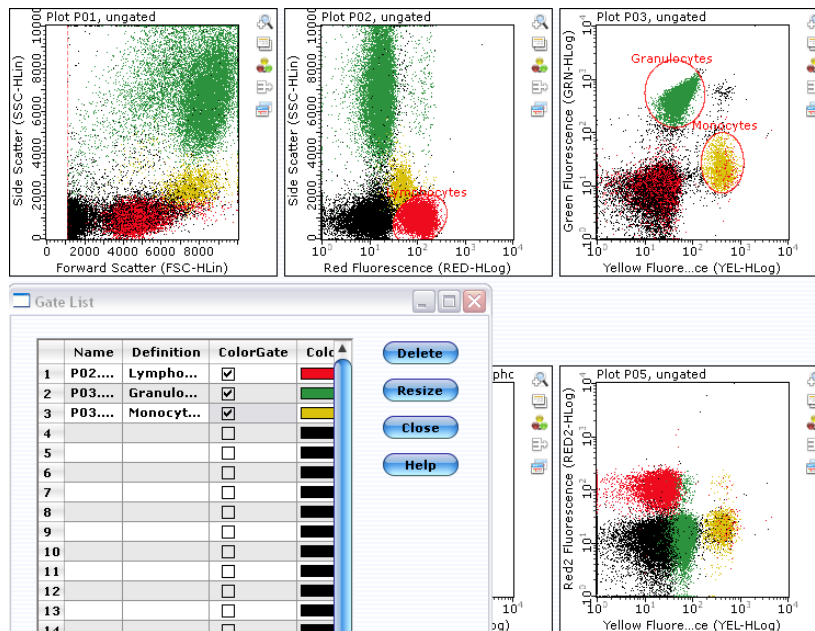
- To display all the columns in the window, click **Resize**.
- To delete a gate from the list, select the gate and click **Delete**. Click **OK** in the confirmation box.
- To change the gate color, double-click the color in the Color column. The ColorPickerDialog appears. See “Changing the Color of Gated Data” on page 7-31 for information on using this dialog box to change the color.
- You can turn a region into a gate by entering a name for the gate in the Name field and typing the region name (for example, R1) in the Definition field.
- You can also define a gate using the operators AND, OR, and NOT. Create regions to identify the subpopulations of interest. Open the gate list and type an appropriate name for the gate. Type a definition, for example:
 - R1 AND R2 means the event must be in *both* the R1 and R2 regions to be included in the gate.
 - R1 OR R2 means the event must be in *either* the R1 or R2 region to be included in the gate.
 - R1 AND (NOT R2) can be used if R1 and R2 overlap and you want to include events in R1 but not in R2.

The gate can now be applied to any plot by clicking the **Plot gate** icon to the plot tool bar and selecting the gate from the list.



- The ColorGate box allows you to view a backgate. Start by creating a gate in one plot. Then, drag that gate to the plot interest. This applies the gate to the second plot and shows only the gated events. Select **Tools > Show Gate List** and check the ColorGate box for the gate. The gated events appear in both plots in the selected

color. View the second plot without a gate (select <ungated> from the plot tool bar) to see the color gated events in relationship to all events.

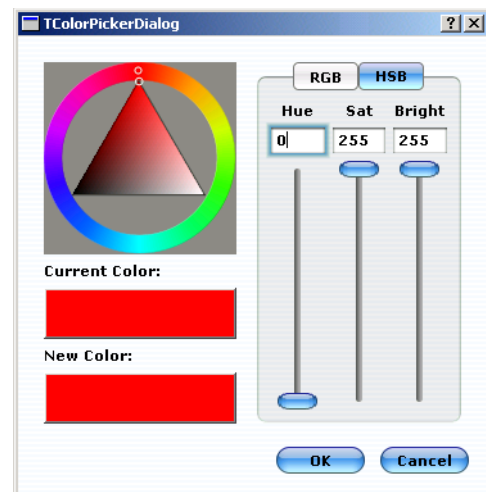


A lymphocyte gate was created in the RED (CD3 PE-Cy5.5) vs SSC plot, then dragged to the YEL (CD14 PE) vs GRN (CD15 FITC) plot. Granulocyte and monocyte gates were created in the YEL vs GRN plot and dragged to the RED vs SSC plot. ColorGate checkboxes were selected for all gates to apply color to the gated events. All plots are viewed ungated to show all data and gated data in colors.

Changing the Color of Gated Data

You can change the color of data within a gate. Open the Gate List and double-click the color in the Color column to open the ColorPickerDialog. Use this dialog to change the color, hue, saturation, and brightness.

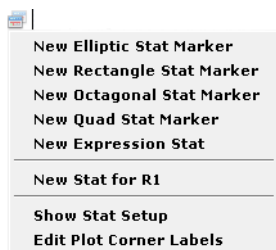
- 1 To change the color, click the RGB button and use the sliders to adjust each color. Or, click to select a color in the outer circle. Drag the pointer to change the color.
- 2 To adjust the hue, saturation, or brightness, click the HSB button and use the sliders to make the appropriate adjustments. Or, click a shade in the inner triangle.
- 3 Click **OK** when you are finished.



Stat Markers

The Stats feature allows you to derive, display, and export statistics for single wells, groups, or whole data sets. Each stat is derived from a region/marker. Stats can be assigned to existing regions, or new regions can be created. A single Analysis Method can contain a maximum of 24 regions/markers.

- 1 Click the **New Stat Marker** icon in the plot tool bar and select the appropriate option. You can obtain statistics for any existing regions in the plot. Or, you can create a new region and obtain statistics for that region.
 - Select **New Elliptic/Rectangle/Octagonal/Quad/Histogram Stat Marker** to add a new region and obtain stats for it. Histogram Stats appear only in a histogram plot.
 - Select **New Stat for R1** (or name of existing region) to obtain stats for an existing region.
 - Select **New Expression Stat** to create an expression statistic. See "Creating Expression Statistics" on page 7-35.
 - Select **Show Stat Setup** to display the Current Run Stats. See "Current Run Stats Window" in the following section.
 - Select **Edit Plot Corner Labels** to add custom labels to your plots. See "Labeling Plot Corners" on page 7-37.



The Add New Stat Marker window appears for the stat type you selected. The default base stat name is the same as the gate name. Base stat implies it is the base name

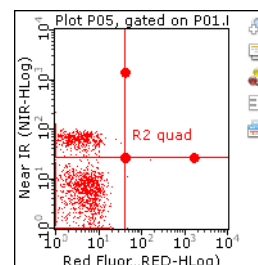
before you select a specific metric (stat), then the stat name becomes *BaseStatName.Count*, for example.

If a stat with the default name already exists, the field will appear red. You must enter a new stat name.

- 2 Select the metric—**Count**, **Concentration**, **Percent**, **%CV**, **Mean**, and/or **Median**.
- 3 For quad stats only, select the quad region(s) of interest. Select **Show on plot** if you wish to see the stat for each quadrant appear in the corners of the plot. If multiple parameters are selected, the count appears by default. Quadrant markers will appear on the plot after you click **OK**.
- 4 Click **OK**.

The Current Run Stats window (see below) appears with the newly created stat. The value listed corresponds to the sample well highlighted in the plate map. Each stat is automatically applied to all sample wells in a given data set. To see the results for any well, click on the well in the plate map. If you adjust the marker/region or any region that is part of the gating strategy, the statistics are automatically updated.

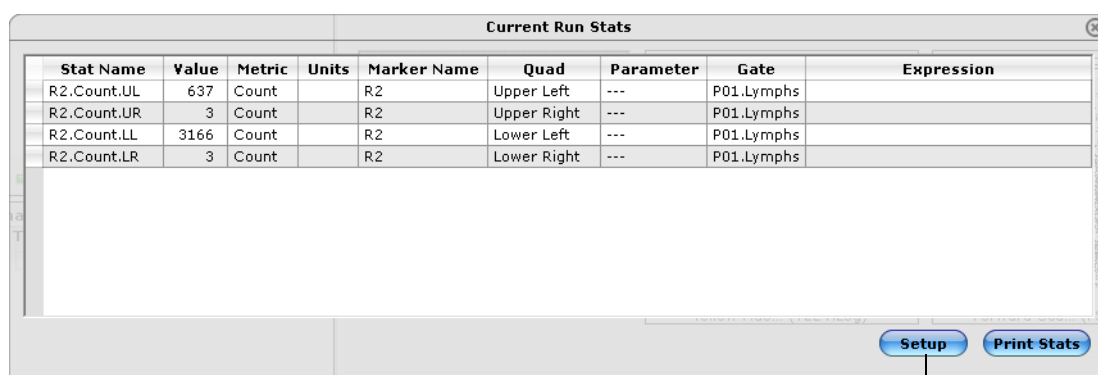
To adjust quadrant markers, click to select the markers. The handles will appear solid. Position the cursor over the handle at the intersection and drag to the desired location. You can adjust the angle of the markers $\pm 44^\circ$ from their original locations. Drag the handle (solid circle) towards the end of the marker and tilt it to the desired location.



Current Run Stats Window

The Current Run Stats window appears when you create a stat. You can also click the **Show Current Run Stats** icon in the main tool bar or select **Tools > Show Current Run Stats** from the menu bar to display the window. For each Analysis Method, all stats for all plots are listed in this window. The Values displayed correspond to the sample well highlighted in the plate map. Simply click any well to see the values for that sample. Stats are listed in the order they were created. To reorder the stats in the list, see “Current Run Stats Setup Window” on page 7-34.

For each Analysis Method, all stat markers are listed in the Region List, as well as the Analysis Methods pane. All stats are saved with the Method.



Stat Name	Value	Metric	Units	Marker Name	Quad	Parameter	Gate	Expression
R2.Count.UL	637	Count		R2	Upper Left	---	P01.Lymphs	
R2.Count.UR	3	Count		R2	Upper Right	---	P01.Lymphs	
R2.Count.LL	3166	Count		R2	Lower Left	---	P01.Lymphs	
R2.Count.LR	3	Count		R2	Lower Right	---	P01.Lymphs	

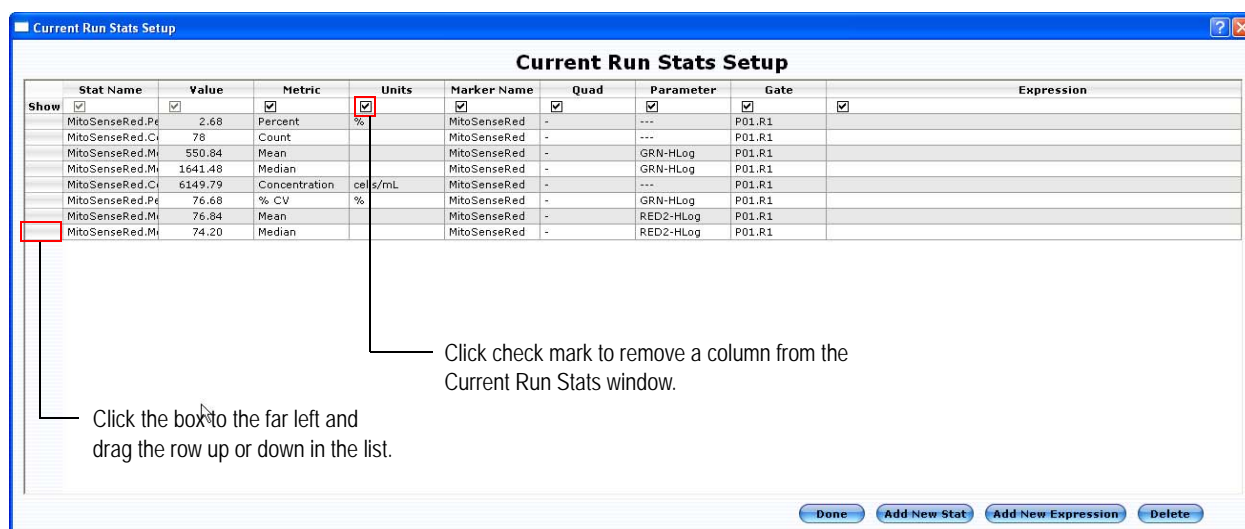
Setup Print Stats

customize statistics

- Click **Setup** to customize the stats listed in the Current Run Stats window.
- Click **Print Stats** to print the statistics listed in the table.
- To copy the window to the clipboard, right-click in the window and select **Copy to Clipboard**.

Current Run Stats Setup Window

To customize the statistics that are listed in the Current Run Stats window, click **Setup**. The Current Run Stats Setup window appears.



Show	Stat Name	Value	Metric	Units	Marker Name	Quad	Parameter	Gate	Expression
<input checked="" type="checkbox"/>	MitoSenseRed.Pe	2.68	Percent	%	MitoSenseRed	-	---	P01.R1	
<input checked="" type="checkbox"/>	MitoSenseRed.Ci	78	Count		MitoSenseRed	-	---	P01.R1	
<input checked="" type="checkbox"/>	MitoSenseRed.Mi	550.84	Mean		MitoSenseRed	-	GRN-HLog	P01.R1	
<input checked="" type="checkbox"/>	MitoSenseRed.Ci	1641.48	Median		MitoSenseRed	-	GRN-HLog	P01.R1	
<input checked="" type="checkbox"/>	MitoSenseRed.Ci	6149.79	Concentration	cells/mL	MitoSenseRed	-	---	P01.R1	
<input checked="" type="checkbox"/>	MitoSenseRed.Pe	76.68	% CV	%	MitoSenseRed	-	GRN-HLog	P01.R1	
<input checked="" type="checkbox"/>	MitoSenseRed.Ci	76.84	Mean		MitoSenseRed	-	RED2-HLog	P01.R1	
<input checked="" type="checkbox"/>	MitoSenseRed.Mi	74.20	Median		MitoSenseRed	-	RED2-HLog	P01.R1	

Click the box to the far left and drag the row up or down in the list.

Click check mark to remove a column from the Current Run Stats window.

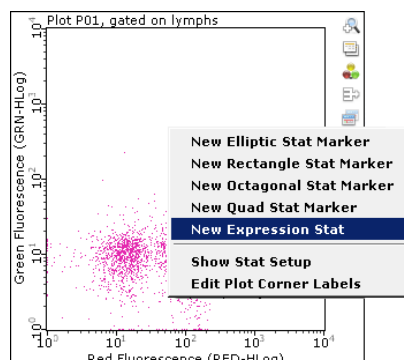
Done Add New Stat Add New Expression Delete

- To change the Stat Name, double-click the name and enter a new name. To change the metric, gate, quadrant, marker name, or parameter, double-click the item and select the new one from the drop-down list. Expression stats cannot be modified. They can only be deleted.
 - **NOTE:** If you wish to change the parameter, double-click to display the parameters for that plot. If you wish to see all available parameters for the data set, press Ctrl and double-click the parameter value. For a parameter to be displayed, you must select it in the Add New Stat Marker dialog.
- To remove a column, remove the “Show” check mark for the selected parameter(s) and click **Done**. The Current Run Stats window is updated.
- To reorder the rows and/or columns in the Current Run Stats Setup window, click the column header and drag the column to the left or right. You cannot move the Stat Name or Value column. They are fixed.
- You can also create stats directly from the Current Run Stats Setup window. Click **Add New Stat** to display the Add New Stat Marker window. This window is similar to the window that appears when you select **Show Stat Setup** from the New Stat Marker icon in the plot tool bar (see page 7-32). However, this stat marker window allows you to create a stat independent of a plot and based on any gate. Enter a stat name; select the marker/region, gate, parameter, and metric, and click **OK**.
- To create an expression, click **Add New Expression** (see "Creating Expression Statistics" in the following section).
- To delete a stat, select the stat from the list and click **Delete**.

Creating Expression Statistics

Expression stats allow you to create custom statistics. For example, you can apply the dilution factor to a count or concentration value of a specific population to get the actual value for your diluted sample. Or, you can create an expression statistic that adds the events in two quadrants of quad markers.

- 1 Create statistics as you normally would.
- 2 Click the **New Stat Marker** icon in the plot tool bar and select **New Expression Stat**.

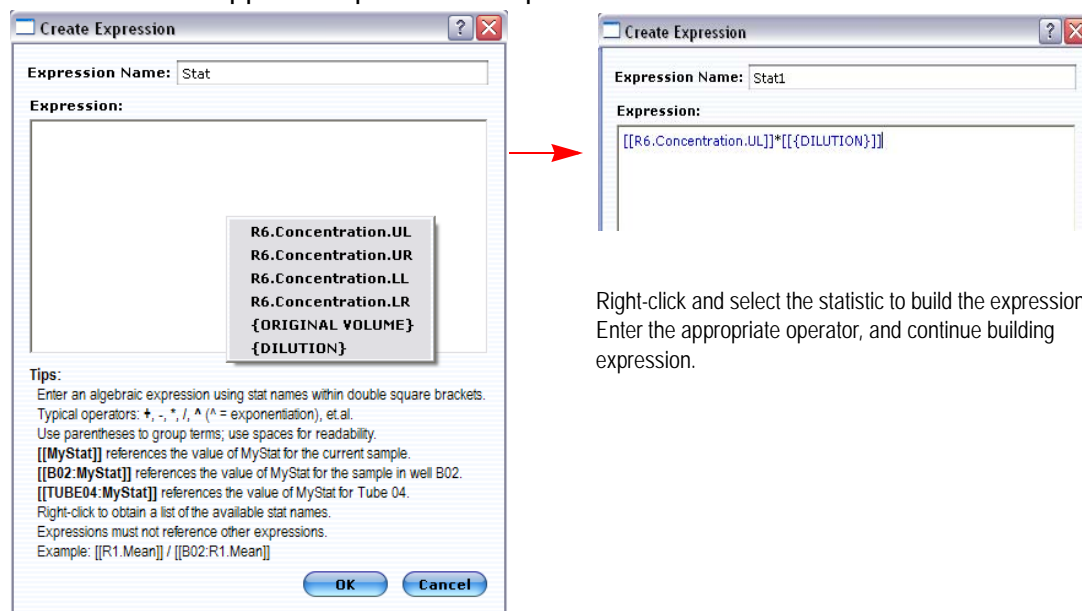


- 3 Enter a stat name for the expression.

- 4 Right-click in the Expression area of the window and select the stat. Type an operator (+, -, *, /, or ^ for exponentiation). Complete the expression.

■ **NOTE:** To create an expression for a specific well/tube, press Ctrl and right-click the Expression area. The pop-up menu allows you to select a specific tube or well.

The following example shows an expression created to apply the dilution factor of 20 to events in the upper-left quadrant of a plot.



- 5 Click **OK**.

The expression appears in the Current Run Stats window.

Current Run Stats									
Stat Name	Value	Metric	Units	Marker Name	Quad	Parameter	Gate	Expression	
R6.Concentration.UL	8.52E+04	Concentration	cells/mL	R6	Upper Left	---	P01.R1		
R6.Concentration.UR	1193.91	Concentration	cells/mL	R6	Upper Right	---	P01.R1		
R6.Concentration.LL	122.45	Concentration	cells/mL	R6	Lower Left	---	P01.R1		
R6.Concentration.LR	3091.91	Concentration	cells/mL	R6	Lower Right	---	P01.R1		
Stat	8.52E+04	Custom Expression						[[R6.Concentration.UL]]*[[{DILUTION}]]	

- 6 Click the Show Sample Info icon in the tool bar to the left of the data panes to open the sample information window. Enter the dilution factor for the sample and press Enter.

Sample Info

Sample ID: A01

Sample No: 1

Dilution Factor: 20

Original Volume: 10

Quantity: 0

Threshold Parameter: FSC-HLin

Threshold Value: 151

Count Gate: All Events

- The updated concentration value, based on the new dilution factor, appears in the Current Run Stats window.

Analysed Data		Current Run Stats						
Stat Name	Value	Metric	Units	Marker Name	Quad	Parameter	Gate	Expression
R6.Concentration.UL	8.52E+04	Concentration	cells/mL	R6	Upper Left	---	P01.R1	
R6.Concentration.UR	1193.91	Concentration	cells/mL	R6	Upper Right	---	P01.R1	
R6.Concentration.LL	122.45	Concentration	cells/mL	R6	Lower Left	---	P01.R1	
R6.Concentration.LR	3091.91	Concentration	cells/mL	R6	Lower Right	---	P01.R1	
Stat	1.70E+06	Custom Expression						[[R6.Concentration.UL]]*[[{DILUTION}]]

Labeling Plot Corners

You can add custom labels to the corners of any plots. Default labels already appear in plots with quadrant markers, if you select **Show on plot** when setting up the quadrant using the New Quad Stat Marker option.

- Click the **New Stat Marker** icon in the plot tool bar and select **Edit Plot Corner Labels**.

The Edit Plot Corner Labels dialog appears.

[[R2.Count.UL]]

[[R2.Count.UR]]

[[R2.Count.LL]]

[[R2.Count.LR]]

Tips:
Enter text to display in each corner of the plot. You may use multiple lines.
Right-click to obtain a list of the available stat names, Ctrl+right click for well-specific stats.
[[MyStat]] references the value of MyStat for the current sample.
[[B02:MyStat]] references the value of MyStat for the sample in well B02.
[[TUBE04:MyStat]] references the value of MyStat for Tube 04.

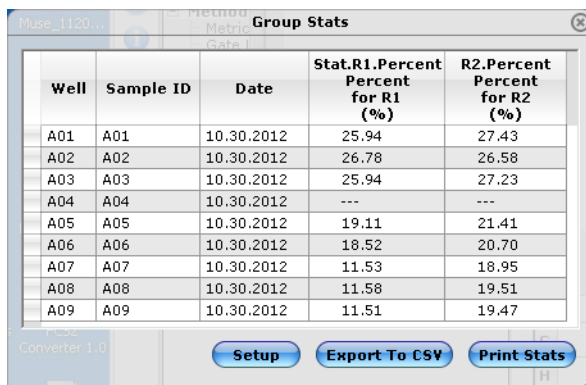
OK Cancel

Labels for quadrants.

- If you are adding labels to a plot with quadrant markers, click the default [count] label and type a new label. If you are adding labels to a plot without quadrant markers, simply type the label in the corresponding box.
- Click **OK**. The labels appear in the corners of the plot.

Group Stats Window

The Group Stats window displays the statistical values for *all* samples in the data set (or group). To display the Group Stats window, click the **Show Group Stats** icon in the main tool bar or select **Tools > Show Group Stats** from the menu bar.



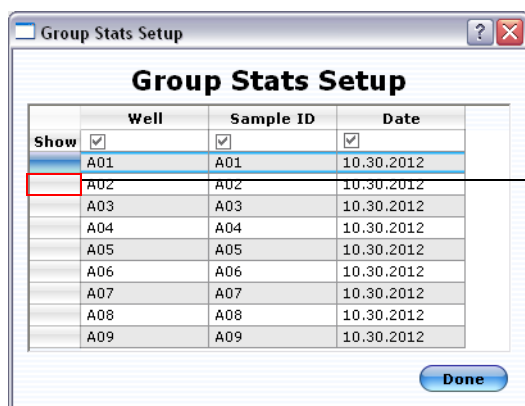
Well	Sample ID	Date	Stat.R1.Percent Percent for R1 (%)	R2.Percent Percent for R2 (%)
A01	A01	10.30.2012	25.94	27.43
A02	A02	10.30.2012	26.78	26.58
A03	A03	10.30.2012	25.94	27.23
A04	A04	10.30.2012	---	---
A05	A05	10.30.2012	19.11	21.41
A06	A06	10.30.2012	18.52	20.70
A07	A07	10.30.2012	11.53	18.95
A08	A08	10.30.2012	11.58	19.51
A09	A09	10.30.2012	11.51	19.47

- Click **Setup** to customize the stats listed in the Group Stats window.
- Click **Export To CSV** to export the group stats to a comma-separated values file for analysis using a spreadsheet program. Select the location, enter a file name, and click **Save**.
- Click **Print Stats** to print the statistics listed in the table.
- To copy the window to the clipboard, right-click in the window and select **Copy to Clipboard**.

Group Stats Setup Window

To customize the statistics that are listed in the Group Stats window, click **Setup**. The Group Stats Setup window appears. To remove a column, remove the “Show” check mark for the selected parameter(s) and click **Done**. The Group Stats window is updated.

You can reorder the rows by clicking the far-left box of the row and dragging the row up or down.



	Well	Sample ID	Date
Show <input checked="" type="checkbox"/>	A01	A01	10.30.2012
<input type="checkbox"/>	A02	A02	10.30.2012
<input type="checkbox"/>	A03	A03	10.30.2012
<input type="checkbox"/>	A04	A04	10.30.2012
<input type="checkbox"/>	A05	A05	10.30.2012
<input type="checkbox"/>	A06	A06	10.30.2012
<input type="checkbox"/>	A07	A07	10.30.2012
<input type="checkbox"/>	A08	A08	10.30.2012
<input type="checkbox"/>	A09	A09	10.30.2012

To reorder the rows, click the box to the far left of the row and drag the row up or down in the list.

Exporting guava InCyte Results to a Spreadsheet File

For each Analysed Group, you can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel. The CSV file will contain the following statistics: Group name, Stat Type, Parameter, Stat gate, Control gate, Log Display, Max, Threshold Max%, Threshold Max, Threshold Min%, Threshold Min. In addition, each sample well will include a derived statistical value, its unit of measurement, a control well (if applicable), the well's sample ID, and the FCS 3.0 file from which it was derived.

- 1 Right-click on any well and select **Export Heatmap Stats to CSV**.

If the HeatMap legend is divided into multiple sectors, then data from each sector will be exported. If you wish to export results for a single sector (or Analysed Group), first click on the specific sector in the legend, then right-click the well and select **Export Heatmap Stats to CSV**.

To export the group stats to a CSV file, refer to “Group Stats Window” on page 7-38.



- **NOTE:** You can copy and save any of the plots, HeatMap, or IC-50 graphs by right-clicking, then selecting **Copy To Clipboard**.

7.6 Compensation

This section provides details on specific compensation adjustments for your stained samples during acquisition. It also includes information on performing post-acquisition compensation and automated compensation on data files acquired with ExpressPlus, ExpressPro, and InCyte only.

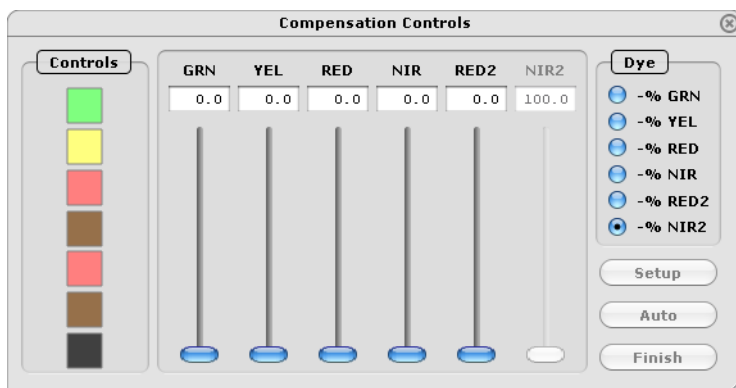
Performing Compensation During Acquisition

Use the **Compensation Controls** to select the radio button for the signal you wish to subtract, then use the slider to adjust the percentage of the overlapping signal to be removed from the detector/channel.

For example, if using FITC (GRN) and PE (YEL) fluorochromes:

- 1 Click the **Show Compensation Controls** icon in the tool bar to open the Compensation Controls window.
- 2 Using a FITC single-color control, select the **-% GRN** radio button under Dye. Then adjust the YEL slider to remove the FITC signal from the YEL channel (YEL-%GRN).

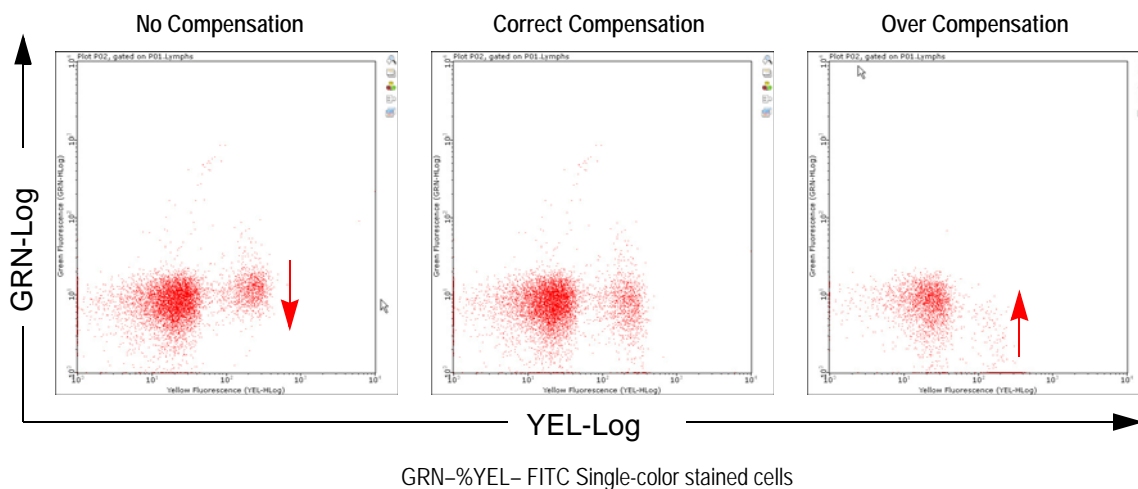
- Next, using the PE single-color control, select the **-% YEL** button, and adjust the GRN slider to remove the PE signal from the GRN channel (GRN-%YEL).

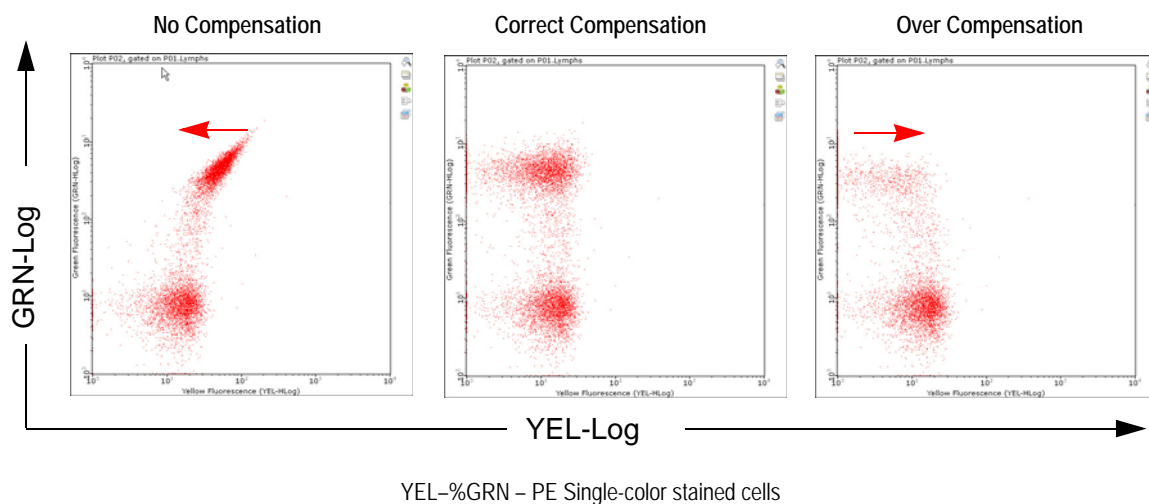


■ NOTE: The Controls at the left of the window are for semi-automated compensation only.

You must check the compensation for each fluorochrome combination you are using. Compensation settings are correct when the center of the stained population is aligned with the center isotype or negative control population. Avoid having too many cells touching the axis (overcompensated).

- NOTE: Some fluorochromes require very little compensation because they have little overlap, such as PE into the GRN channel (GRN-%YEL), as shown in the first three plots below; whereas others require much more compensation because they have more overlap, such as FITC into the YEL channel (YEL-%GRN), as shown in the last three plots below.





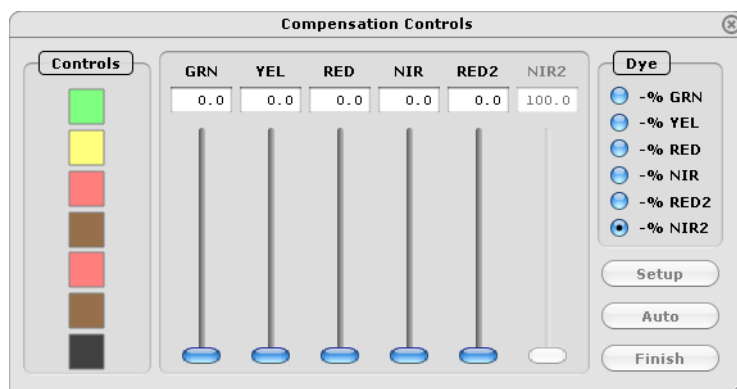
Performing Post-Acquisition Compensation

InCyte allows you to perform post-acquisition compensation on data files acquired using software modules that have the compensation feature and allow you to adjust compensation during acquisition, such as ExpressPlus, ExpressPro, and InCyte.

■ **NOTE:** You can adjust compensation only; you cannot adjust gain settings.

- 1 To adjust compensation, open the FCS file from the Analyse control panel and set up the plots.
We recommend using single-color samples, then checking the compensation again with a sample stained with all fluorochromes to fine-tune, if necessary.
- 2 Click the **Show Compensation Controls** icon in the tool bar to open the Compensation Controls window.
- 3 Select the radio button for the signal you wish to subtract, then use the slider to adjust the percentage of the overlapping signal to be removed from the detector. For example, to remove the FITC signal from the YEL detector, select the – %GRN radio button and adjust the YEL slide (YEL-%GRN).

- 4 Repeat for the remaining compensation adjustments for your samples.



■ NOTE: The Controls at the left of the window are for semi-automated compensation only.

Settings are applied to all samples in the data set, as well as all groups derived from the data set. Adjustments made to a sample within a group, are applied to all samples in the originating data file.

Performing Semi-automated Compensation

InCyte allows you to perform semi-automated compensation on data files acquired using software modules that have the compensation feature and allow you to adjust compensation during acquisition, such as ExpressPlus, ExpressPro, and InCyte. You must have acquired single-color control samples to use the semi-automated compensation feature.

■ NOTE: You can adjust compensation only; you cannot adjust gain settings.

- 1 To adjust compensation, open the FCS file from the Analyse control panel and set up the plots.

We recommend using single-color samples, then checking the compensation again with a sample stained with all fluorochromes to fine-tune, if necessary.

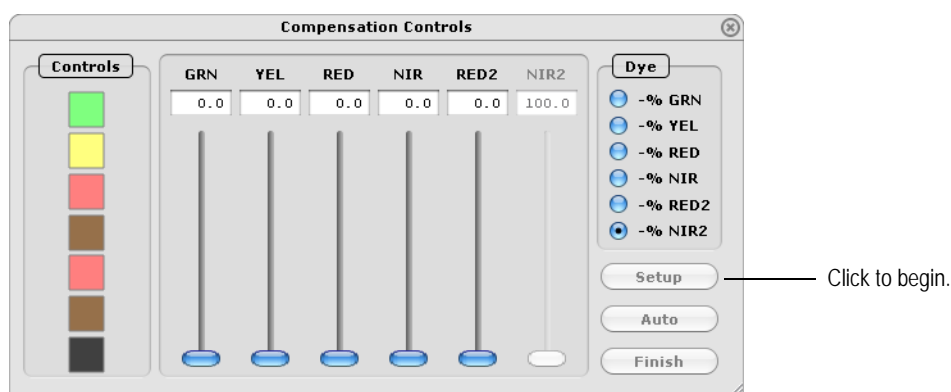
- 2 Click the **Show Compensation Controls** icon in the main tool bar to open the Compensation Controls window.

This window is specific for compensation during analysis. It contains a slider for each parameter and a list of controls in the far left panel. These controls correspond to each parameter and the fluorochrome used:

- green square is the GRN channel
- yellow square is the YEL channel
- 1st red square is the RED channel from the blue laser
- 1st brown square is the NIR channel from the blue laser
- 2nd red square is the RED2 channel from the red laser
- 2nd brown square is the NIR2 channel from the red laser
- black square is negative or isotype

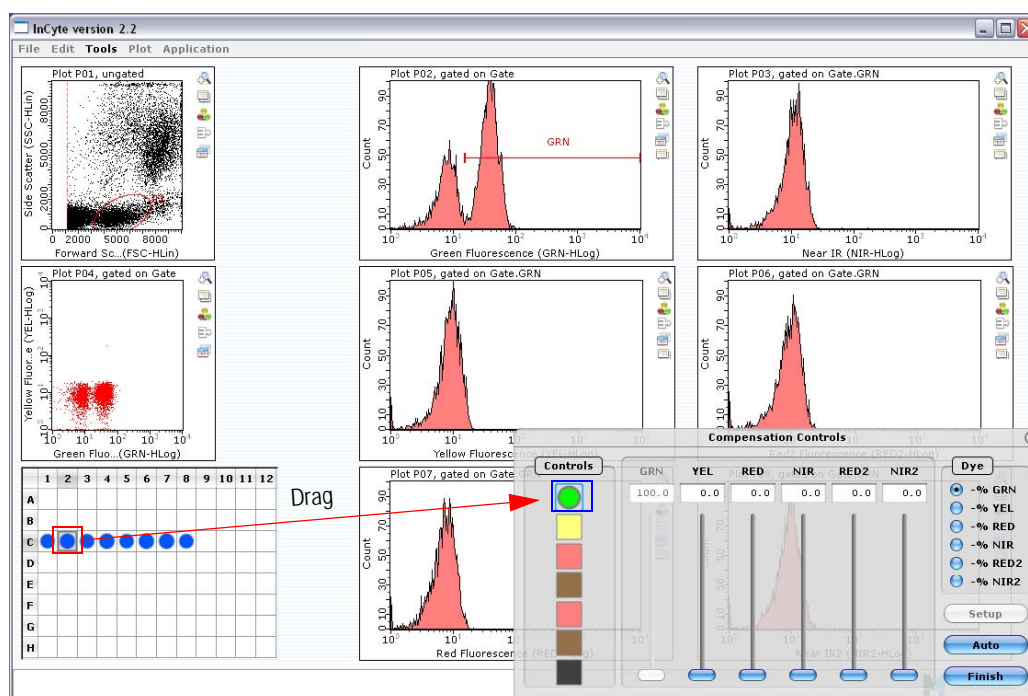
■ NOTE: If it is necessary to gate the data, pair a Method with the data file to create an Analysed Group and set regions and gates. Refer to “7.5 Regions, Gates, and Statistics” on page 7-25. Otherwise, it is not necessary to create an Analysed Group

to adjust post-acquisition compensation.



- 3 Click **Setup** in the Compensation Controls window.
The application screen changes to display two dot plots on the left with the plate map below, and up to six histograms for each fluorescence channel.
- 4 Adjust the default region in plot 1 so that it encompasses the events of interest.
- 5 Drag the isotype (or negative) control from the plate map to the black square at the far left in Compensation Controls window
- 6 Drag the next control, for example the GRN control (FITC-positive control) from the plate map to the green square at the far left in the Compensation Controls window (see example in figure below).
For this example, the green square turns into a circle and the FITC control data is displayed in the GRN histogram.

- 7 Adjust the GRN histogram marker to include the positive population.



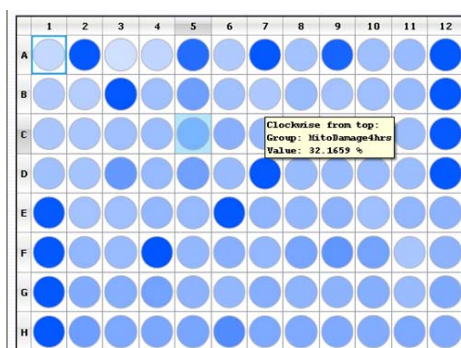
- 8 Drag the next control, for example YEL control (PE-positive control) to the yellow square. Continue dragging the remaining controls to the corresponding Controls squares and adjusting the markers to include the positive population.
- 9 Check that the markers are correct for the isotype control.
- 10 Click **Auto**. The software automatically calculates the compensation values for each parameter.
Settings are applied to all samples in the data set, as well as all groups derived from the data set. Adjustments made to a sample within a group are applied to all samples in the originating data file.
- 11 If you need to adjust compensation further, refer to “Performing Post-Acquisition Compensation” on page 7-41.
- 12 Click **Finish** to return to the analysis display.

7.7 HeatMap

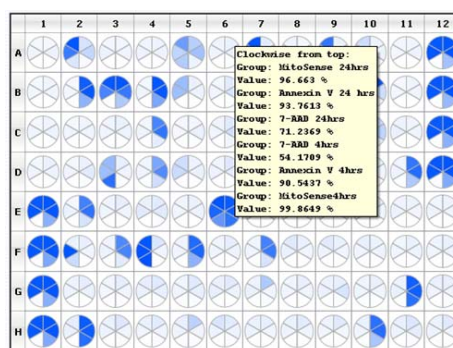
HeatMapping provides a colorimetric representation of comparative results at the experiment level. Each Analysed Group is represented by an individual sector in the HeatMap legend. After the completion of sample analysis, individual parameters can be compared over the entire experimental run. A visual representation of the data and the results will appear in the plate map. Well-to-well variations in blue (dark blue = maximum value, white = minimum value) are based on relative differences in the data as measured on a linear scale.

The plate map can simultaneously display results for up to six Analysed Groups—the same number of sectors that the legend is displaying. Place the cursor over any well to see the result for that Analysed Group. When the HeatMap legend displays more than one sector, the results for every sector will appear within the well pop-up.

- **NOTE:** If the Analysis Method used to create the Analysed Group is new (no regions and/or gates have been defined), the plate map will initially display all wells as dark blue. Perform the analysis on the data before proceeding to HeatMapping.



One sector in HeatMap Legend



Six sectors in HeatMap Legend

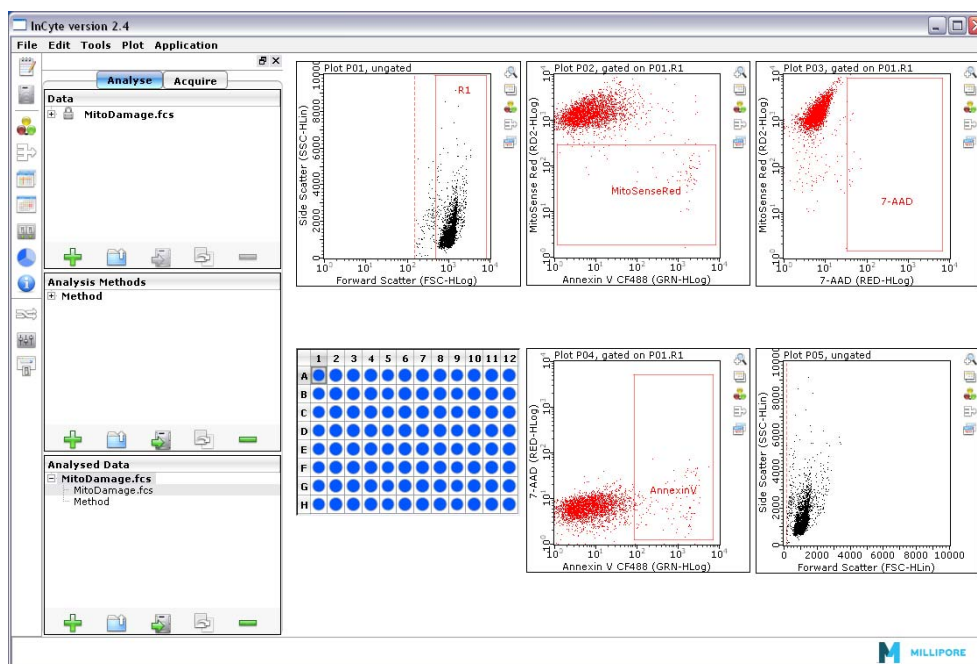
Creating a HeatMap

The following examples show how to use the HeatMap to get a visual and numerical representation of results for a single or multiple parameters from one data file. This example shows a sample stained to detect mitochondrial membrane potential, cells undergoing apoptosis, and dead cells.

Single-Parameter HeatMap Using a Region or Gate

1 Open the Analysed Group.

You can also open an FCS data file and perform the analysis for the populations you are interested in. See “7.4 guava InCyte Analysis” on page 7-19, for information.



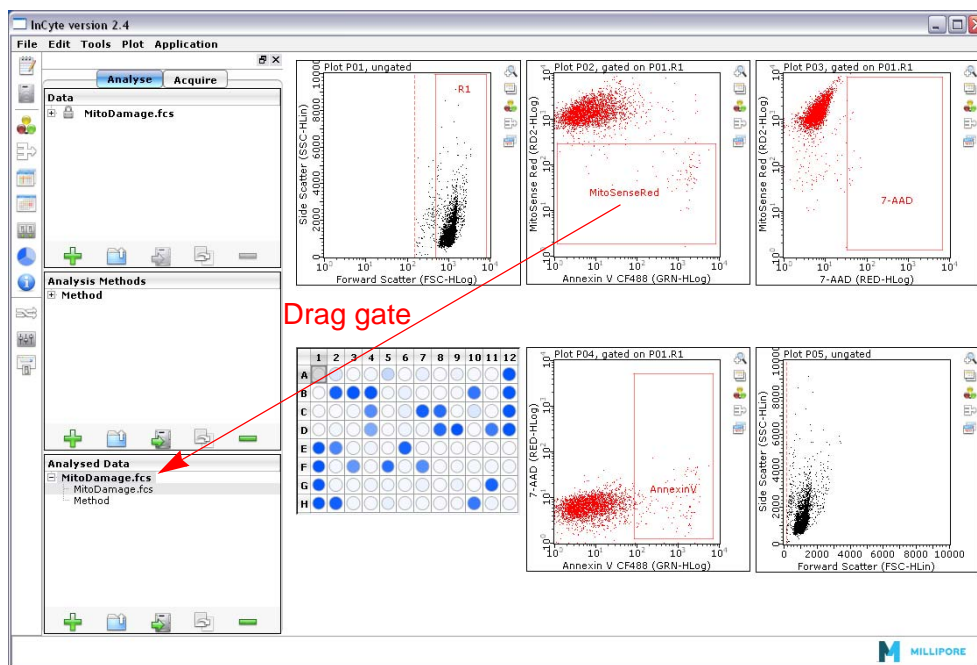
Jurkat cells were stained with FlowCelect MitoDamage kit to look at mitochondria membrane potential (MitoSense Red), apoptosis (Annexin V CF488), and cell death (7-AAD). R1 was created for cell population, then applied to plots 2-4. Three regions were created for each population of interest.

2 Select the gate of interest, then click and drag it to the Analysed Group in the Analysed Data pane. Click **OK** to accept the new gate name, or change the name.

As you start to drag the gate, a gray box appears at the cursor. Drag the gate to the Analysed Group and a plus sign (+) appears on the cursor. Drop the gate in the Analysed Group.

- **NOTE:** You can also use the HeatMap legend by dragging the Analysed Group and the gate to the legend. Then, to update the analysis, simply drag a new gate to the legend. The plate map will update automatically.

After the gate is applied to the Analysed Group, the wells will change to varying shades of blue, depending on the population percentages. Place the cursor over any well to see the value for that well.



MitoSense Red region is dragged to the Analysed Group. Plate map displays well-to-well variation of relative percentage of mitochondrial membrane potential among samples.

- 3 To change the metric and/or threshold, see “Metrics” on page 7-49 and “Thresholds” on page 7-49.
- 4 To save the HeatMapped Analysed Group, select it and click the Save button (3rd) at the bottom of the pane. Navigate to the location where you wish to save it, enter a file name, and click **Save**. The Analysed Group will be saved as an FCS file and will contain the Method (regions, gates, metrics, and stats), as well as the data and the instrument settings.

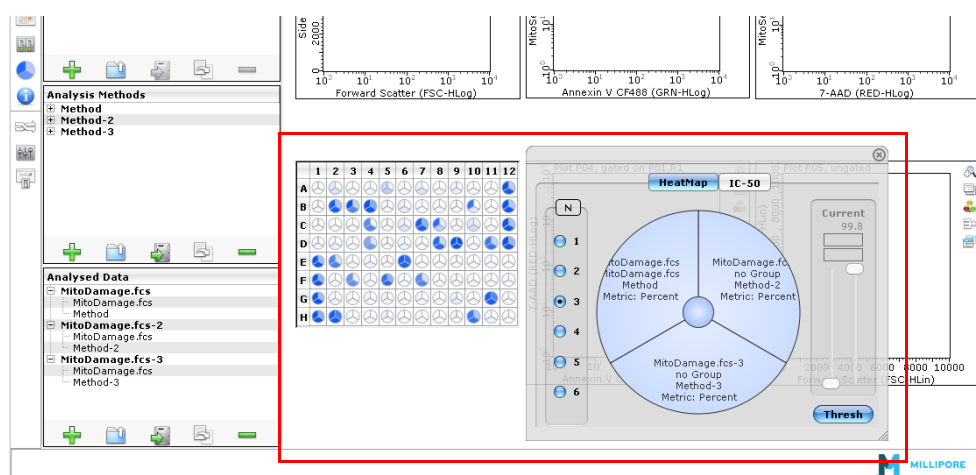
Multi-Parameter HeatMap Using a Region or Gate

The following example is the same as the single-parameter HeatMap shown above. In this example, three Analysed Groups are created to view and compare the results for three different populations simultaneously.

- 1 Open the Analysed Group.
- 2 To compare multiple parameters from one FCS file, duplicate the Analysed Group. Select the Analysed Group, then click the Duplicate button (4th button) in the pane.
For this example, the Analysed Group was duplicated twice to obtain results on three populations.
- 3 Select the first Analysed Group, then click to select and drag the gate of interest to this Analysed Group. Click **OK** to accept the new gate name, or change the name.

- 4 Select the second Analysed Group, then click to select and drag the next gate of interest to this Analysed Group.
 - 5 Repeat by dragging the remaining gates to the Analysed Groups.
 - 6 (Optional) To change the name of an Analysed Group, double-click it and type the new name.
 - 7 Click the Pie Legend icon in the tool bar on the left side of the application window to display the HeatMap legend. Select the number of sectors (N) to display.
 - 8 Click and drag the Analysed Groups to the appropriate sectors. Then, click the center circle in the legend to display the results for all populations of interest in the plate map.
- **NOTE:** When multiple sectors are displayed, no data is shown in the plots and you cannot modify any Methods (add regions, move gates, etc). To modify individual Methods, click on the sector you wish to update.

For more information on the HeatMap legend, see “Pie Legend” on page 7-10.



- 9 The plate map shows each well with the number of sectors used for analysis. Place the cursor over any well to see all the values for that well.
- 10 (Optional) To clear a sector, right-click on the sector in the HeatMap legend and select **Clear sector**.
- 11 To change the metric and/or threshold, see “Metrics” on page 7-49 and “Thresholds” on page 7-49.
- 12 To save the HeatMapped Analysed Groups, select one and click the Save button (3rd) in the pane. Navigate to the location where you wish to save it, enter a file name, and click **Save**. The Analysed Group will be saved as an FCS file and will contain the Method (regions, gates, metrics, and stats), as well as the data and the instrument settings. Repeat for the remaining groups.

Single-Parameter HeatMap Using a Statistic or Expression Stat

The following example shows how to use the HeatMap to get a visual and numerical representation of results for a single or multiple parameters from one data file.

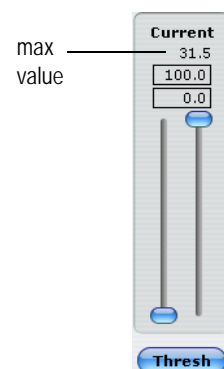
- 1 Open the Analyzed Group.
- 2 If necessary, create a stat region or a statistic for any region created. For more information on creating statistic gates and statistics for regions see “7.4 guava InCyte Analysis” on page 7-19.
- 3 Click the Pie Legend icon in the tool bar on the left side of the application window to display the HeatMap legend.
- 4 Select the statistic of interest, then click and drag it to the Analyzed Group in the Analyzed Data pane. Click **OK** to accept the new gate name, or change the name.

Thresholds

The Workspace contains a dual-barred threshold tool for setting upper and lower limits on statistical values. The threshold bars can be used to set limits on a single Analysed Group at a time (the one that is displayed in the plate map). If the HeatMap is displaying multiple sectors, the threshold feature is disabled.

Above the threshold panel is the maximum derived value for a given data set. Each slider tool is based upon a % scale where the minimum value (0 is lower limit) is Zero and the maximum value (100 in upper limit) is defined by the maximum value derived from the analysis. Simply slide the bars up or down to set boundaries for data display.

In addition, any well can be selected to represent the MAX value. To set a MAX, right-click on a well and select **Use as Max** from the menu. The new max value will also be displayed at the top of the threshold panel.



Metrics

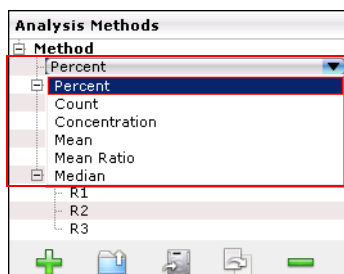
Metrics are the statistical parameters applied to a set of gated events. Applying a metric results in the calculation of a numerical value for each well/sample. These values are compared across a given data set and determine the well-to-well variability in color displayed in the plate map. There are six metrics from which to choose:

- **Percent (%)** - Percentage of events within a given gate relative to the total number of events displayed within the originating plot.
- **Count** - Number of events within a given gate.
- **Concentration** - Event count for a gate as a function of the volume of sample acquired (events/uL).
- **Mean** - Mean fluorescent intensity (MFI) for a set of gated events. If derived from a dot plot, the mean value applies to the parameter associated with the x axis. (For log scale, the mean is geometric; for linear scale, the mean is arithmetic.)

- **Median** - Median fluorescence for a set of gated events. If derived from a dot plot, the median value applies to the parameter associated with the x axis.
- **Mean Ratio** - A measure of the fluorescence intensity for a set of gated events as defined by the following: $\text{ratio} = \text{MFI}_{\text{well } X} / \text{MFI}_{\text{control well}}$. The control MFI may be derived from a single well or represent the average value from a set of control wells. To apply a mean ratio, select **Mean Ratio**. Using the plate map, select the appropriate control well(s) and drag to the corresponding legend sector. This well's value is used as the denominator in the above equation. You must select a control well as the denominator to get a mean ratio.

Changing the Metric

The default metric for a new Method is percent. To select a different metric, double-click the metric in the Analysis Methods pane, then open the drop-down list to display the Metric list. Select a metric to apply it as the statistical parameter for that Method. Once selected, the new metric will replace the previous one. If you are heatmapping and the corresponding Method occupies a legend sector, the plate map will automatically update to reflect the change.



To change the metric, double-click the metric in the Method and select the new metric.

7.8 IC-50/EC-50

Understanding the kinetic characteristics of a given compound's mode of action is important for determining efficacy, toxicity, and dosage. Assays measuring parameters such as apoptotic status or protein expression are commonly used to obtain this information. Use the IC-50 application to create curves based on the relationship between concentration and response, where response is measured by changes in the statistical parameter for the applied Analysis Method. IC-50 (or EC-50) values are derived directly from the calculated curves.

- IC50 is the measure of the effectiveness of a compound inhibiting biochemical function. This measure indicates how much of a particular drug is needed to inhibit a given biological process by half.
- EC50 is the measure of the half-maximal effective concentration of a drug, antibody, or toxicant at some specified exposure time. It is commonly used as a measure of a drug's potency.

Creating an EC-50 or IC-50 Curve

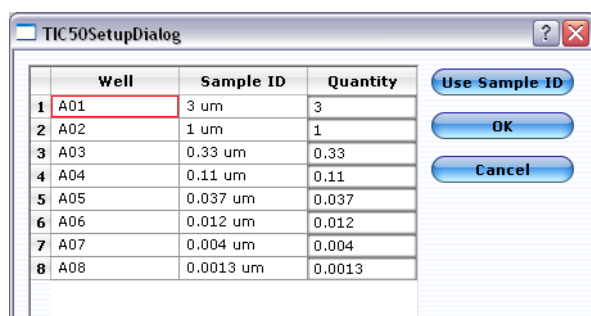
- 1 To determine the EC50 value for a given compound, perform the appropriate gating. If the compound-specific sample set is part of a larger data set, create a group to define this specific subset (see “Creating a Group” on page 7-52). Then drag the group to the legend sector.
- 2 Click the **Show Pie Legend** icon in the tool bar to open the workspace. Click **IC50** at the top of Workspace.

A graph appears where x and y axes are defined as Concentration (log10 scale) and response level % (linear), respectively.

The y coordinates correspond to the numerical values derived from the Analysed Group. Values are converted to a relative scale where the maximum derived value is set to 100% on the y axis.

The x coordinates represent the actual compound concentrations used at each step in the titration. The axis display is open and set automatically from the range of concentrations used.
- 3 To set the x-axis values for the curve, click the **Setup** button.

The setup dialog appears, displaying the wells used in the analysis, the Sample ID, and Quantity.



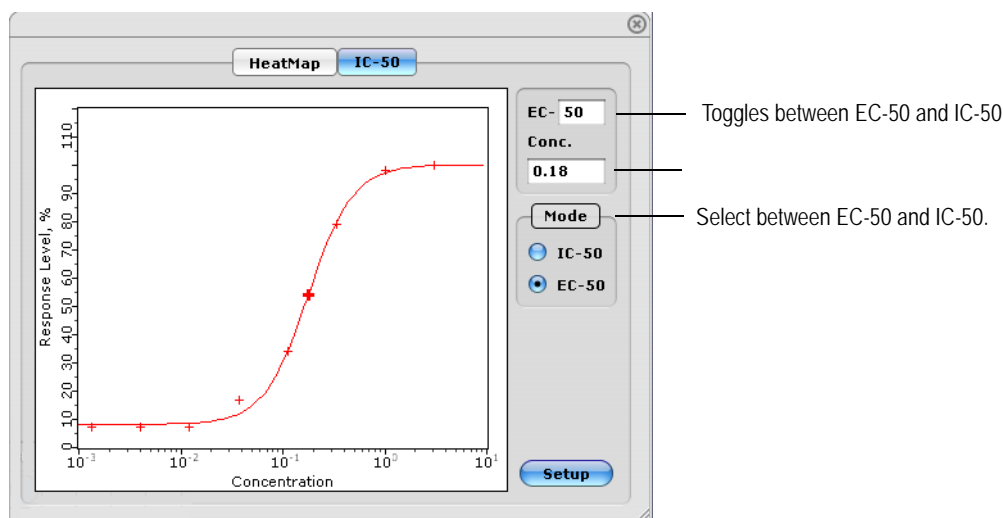
	Well	Sample ID	Quantity
1	A01	3 um	3
2	A02	1 um	1
3	A03	0.33 um	0.33
4	A04	0.11 um	0.11
5	A05	0.037 um	0.037
6	A06	0.012 um	0.012
7	A07	0.004 um	0.004
8	A08	0.0013 um	0.0013

The Sample ID is derived from the original FCS file. If a sample ID was not entered for each well, the default well name appears (A01, A02).

- 4 Enter the Quantity (concentration) for each well. Quantity defines the x-axis values for the graph. These are user-defined and must be entered for each curve.

If, during acquisition, you enter a concentration as the sample ID, you can click the **Use Sample ID** button to enter the Quantity. Characters before the “space” in the ID are used for the quantity. For example, if your sample ID is “3 um staurosporine,” the 3 is used for the quantity. You can also type the values into the Quantity fields. Enter the actual number (.01, .001, .0001) or use scientific notation (1e-2, 1e-3, 1e-4).
- 5 Click **OK** to display the graph.

The EC-50 (IC-50) value is marked by a bold + on the curve.



You can make the following adjustments to the data:

- Change the target value (from 20–80). For example, instead of viewing a graph where 50% of the effect is observed, set the EC or IC value to 25 to see where 25% of the effect is observed.
- The Conc field provides the numerical value for the EC-50 point, in this case 0.16.
- Select between EC-50 and IC-50.

7.9 Special Features

Creating a Group

A group is a user-defined set of sample wells from one or more FCS files (or data sets). Grouping allows you to extract and analyze a subset of samples from the data set(s). You can also use grouping to apply different Methods to subsets of data that are part of a single FCS file, to get different statistics, for example.

To create a group:

- 1 Select the FCS data file in the Data pane.
The plate map displays the wells acquired in that file.
- 2 Drag the cursor across the appropriate wells in the plate map, right-click the selected wells, and choose **Create group** from the menu.

To select non-consecutive wells, press the Ctrl key while clicking to select wells. The order that you select and add the wells to the group is the order that they will be in the group.

The group name appears under the original FCS file in the Data pane. To rename the group, double-click the name and type in the new name.

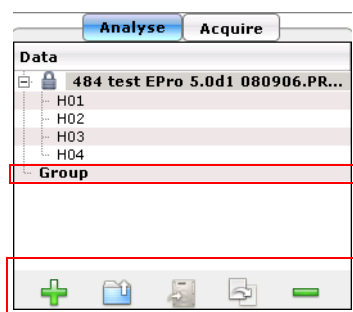


To add additional samples to an existing group, select the wells and drag them to the group in the Data pane.

- **NOTE:** You can combine samples from different FCS files; however, if you select the same/overlapping wells, the plate map and plots will display only the first of the overlapping well(s) selected (eg, the first A01 selected).

- 3 To remove an individual well, right-click it and select **Remove Item**. The deleted well is removed from the group and appears as an empty square in the plate map. Wells can be deleted only from groups, not from the original FCS file. To delete a group, select it and click the **Delete Group** icon in the Data pane.

- **NOTE:** Use the **Duplicate Group** icon in the Data pane to make a copy of a group.



Group under the data file from which it was created.

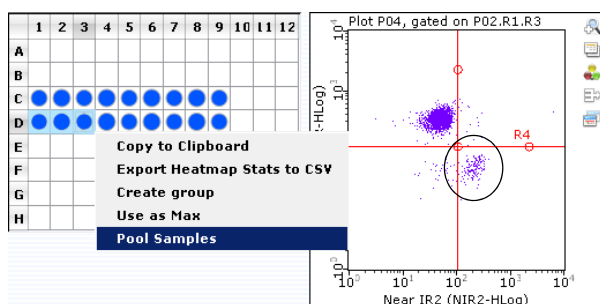
You can also use the icons at the bottom of the Data pane to create, open, save, duplicate, and delete a group.

Data Pooling

During analysis you can combine the data from multiple wells into a single sample. This can be useful when analyzing rare events.

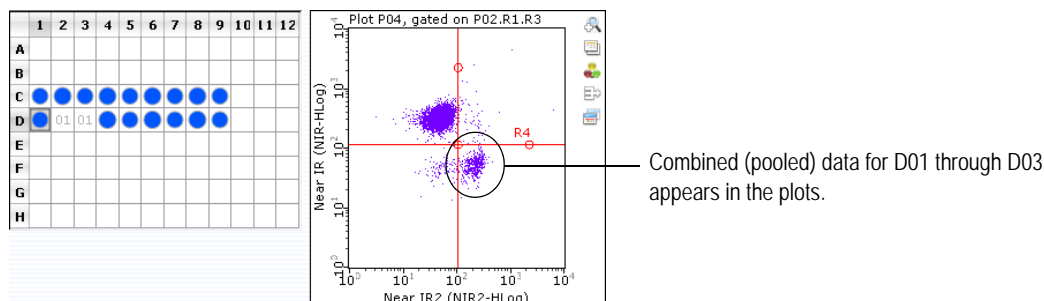
To pool samples, you must first pair a data file with a Method to create an analyzed group, if an analyzed group does not already exist.

- 1 Click a well that you wish to pool data from other wells into, then drag to select the other wells.
- 2 Right-click the selected wells and choose **Pool Samples** from the menu.



Example:
D01 is selected and cursor is dragged through D03.
Right-click and select Pool Samples. Wells D02 and D03 now appear with 01 label.

The data combined from all select wells appears in the plots. The wells appear in the plate map with the label number of the first selected well.



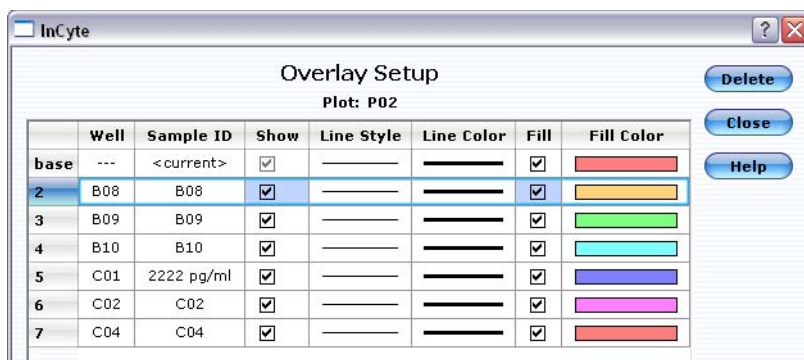
To see the new combined number of events for a given population, open the Current Run Stats window by selecting **Tools > Show Current Run Stats**. If stats have not been created, click the New Stat Marker icon to the right of the plot and select **New Stat for Rx**. Select Count as the Metric and click **OK**.

- 3 To “unpool” the data, click and drag to select the wells (in any order). Right-click the selected wells and choose **Cancel Pooled Samples**.

Overlaying Histograms

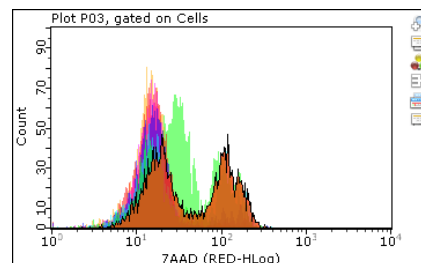
The histogram overlay feature allows you to superimpose histograms from multiple samples derived from one data file. By selecting a different color for each sample, you can see the different sample data overlaid within the same plot. Each histogram plot has its own overlay list.

- 1 Click the **Edit Overlay List** icon (last icon in the tool bar of the histogram plot). The Overlay Setup dialog box appears with the currently displayed sample well listed as base in the far-left column and *<current>* under Sample ID.



- 2 To add samples to the overlay list (and plot), drag a well directly from the plate map to either the histogram plot or the Overlay Setup dialog box. To select multiple wells, drag to select wells or use the Ctrl key to select non-consecutive wells.

The plot will display the selected sample data in the designated colors. When you overlay plots, any markers you had previously set, as well as the stats, apply to the base plot only. The overlays are derived from the same gate as the initial plot. You can also change the parameter displayed by clicking the x-axis label and choosing a different parameter.



- To change the overlay order, click the number (or “base”) in the left column and drag it to a new location in the list.
 - To remove data for an individual sample, click to select the well in the list and click **Delete**.
 - To hide the data for an overlay from the plot, click to remove the check mark from the Show check box.
 - Click Line Style, Line Color, and Fill Color to change the appearance of the overlay.
- **NOTE:** You cannot overlay sample data with different plot parameters. A message will appear informing you that some samples do not have the required parameters.

7.10 Exporting guava InCyte Results

You can export the results from the current run or any sample run to both FCS 2.0 and FCS 3.0 files. The data must have an Analysed Group associated with it. One FCS file is saved for each sample acquired. You can analyze both FCS 2.0 and 3.0 files using a third-party flow cytometry analysis application. FCS 3.0 files are exported with all parameters included. FCS 3.0 files with semi-automated or post-acquisition compensation may be compatible with some third-party applications. For more information, contact EMD Millipore Technical Support.

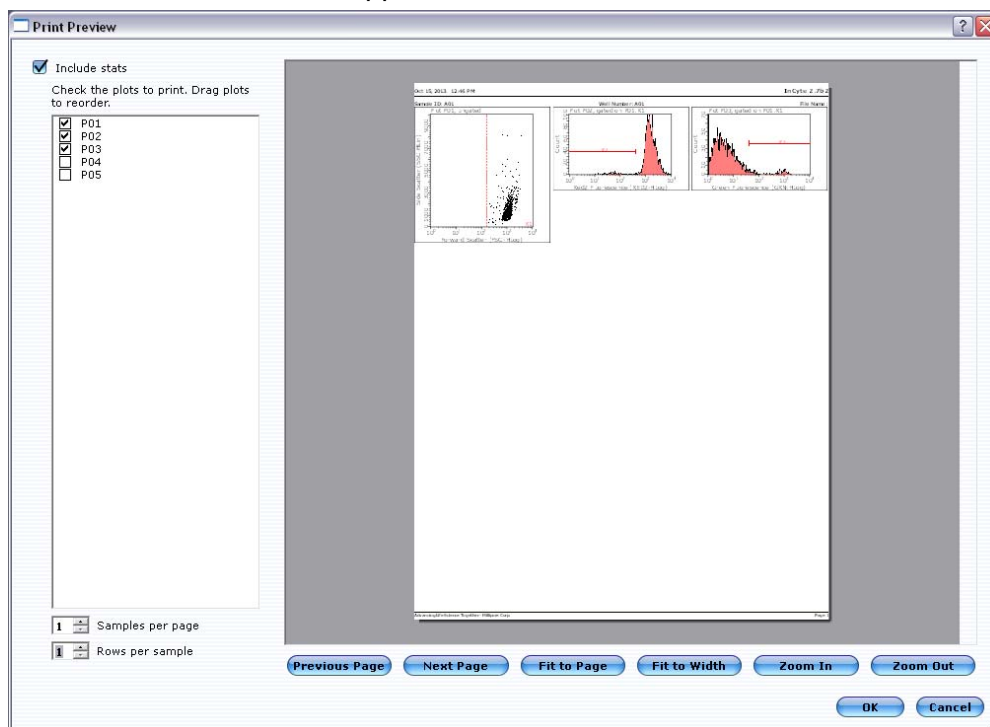
- 1 To export analysis results for all sample data within the run to individual FCS 2.0 or 3.0 files, select **File > Export to FCS 2.0**, **Export to FCS 3.0**, or **Export to FCS 3.0 (No Comp)**, if you do not want the post-acquisition compensation changes included. You can also select a specific Analysed Group from the Analysed Data Pane and select the appropriate export option from the File menu.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**. The samples are numbered sequentially and a number is automatically appended to the file name. For example, if the sample number is A01, the file will be named *filename-1.FCS*, A02 is *filename-2.FCS*, etc.

7.11 Printing Results

Before printing results, use the Print Preview option to customize the information that prints.

- 1 If necessary, set up the page orientation, paper size, and margins by selecting **File > Page Setup**.
- 2 Select **File > Print Preview**.

The Print Preview window appears.



- 3 Select how you wish the results to print:
 - Select the plots to print by clicking the check box next to the plots. The selected plots will print in the order they are listed. If you wish to reorder the plots in the list, click and drag a plot to a new location in the list.
 - Check to includes the statistics. The stats will appear in a table at the end of the printout.
 - Select how many samples you wish to print per page.
 - Select how many rows you wish to print per sample.
 - To scroll through the pages, place the cursor on the page, then click and drag (the hand) to move the page.

7.12 Instrument Settings

InCyte saves the instrument settings for each sample acquired. To access the settings window select the Analysed Group from the Analysed Data pane, then click the Show Instrument Settings tool in the tool bar, or select **Tools > Show Instrument Settings**. In addition to the threshold, gains, and compensation, all of the acquisition information, such as date, time, number of events, total volume, concentration, and flow rate, along with sample-specific information, like sample ID and number, dilution factor, and original volume are saved.

To copy the window to the clipboard, right-click in the window and select **Copy to Clipboard**.

Instrument Settings										
Sample Number	Sample ID	Number of Events	Termination Count	Count Gate	Dilution Factor	Original Volume	Cells/ μ L	Total Volume (μ L)	Acquisition Time (s)	Date o
13	Set 5-WLB 1	15341	0	All Events	1.00	10.00	4.828e+02	31.78	53.86	09-OC1
14	Set 5-WLB 2	7994	0	All Events	1.00	10.00	2.656e+02	30.10	51.02	09-OC1
15	Set 5-WLB 3	7933	0	All Events	1.00	10.00	2.516e+02	31.53	53.44	09-OC1

Exporting Instrument Settings

You can export these values to a comma-separated values (CSV) file for use in a spreadsheet program. Click **Export to CSV**. Select the folder where you want to store the file, enter a file name, and click Save.

You can also print the settings. Click **Print Stats** to print the instrument settings.

7.13 guava InCyte Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava InCyte spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava InCyte FCS file to another directory or give it a new name.
InCyte Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Gates and/or events in plots disappear.	1. Data file or Method is selected. 2. Data was acquired in tubes. 3. More than 96 samples were acquired.	Click on the AnalyzedGroup in the Analysed Data pane to display data and gates.

Problem	Possible Cause	Solutions
Few events, as indicated in Cell Count section of Sample Information control panel.	1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled.	1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate).	1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris.	1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but the events appear in the wrong places in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	1. Sample was not stained. 2. Cell lysis.	1. Check sample. If necessary, restain sample from original suspension. 2. Check buffers used to process cells.
Events appear in some plots but not in others.	Ensure correct gate is selected for plot in question.	1. Open plot menu, point to Apply Gates and select gate. 2. Check gate definition to ensure it includes the correct regions and operators.

Problem	Possible Cause	Solutions
Few events, as indicated in Cell Count section of Sample Information control panel.	1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled.	1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate).	1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris.	1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but the events appear in the wrong places in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	1. Sample was not stained. 2. Cell lysis.	1. Check sample. If necessary, restain sample from original suspension. 2. Check buffers used to process cells.
Events appear in some plots but not in others.	Ensure correct gate is selected for plot in question.	1. Open plot menu, point to Apply Gates and select gate. 2. Check gate definition to ensure it includes the correct regions and operators.

Problem	Possible Cause	Solutions
Events appear off scale in dot plots or histograms.	FSC SSC, GRN, YEL, RED, NIR, RED2, and/or NIR2 gains set incorrectly, or samples staining brightly.	Adjust gain setting so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Gains too low to detect fluorescent signals. 2. Incomplete staining with fluorescent probe, or fluorescent probe inappropriate for cell type. 3. Fluorescent probes over-exposed to light, stored improperly, or expired. 4. Non-specific binding of fluorescent probes. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Ensure positive control is staining adequately and with correct reagent. 3. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 4. If using antibody-based probes, try Fc blocking reagent during staining to minimize non-specific binding. Otherwise, titer the fluorescent probes down to reduce the nonspecific staining. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.
Cannot resolve dim positive staining from background signal.	Dirty capillary.	Perform at least one cycle of Guava Clean. While acquiring samples, select Clean & Rinse instead of Quick Clean, and if necessary, run it frequently.

Problem	Possible Cause	Solutions
Poor resolution between positive populations in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s). 2. Too much reagent in staining tube. 3. Fluorescence background too high. 4. Gain too high causing signal to bleed into other parameters. 5. Gain too low to optimally detect positive signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. 2. Washing cells may remove residual reagent. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce gain. Adjust compensation settings. 5. Adjust settings to increase gain. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris. Or, select one of the fluorescence parameters as the threshold.

CHAPTER 8

guava Nexin Assay

Introduction

Apoptosis, or programmed cell death, is an important regulatory pathway of cell growth and proliferation. In apoptosis, cells respond to specific induction signals by initiating intracellular processes that result in physiological changes. Among these changes are:

- externalization of phosphatidyl serine (PS) to the cell surface
- cleavage and degradation of specific cellular proteins
- compaction and fragmentation of nuclear chromatin
- loss of membrane integrity (in late stages)

Early in the apoptotic pathway, the PS molecules, normally localized on the inside of the cell membrane, move to the outer surface of the membrane. In the later stages of apoptosis, the cell membrane loses its integrity and begins to break down.

The Guava PCA-96 Nexin Kit includes Annexin V, a calcium-dependent phospholipid binding protein with a high affinity for PS. The Annexin V-PE in the reagent binds to the PS that has migrated to the outside of the cell membrane. Nexin 7-AAD, a viability stain, is also included in the assay and used as an indicator of membrane structural integrity. 7-AAD binds to the nuclear material within the cell after the membrane breaks down, allowing you to differentiate the apoptotic cells (Annexin V-positive) into early (7-AAD-negative) and late (7-AAD-positive) stages.

To run the assay, follow the instructions in the *Guava PCA-96 Nexin Kit* package insert to stain the cells. Acquire samples on the guava easyCyte HT System using guavaSoft Software. Data are displayed in an Annexin V-PE vs 7-AAD dot plot with quadrant markers that you can adjust for immediate on-screen results.

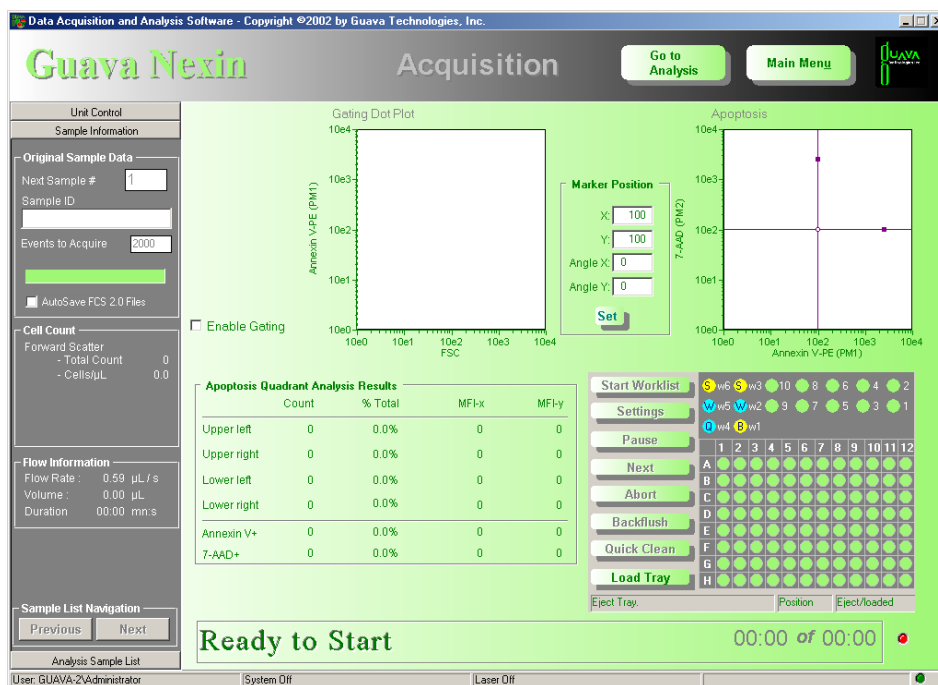
Statistics for each quadrant include the cell count, percentage of cells in the gate, and the mean fluorescence intensity (MFI). Additionally, statistics for Annexin V-PE–positive and 7-AAD–positive cells are also provided.

The guava Nexin data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0–compatible program, if you selected to save FCS 2.0 files. In addition to the saved data file, all results, instrument settings, and the acquisition summary information are exported to a spreadsheet file.

Running the guava Nexin Assay

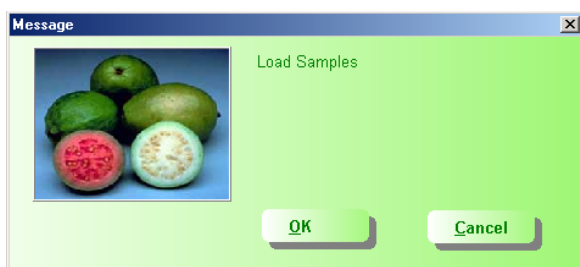
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “guava Nexin Software Module Control Panels” on page 8-14.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.
- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.
- 3 Click **Nexin** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

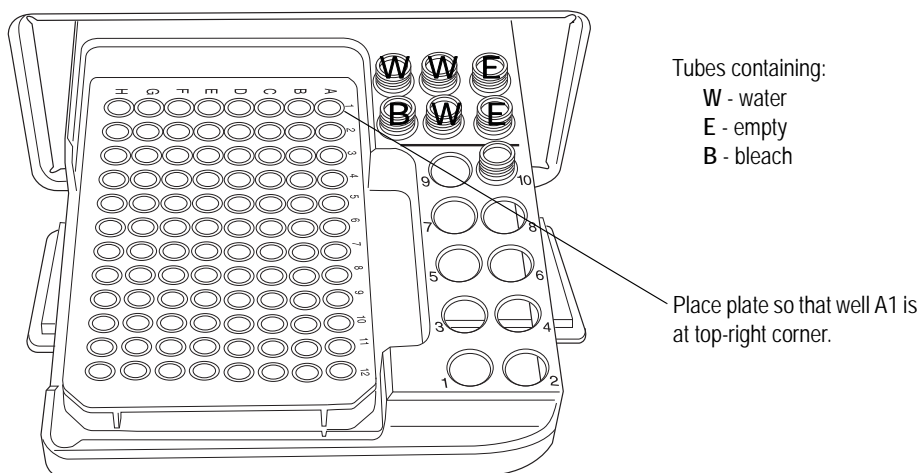


- 4 Prepare samples for analysis in a microplate or 0.5-mL tubes. Refer to the *Guava PCA-96 Nexin Kit* package insert for information.
- 5 Click **Start Worklist** on the guava Nexin screen.
The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

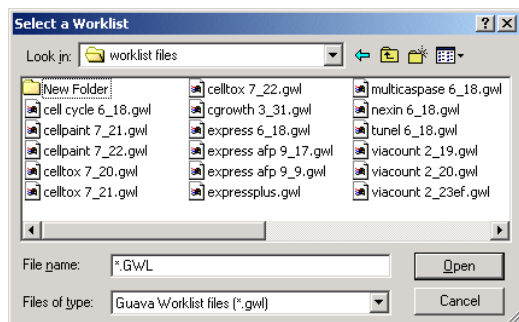


- 6 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

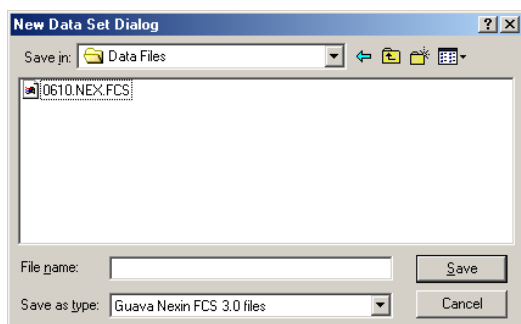
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current plate and click **Open**.



Select worklist file and click Open.

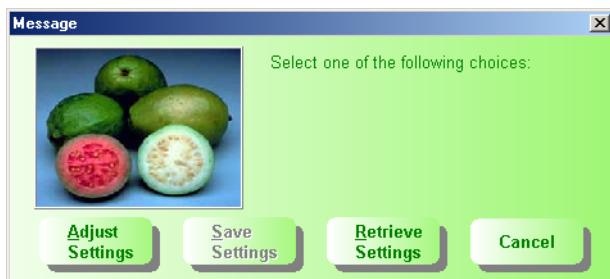
- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.

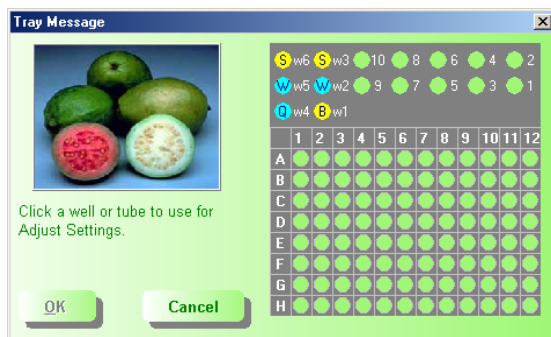


Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.
- To adjust instrument settings, click **Adjust Settings**.
 - To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



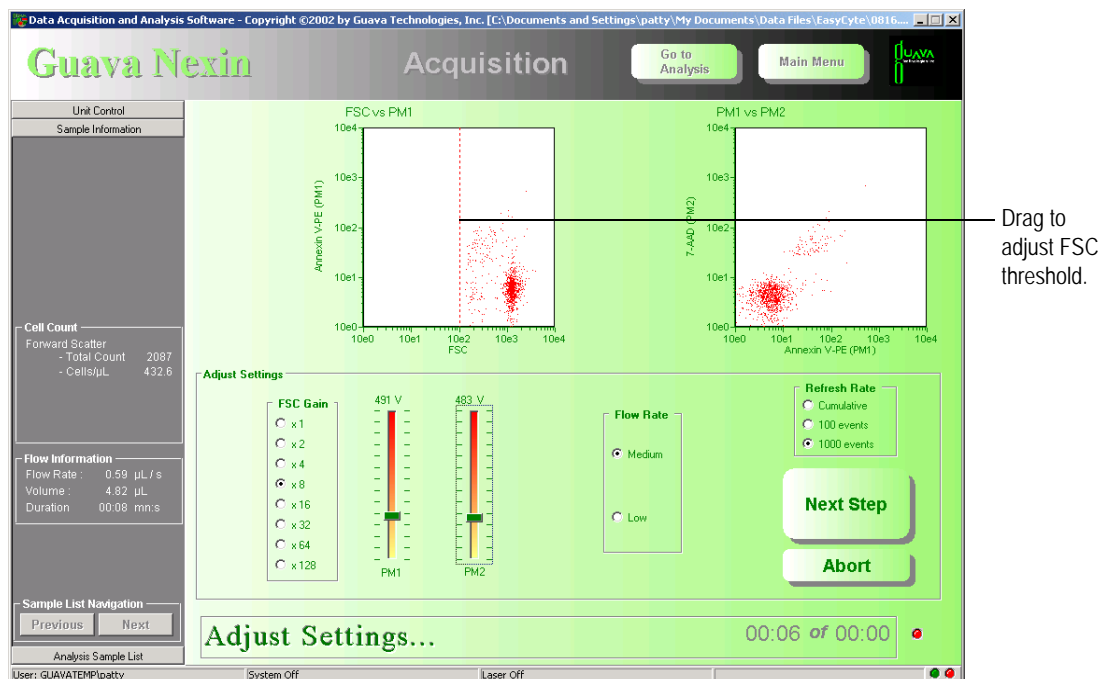
- 11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends using a stained negative (non-apoptotic or uninduced) control sample. Click to select the well/tube used to adjust settings, then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

- 12 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is less than or equal to 500.
- **NOTE:** If the value is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with Guava Nexin Reagent diluted 1:2 with protein-containing medium to lower the concentration to <500 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.
- 13 If you need to fine tune the settings, you can make the following adjustments once events start to appear on the screen:
- Set the **Refresh Rate** to the maximum number of events you want to display.
 - Set the **Flow Rate** to Low (0.24 μ L/s) or Medium (0.59 μ L/s). The recommended flow rate is Medium.
 - Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the cells are visible and on scale.
 - To adjust the FSC threshold, click and drag the threshold marker up or down the FSC axis of the FSC vs PM1 dot plot until the desired amount of debris is eliminated below the threshold.
 - **NOTE:** When setting the threshold, allow for a possible shift in the apoptotic cell population. Any debris that gets acquired can be excluded during analysis with the optional dot plot gate.
 - Adjust the voltages (using the PM1 and PM2 sliders or the arrow keys on the keyboard) so that the live cell population is positioned in the lower-left corner of the

PM1 vs PM2 dot plot. Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.



- 14 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.

If necessary, you can repeat the adjust settings step to ensure that other samples such as the positive control are on scale and appropriately positioned by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

15 Click Resume.

The system acquires the first sample and displays the results. The tube/well currently being acquired is highlighted in red.



ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 2 minutes.

- 16** EMD Millipore recommends adjusting the quadrant markers prior to acquiring the remaining samples. Click **Pause** during the acquisition of the first sample. The system will pause when the acquisition is complete. Once the system is paused, adjust the gate and/or quadrant markers, then click **Resume**. The remaining samples are automatically acquired.

Refer to “guava Nexin Analysis” on page 8-8 for information.

The system automatically performs a Quick Clean at the end of the assay.

guava Nexin Analysis

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

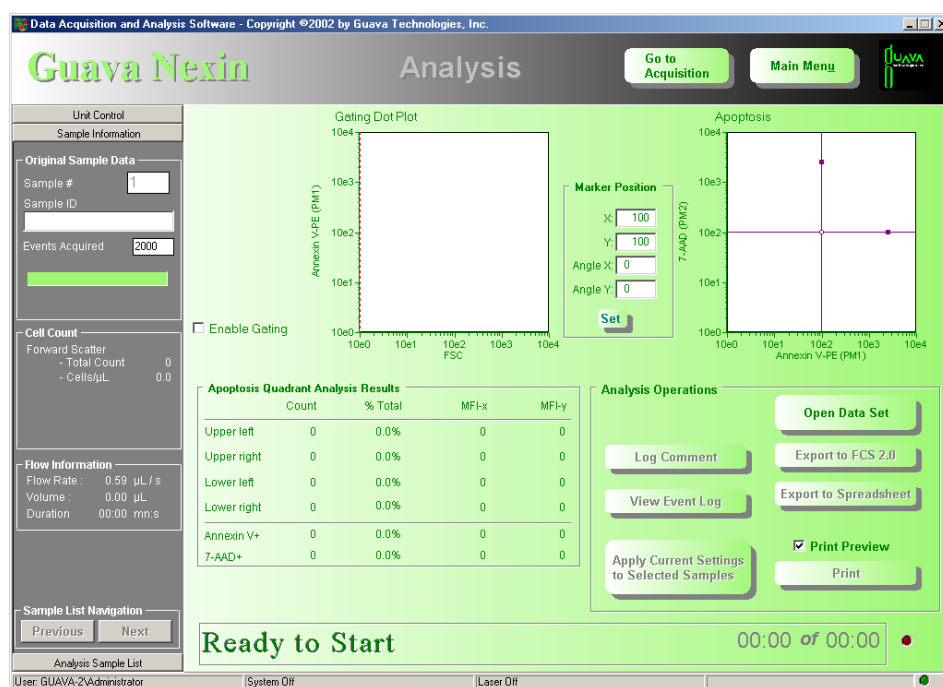
You can save changes made to the sample ID, gate, or markers within Analysis by overwriting the existing file or saving a new file.

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

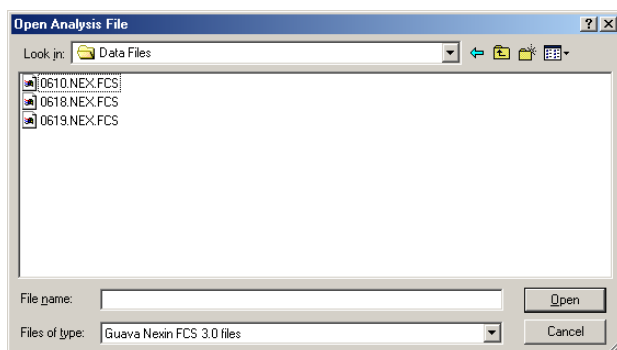
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log.

However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

- 1 Click **Nexin** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



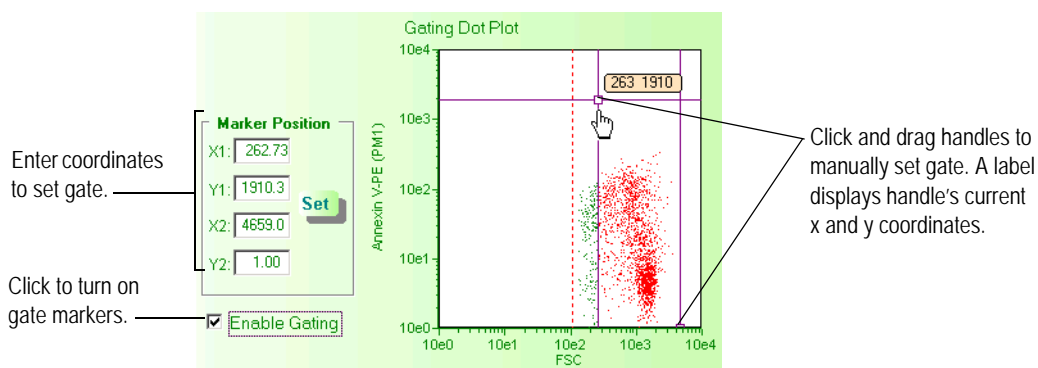
The data and results for the first sample in the data set appear. The marker setting appears as it was when the sample was acquired. Check the positive control to ensure the gate and quadrant markers are set appropriately. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



Optional Dot Plot Gate

To analyze the data for the stained negative control, set the quadrant markers on the Annexin V-PE vs 7-AAD dot plot. If the FSC vs PM1 dot plot shows excessive debris, you can set an optional gate first to exclude the debris from the analysis.

- 1 Click Enable Gating to turn on the gate.
 - 2 To set a gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle and appear in red are included in the gate.
You may also set the gate by entering the coordinates in the Marker Position fields and clicking **Set**. The gated data appears in the Annexin V-PE vs 7-AAD dot plot.
- **NOTE:** If using the stained negative control to set the gate, be sure to extend the gate high enough up the y-axis (as shown) so that PM1-positive events in subsequent samples are included in the gate.



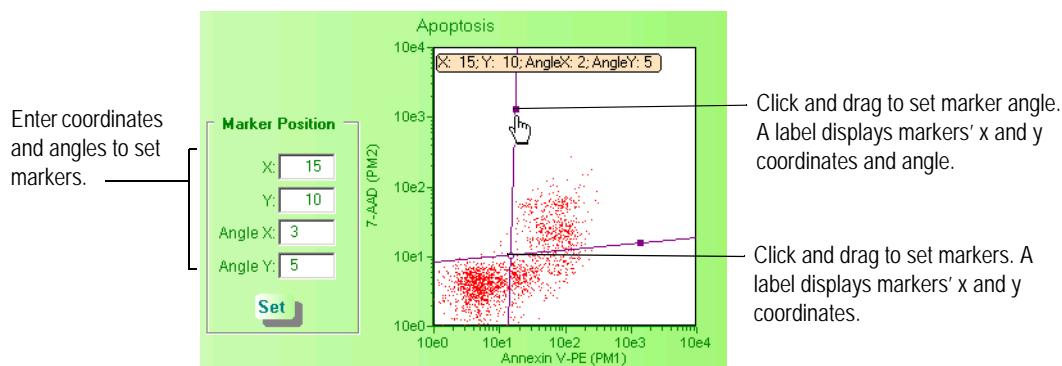
FSC vs PM1 dot plot gate identifying Annexin V-positive (apoptotic) and -negative (live) cells.

Quadrant Markers

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

- 3 To set the markers, position the cursor over the handle at the intersection, then click and drag to the desired location.
You may also set the markers by entering the coordinates in the Marker Position fields and clicking **Set**. The Apoptosis Quadrant Analysis Results are automatically updated each time you move the markers.

- 4 If necessary, you can adjust the angle of the markers $\pm 44^\circ$ from their original locations. Click and drag the handle (solid square) towards the end of the marker and tilt it to the desired location.
You may also set the angles by entering the values in the Angle fields and clicking **Set**.



Annexin V-PE vs 7-AAD quadrant markers differentiating Annexin V-pos cells into 7-AAD-pos and 7-AAD-neg.

- 5 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 6 You can apply gate and/or markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 7 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
 - **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 8 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

guava Nexin Quadrant Results

The statistics for the data within each quadrant are displayed. Results include the count, % of Total (all data in the dot plot) or % Gated if a gate was enabled, and the mean fluorescence intensity (MFI) for each parameter (PM1 and PM2). Additionally, statistics for Annexin V–positive cells (upper-right and lower-right quadrants) and 7-AAD–positive cells (upper-left and upper-right quadrants) are included.

Apoptosis Quadrant Analysis Results				
	Count	% Total	MFI-x	MFI-y
Upper left	35	2.0%	23	32
Upper right	432	24.6%	259	52
Lower left	878	50.0%	10	3
Lower right	411	23.4%	95	5
Annexin V+	843	48.0%	179	29
7-AAD+	467	26.6%	241	51

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

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

guava Nexin apoptosis quadrant statistics.

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

Quadrant	Staining	Population
upper left	Annexin V–negative, 7-AAD–positive	nuclear debris
upper right	Annexin V–positive, 7-AAD–positive	late apoptotic cells
lower left	Annexin V–negative, 7-AAD–negative	live, healthy cells
lower right	Annexin V–positive, 7-AAD–negative	early apoptotic cells

Exporting guava Nexin Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting guava Nexin Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**. The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

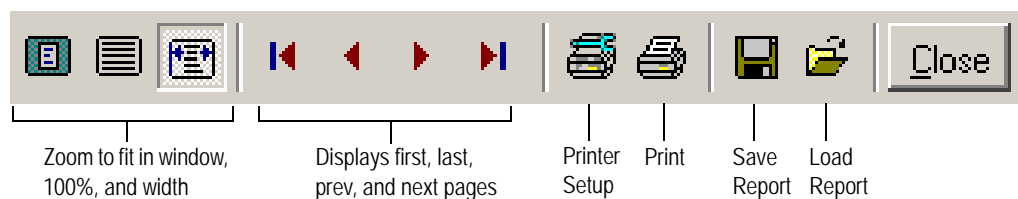
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

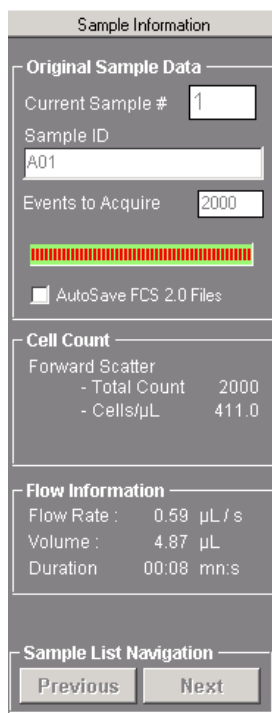
Move the cursor over the icon to display the description.



guava Nexin Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information



The screenshot shows the 'Sample Information' control panel. It has a title bar 'Sample Information'. Below it are four sections: 'Original Sample Data' with fields for 'Current Sample #' (1), 'Sample ID' (A01), 'Events to Acquire' (2000), and a progress bar; 'Cell Count' with 'Forward Scatter' data; 'Flow Information' with 'Flow Rate', 'Volume', and 'Duration' data; and 'Sample List Navigation' with 'Previous' and 'Next' buttons.

Original Sample Data	
Current Sample #	1
Sample ID	A01
Events to Acquire	2000
Progress Bar: [Red bar showing progress]	
<input type="checkbox"/> AutoSave FCS 2.0 Files	

Cell Count	
Forward Scatter	
- Total Count	2000
- Cells/ μ L	411.0

Flow Information	
Flow Rate :	0.59 μ L / s
Volume :	4.87 μ L
Duration	00:08 mn:s

Sample List Navigation	
Previous	Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 2000.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells and cells/ μ L that have exceeded the FSC threshold.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

The screenshot shows the 'Unit Control' software interface with the following sections:

- Detection:** Includes a 'PM Power' indicator (yellow circle), 'Setting' (444 V), 'PM 1' (444 V), 'PM 2' (432 V), and 'FSC Gain: x 16'.
- Pump Status:** Includes 'End of Stroke' (red circle), 'Waste' (red circle), 'Pump Reset' (red circle), 'Position' (0.0 Steps), and 'Actual Speed' (0.0 Steps/s).
- Pump Action:** Includes 'Pump on' (red circle), 'Waste' (yellow circle), 'Pump Reset' (yellow circle), 'Purge' (yellow circle), 'Sample' (green circle), and 'Speed' (5 Half Steps/s).
- Threshold parameters:** Includes 'Offset' and 'Threshold' for 'FSC', 'PM 1', and 'PM 2'. For FSC, Offset is 0.65 V and Threshold is 199 mV. For PM 1 and PM 2, both Offset and Threshold are 0.46 V and 0 mV respectively.

Detection

Displays the laser status and the PM1 and PM2 voltage settings and the FSC gain.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold setting for FSC and the PM1 and PM2 offset settings.

■ **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field. Do not change the voltages from this panel. Use the sliders to adjust the PM1 and PM2 voltages.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava Nexin Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava Nexin spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava Nexin FCS file to another directory or give it a new name.
Nexin Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in PM1 vs PM2 dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM1 dot plot but not in PM1 vs PM2 dot plot.	FSC vs PM1 gate excludes events.	Ensure FSC vs PM1 gate is set to include population of interest.
Events appear off scale in dot plots.	PM1 and /or PM2 voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM1 and/or PM2 voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample.

Problem	Possible Cause	Solutions
Poor resolution between PE ⁺ and PE ⁻ populations.	<ol style="list-style-type: none"> 1. PM1 voltage too low to detect PE signal. 2. Incomplete staining with Annexin V-PE. 3. Incomplete induction of apoptosis in test suspension. 4. Uninduced population reacting with Annexin V because cells have been stressed. 5. Apoptotic bodies included in analysis. 6. Annexin V expired, or not stored properly. 7. Background noise too high. 8. Fluorescence background too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase PM1 signal. 2. Refer to package insert for proper staining instructions. 3. Check induction control. 4. Stabilize unstained cells by resuspending in 10–20% FBS for 30 min and restain. 5. Adjust FSC threshold or set gate to remove debris. 6. Refer to package insert for proper storage instructions. Do not use expired reagent. Do not expose reagent to light. 7. Adjust settings to increase FSC threshold to remove debris. 8. Washing cells may remove residual reagent.
Poor resolution between 7-AAD ⁺ and PE ⁺ populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with 7-AAD. 3. Too much Annexin V-PE in staining tube. 4. Fluorescence background too high. 5. PM1 signal too high causing PE to bleed into PM2. 6. PM2 voltage too low to optimally detect 7-AAD signal. 7. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check 7-AAD expiration date. 3. Washing cells may remove residual reagent. 4. Washing cells may remove residual reagent. 5. Adjust settings to reduce PM1 voltage. 6. Adjust settings to increase PM2 voltage. 7. Adjust settings to increase FSC threshold to remove debris.

CHAPTER 9

guava Caspase Assay

Introduction

Apoptosis, or programmed cell death, is an important regulatory pathway of cell growth and proliferation. Caspase enzymes play a central role in the apoptotic cell death process. Caspases (cysteiny-directed aspartate-specific proteases) form a family of enzymes that initiate the apoptotic cascade, carry out cellular breakdown, and process cytokines. The activation of caspases usually commits a cell to death by the apoptotic pathway.

The Guava Caspase Kits use one or two fluorochrome-conjugated inhibitors of caspases consisting of a fluorophore (sulforhodamine [SR] or carboxyfluorescein [FAM]), a peptide specific for the active site of a particular caspase or many caspases, and a reactive functional group (fluoromethylketone or FMK). These inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the caspase inhibitors bind specifically through the peptide moiety to caspases that have been activated in apoptosis, and the FMK moiety covalently links the inhibitor to the caspase. The resulting signal is proportional to the number of active caspase enzymes that are present in the cell. Cells with significant Caspase Reagent staining are in the mid- or late-apoptotic stages or are dead.

7-AAD, a viability stain, is also included in the assay and used as an indicator of membrane structural integrity. 7-AAD does not bind to live, healthy cells or early- to mid-apoptotic cells, but permeates later stage apoptotic cells and dead cells.

To run the assay, follow the instructions in the appropriate *Guava Caspase Kit* package insert to stain the cells. Acquire samples on the guava easyCyte HT System using guavaSoft Software. Use the gating dot plot to display scatter and fluorescence to delineate cells from debris. For two-color Caspase Reagent Kits, data are displayed in an SR Peptide [YLV] (or FAM Peptide [GRN]) vs 7-AAD (RED) dot plot with quadrant markers that you can adjust for immediate on-screen results. For three-color Caspase Reagent Kits, you can configure two dot plots to display the fluorescence from the two Caspase Reagents and 7-AAD in any combination you choose.

Statistics for each quadrant include the cell count, cells/mL, percentage of cells in the gate, and the mean fluorescence intensity (MFI). Additionally, statistics for Caspase Reagent-positive and 7-AAD-positive cells are also provided.

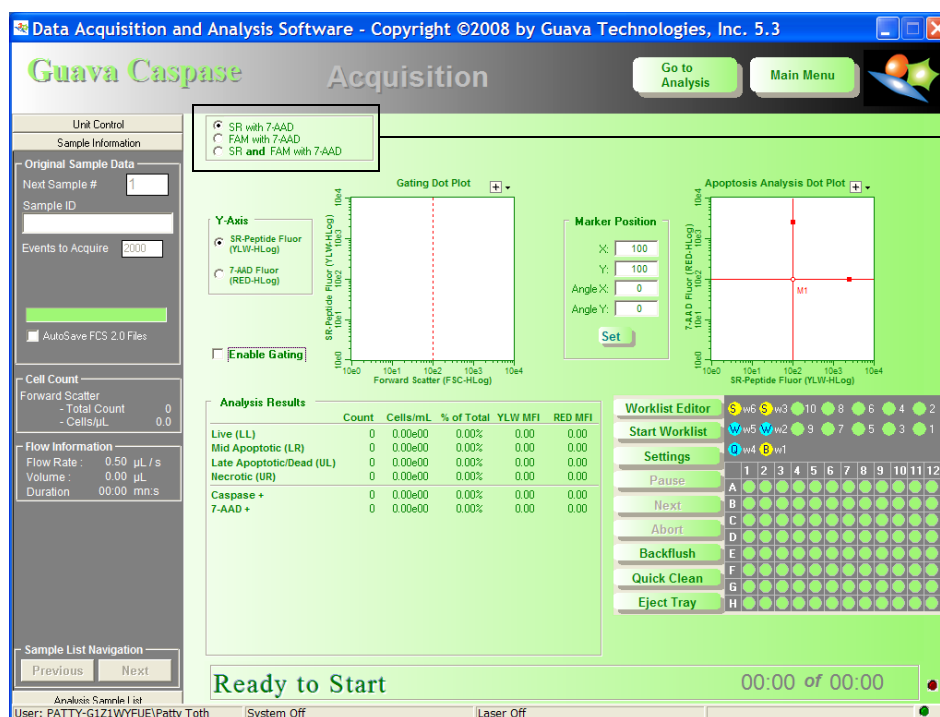
The guava Caspase data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you selected to save FCS 2.0 files. In addition to the saved data file, all results, instrument

settings, and the acquisition summary information are exported to a spreadsheet file.

Running the guava Caspase Assay

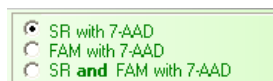
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “Caspase Software Module Control Panels” on page 9-19.

- 1 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop.
- 2 Click **Caspase** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.



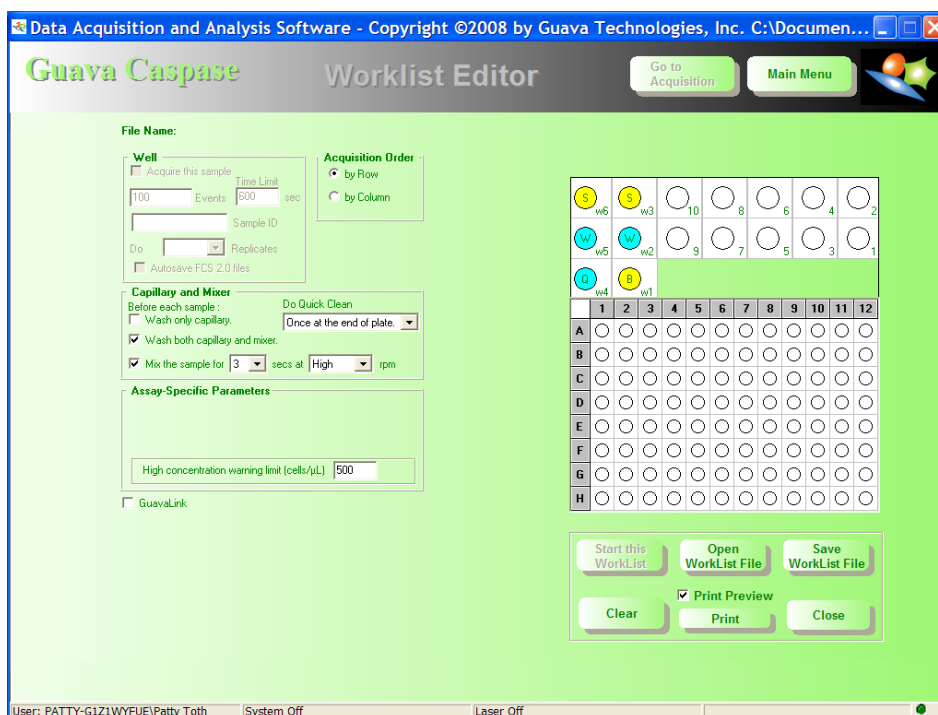
Start by selecting the appropriate reagent combination. See step 4 below.

- 3 Prepare samples for analysis in a microplate or 0.5-mL tubes. Refer to the appropriate *Guava Caspase Kit* package insert for information.
- 4 Select the appropriate reagent combination:
 - SR with 7-AAD
 - FAM with 7-AAD
 - SR and FAM with 7-AAD



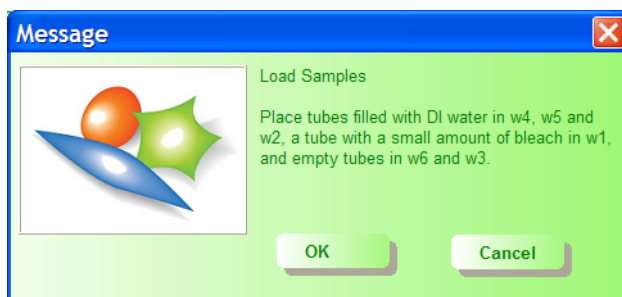
5 Click **Worklist Editor** to open the Worklist Editor application to define the worklist parameters.

- **NOTE:** If you already created a worklist, you can click **Start Worklist**. The tray ejects and dialog boxes appear prompting you to select the worklist file, save the data set, and select the sample to adjust instrument settings.
- **NOTE:** The default number of events to acquire for the two-color assay (SR with 7-AAD or FAM with 7-AAD) is 2000; while the default number of events for the three-color assay (SR **and** FAM with 7-AAD) is 3000. If you are using a previously defined worklist, ensure that the correct number of events is entered for the appropriate reagent combination you will be running.

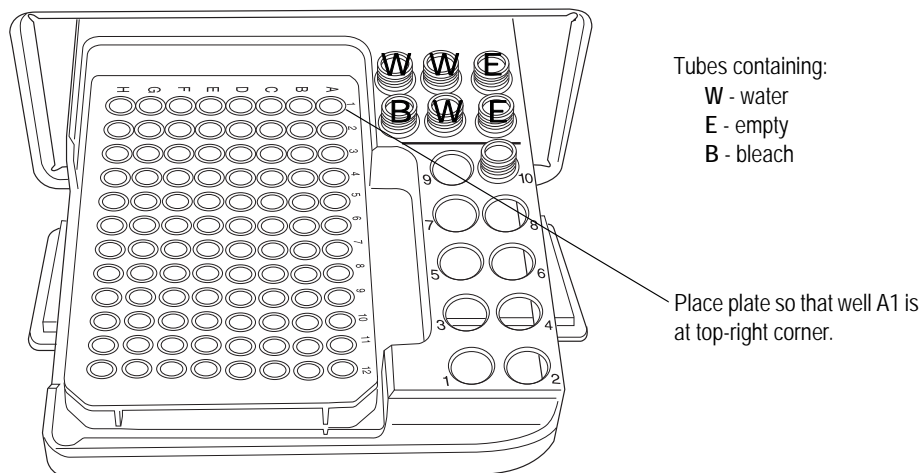


6 Define the worklist parameters for the run and click **Start this Worklist**. See “Creating a Worklist” on page 1-29 for more information on setting up a worklist. See “Worklist Editor Software” on page 1-27 for information on the buttons below the plate map. When you start a worklist, the sample tray ejects. A dialog box appears prompting you to load samples.

◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.



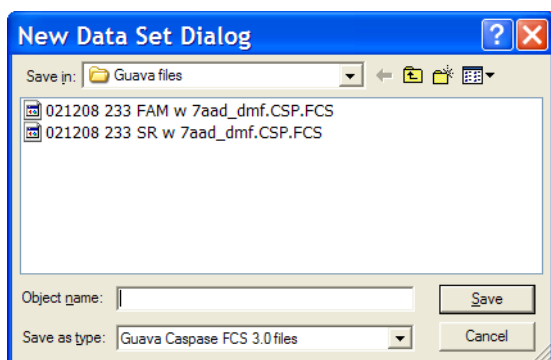
- 7 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
- Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 8 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.

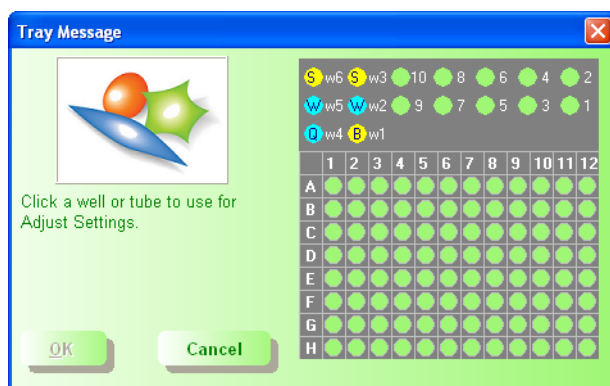
- To adjust instrument settings, click **Adjust Settings**.
- To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



- 11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends using a stained negative (non-apoptotic/uninduced) control sample for two-color assays (SR + 7-AAD or FAM + 7-AAD). For three-color assays, use a stained negative control sample stained with all three reagents (SR, FAM, and 7-AAD). Click to select the well/tube used to adjust settings, then click **OK**.

■ **NOTE:** If you are performing a three-color assay, you will need to perform compensation as well. Ensure **SR and FAM with 7-AAD** is selected to display three plots along with the compensation sliders at the adjust setting screen. Compensation

is not necessary for the two-color assay. However, if you still wish to perform compensation for your two-color assay, select SR **and** FAM with 7-AAD.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

- 12 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is between 10 and 500 cells/ μ L.

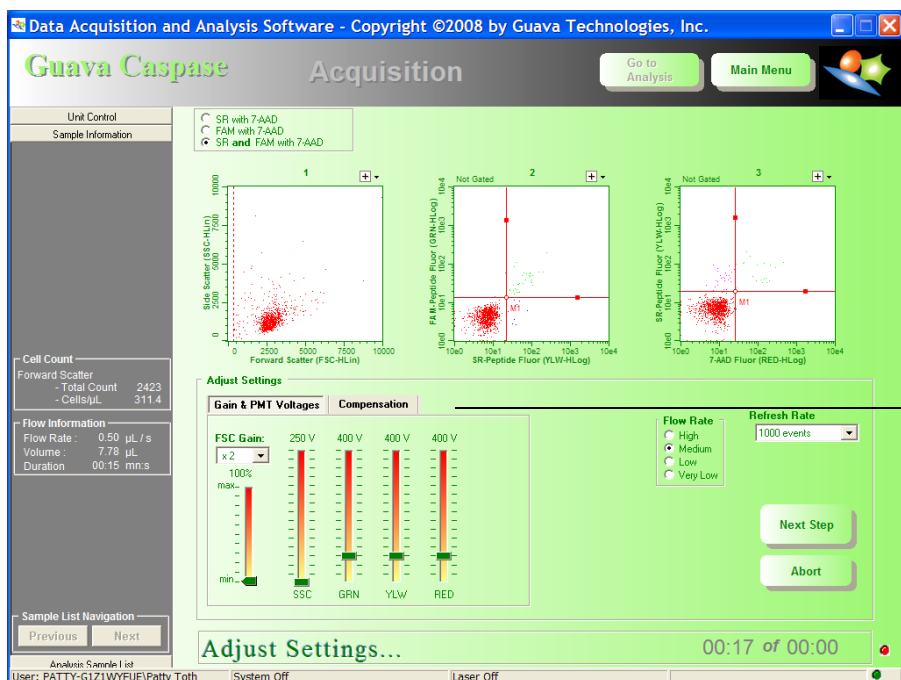
■ **NOTE:** If the value is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with 7-AAD Reagent Working Solution to lower the concentration to <500 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

- 13 If you need to fine tune the settings, you can make the following adjustments once events start to appear on the screen:
 - Set the **Refresh Rate** to the number of events you want to display.
 - Set the **Flow Rate** to High (1.2 μ L/s), Medium (0.59 μ L/s), Low (0.24 μ L/s), or Very Low (0.12 μ L/s).
 - Select the y-axis parameter for the Gating Dot Plot (SR-Peptide [YLW], FAM-Peptide [GRN], or 7-AAD [RED]). For the y-axis parameter of the first plot for three-color assays, you can also select SSC (Lin or Log), as well as select FSC Lin or FSC Log for the x-axis parameter.
 - Use the **FSC Gain** setting to reduce or amplify the FSC signal so that the cells are visible and on scale. Use the drop-down menu to change the FSC signal by a factor of 2. Use the FSC slider to change the FSC signal by steps of 1% between two coarse gain levels. If the slider is set to 100% (min), the gain is equal to the value appearing in the drop-down box. If the slider is set to 200% (max), the gain is 2 times the value appearing in the drop-down box.
 - To adjust the FSC threshold, click and drag the vertical marker up or down the FSC axis of the left-most dot plot until the desired amount of debris is eliminated below the marker.
- **NOTE:** When setting the threshold, allow for a possible shift in the apoptotic cell population. Any debris that gets acquired can be excluded with the optional dot plot gate during analysis.
- Adjust the voltages (using the RED and YLW and/or GRN sliders or the arrow keys on the keyboard) so that the live cell population is positioned in the lower-left corner of the SR-Peptide (or FAM-Peptide) vs 7-AAD dot plot for two-color assays;

or, dot plots 2 and 3 for three-color assays. Adjust the voltages by starting from a lower voltage setting and gradually increasing the voltage.



SR with 7-AAD stained sample.



Perform compensation for three-color assays.

SR and FAM with 7-AAD stained sample.

- Check these settings with a sample containing some live, mid-apoptotic, and dead/late-apoptotic cells.

- If you are running a three-color assay, you must perform compensation after you have adjusted the gain, threshold, and voltages. Click **Next Step**, **Settings**, **Adjust Settings**, then select the location of the following stained samples:
 - Start with an induced sample (with live, mid-, and at least 10% dead/late-apoptotic cells) stained with 7-AAD only. Select the Compensation tab and adjust the YLW—%RED slider until the 7-AAD (RED) signal is removed from the SR-peptide (YLW) parameter. (see Figures 1a and 1b) .

Figure 1a

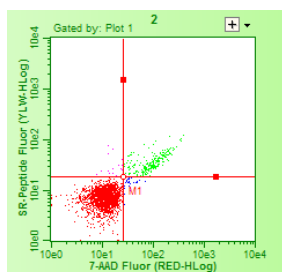
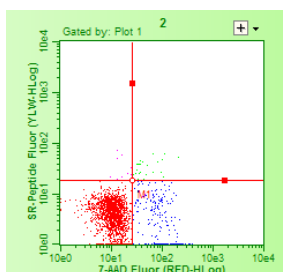


Figure 1b



YLW—%RED uncompensated (Fig 1a) and compensated (Fig 1b)

- Then, run the same sample, this time stained with SR-Peptide and 7-AAD. Adjust the GRN—%YLW and RED—%YLW sliders to remove the SR-Peptide (YLW) signal from the GRN and RED parameters (see Figures 2a and 2b).

Figure 2a

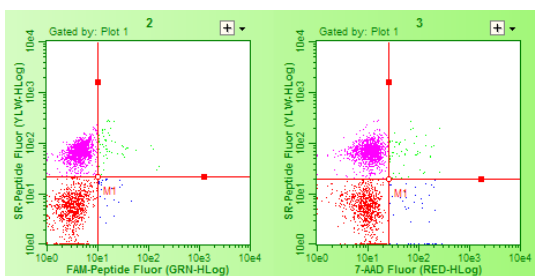
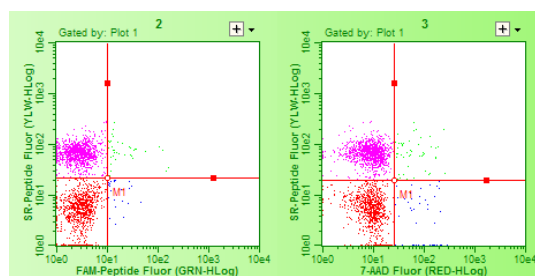


Figure 2b



GRN—%YLW and RED—%YLW uncompensated (Fig 2a) and compensated (Fig 2b)

– Then, run the same sample, this time stained with FAM-Peptide and 7-AAD. Adjust the YLW-%GRN slider to remove the FAM-Peptide (GRN) signal from the YLW parameter (see Figures 3a and 3b).

Figure 3a

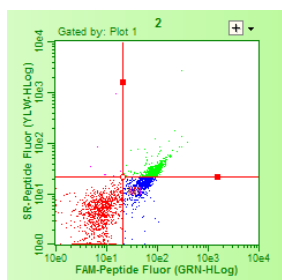
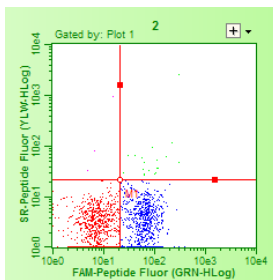


Figure 3b

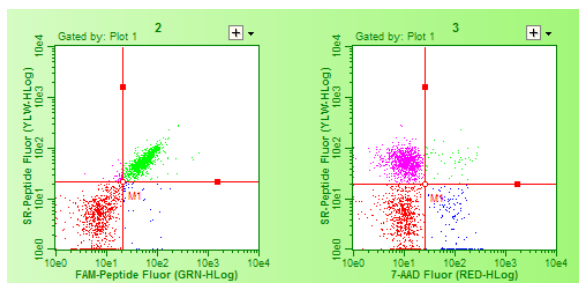


YLW-%GRN uncompensated (Fig 3a) and compensated (Fig 3b)

- **NOTE:** GRN-%RED and RED-%GRN compensation adjustments are generally not necessary in the three-color assay, as 7-AAD does not overlap into the GRN parameter, and FAM does not significantly overlap into the RED parameter. However, you may find that a RED-%GRN adjustment may be needed.

– Finally, check the compensation with a sample stained with all three reagents—SR, FAM, and 7-AAD (see Figure 4). Make any adjustments, if necessary.

Figure 4



Check compensation with three-color sample.

NOTE: The staining pattern in Figure 4 was obtained from a culture treated with an apoptotic inducer that activates both caspases equally in the majority of cells. You may get different staining patterns, depending on the stain you use and how the cells proceed through apoptosis.

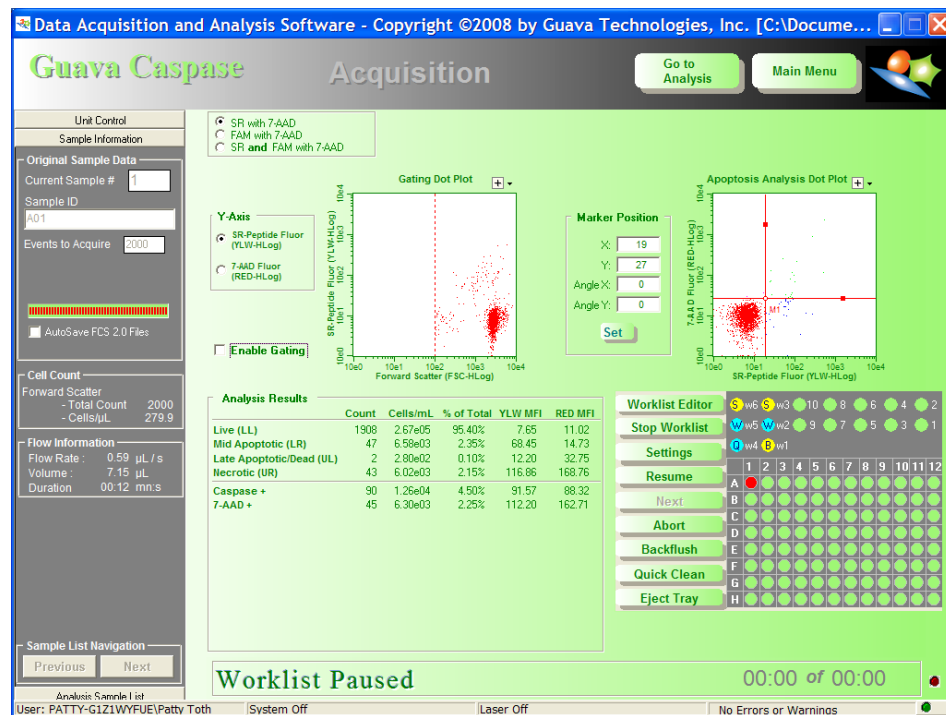
- 14 To use a gate for the two-color assay, click **Enable Gating**. For the three-color assay, select **Add Gate Type** from the plot menu. This enables the counting gate. Refer to “Dot Plot Gate” on page 9-13 for information on setting a gate. When a gate is enabled during acquisition, it acts as a counting gate—all events above the FSC threshold are acquired, and only events that are included in the gate are counted toward the number of Events to Acquire. The counting gate can be enabled during acquisition from the Adjust Settings screen only. If you wish to adjust it or delete it later during acquisition, you must return to the Adjust Settings screen. If you do not enable a counting gate during the adjust settings step of acquisition, you can enable an analysis gate later during data analysis.
- 15 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen. If necessary, you can repeat the adjust settings step to ensure that other samples such as the positive control are on scale and appropriately positioned by clicking

Settings, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

If you made changes to an existing settings file, when you click **Next Step** you will be prompted to save it as a new file or overwrite the existing file.

16 Click **Resume**.

The system acquires the first sample and displays the results. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go To Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 1.75 minutes (high flow rate), 3.5 minutes (medium flow rate), 7 minutes (low flow rate), or 10 minutes (very low flow rate).

The system automatically performs a Quick Clean at the end of the assay.

guava Caspase Analysis

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

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

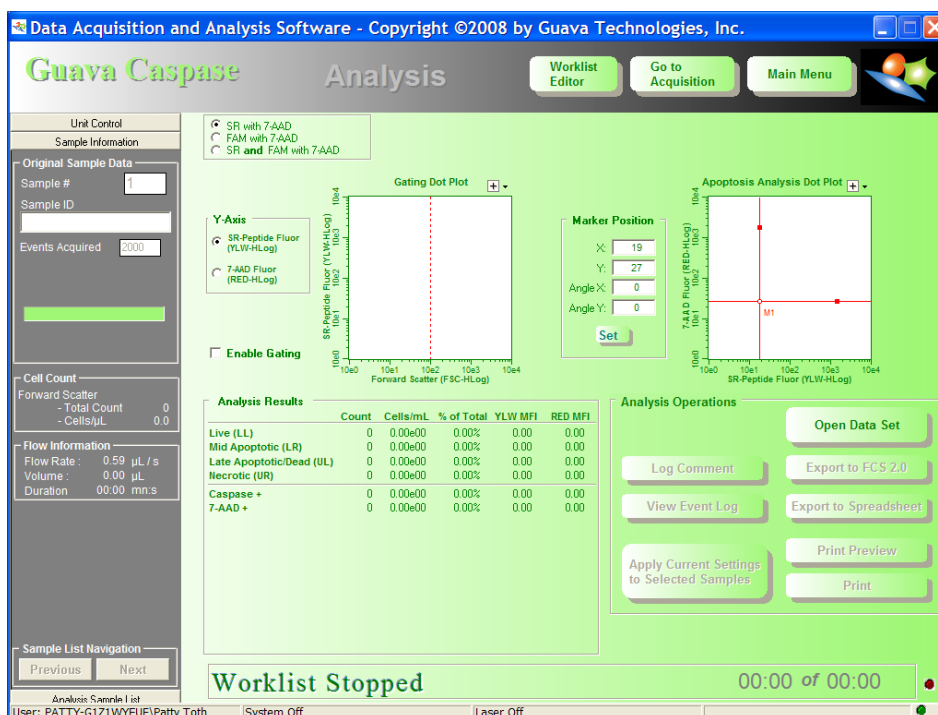
- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

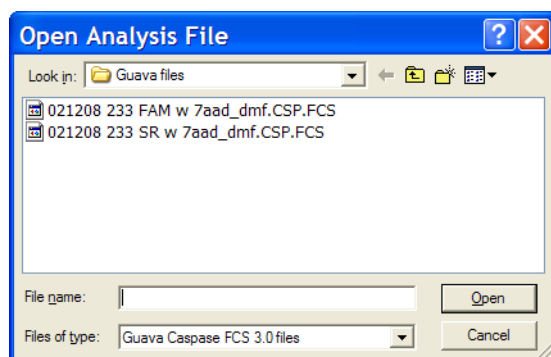
You can select the SR **and** FAM with 7-AAD reagent option at the top of the screen to analyze your two-color data files, allowing you an additional dot plot to view.

This version of the Caspase Software Module within guavaSoft Software, version 2.7 also allows you to open and analyze files acquired using the MultiCaspase Software Module.

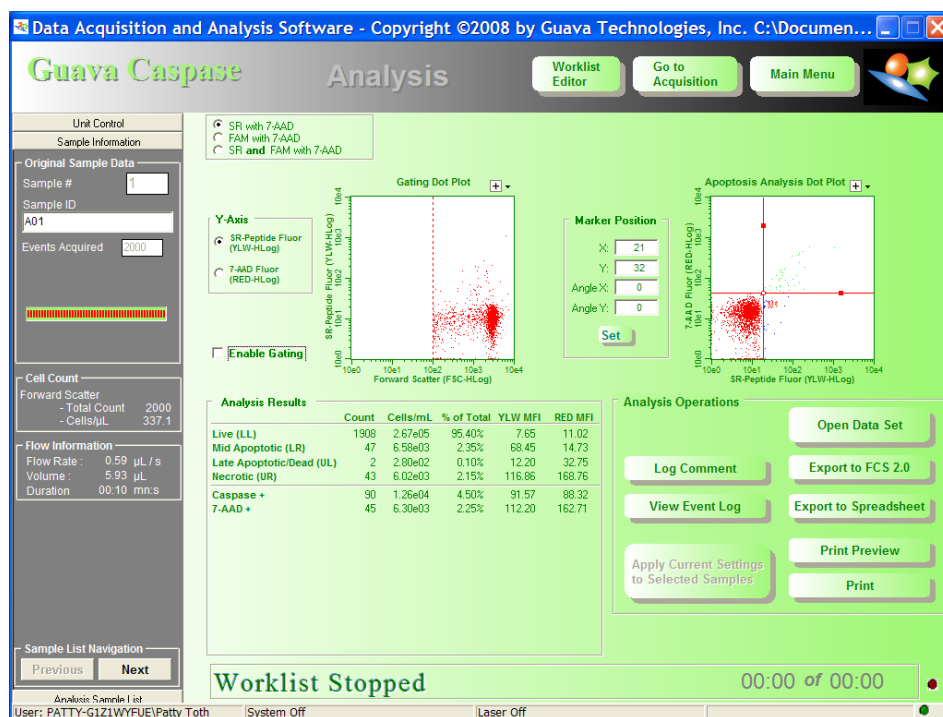
- 1 Click **Caspase** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- Click **Open Data Set**. Select an FCS file for analysis and click **Open**.

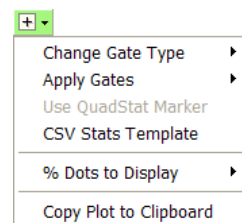


The data and results for the first sample in the data set appear. The marker setting appears as it was when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



Plot Menu

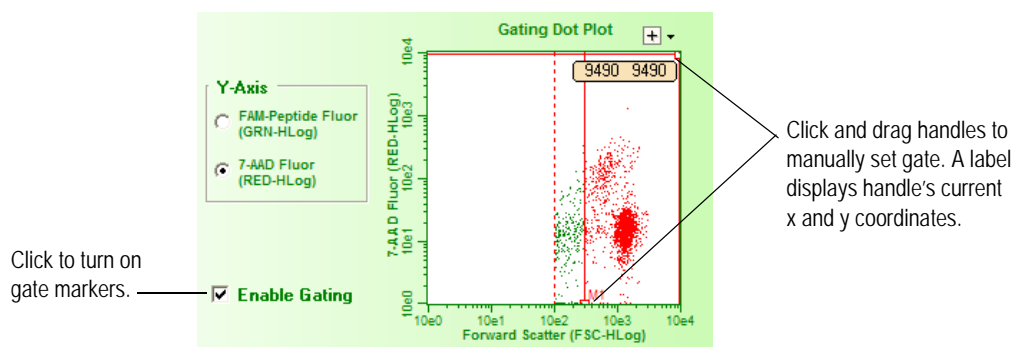
Most of the analysis features are available from the plot menu. Click the plus sign (+) in the upper-right corner above the plot or right-click the plot to open the plot menu (shown at right for three-color data). The options available within the menu vary depending on whether you are viewing two- or three-color data or the gating plot versus the analysis plot. For two-color data you can view quadrant markers only in the analysis plot. For three-color data you can set elliptical, octagonal, or rectangular gates in the analysis plots, and choose whether to display gated or ungated data. To select the statistics to export for three-color data, choose CSV Stats Template (see "Selecting Statistics for the Spreadsheet File" on page 9-17).



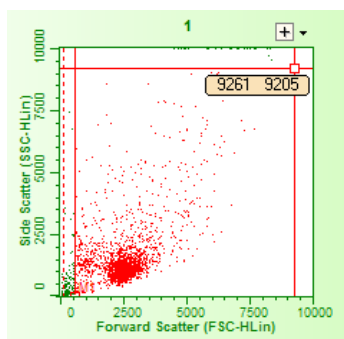
Dot Plot Gate

The dot plot gate is optional. It allows you to remove excess debris from the FSC vs SR-Peptide, FAM-Peptide, or 7-AAD plot for two color data; or FSC (Lin or Log) vs SSC (Lin or Log), SR-Peptide, FAM-Peptide, or 7-AAD plot for three-color data. You can set a rectangular, octagonal, or elliptical gate. The Analysis Results are automatically updated each time you adjust the gate for two-color data. For three-color data, you must use the plot menu to apply the gate to the analysis plots.

- 4 If you did not set a counting gate during the adjust settings step, you can set a gate now during analysis. Click Enable Gating (for two-color data) to turn on the rectangular gate (you can change the gate type using the plot menu), or select **Add Gate Type** from the plot menu and choose the gate type you wish to set.
- 5 Set the analysis gate on the viable and apoptotic cells to exclude the debris.
 - To set a *rectangular gate*, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle are included in the gate. The following examples show gates for two- and three-color assays.

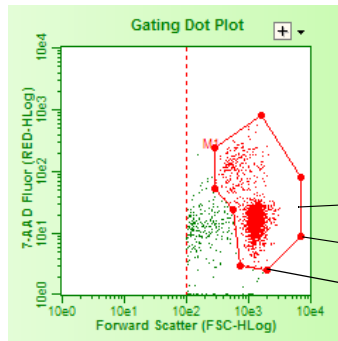


FSC vs SR-Peptide (YLV) gate for two-color data, identifying SR-Peptide-positive (mid- to late-apoptotic) and -negative (live and dead) cells.



FSC vs SSC gate for three-color data, identifying 7-AAD-positive (dead/late-apoptotic cells) from live and mid-apoptotic cells.

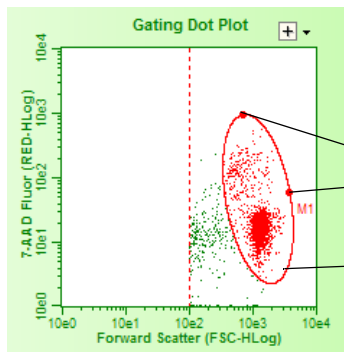
- To set an *octagonal gate*, click on a line of the octagon to drag it to a new location. Click on a handle to change the size of or rotate the octagon. To move one of the handles, press and hold the shift key while clicking and dragging a handle.



- Click and drag a line to move octagon.
- Click and drag handle to change size of or rotate octagon.
- Hold down Shift key while clicking and dragging to move one handle.

Octagon gate.

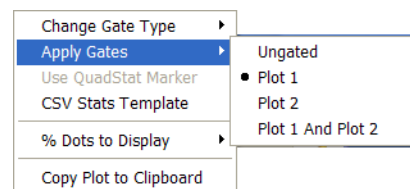
- To set an *elliptical gate*, click anywhere on the edge of the ellipse, except on a handle, and drag it to a new location. Click on a handle to change the size and angle of the ellipse.



- Click and drag handles to change size and angle of ellipse.
- Click and drag edge to move ellipse.

Elliptical gate.

- For three-color data, you must apply the gate(s) to the other plot(s). Select **Apply Gates** from the plot menu (see figure at right), then select the plot containing the gate you wish to apply. You can set a gate in plot 1 and another gate in either plot 2 or 3, then apply the gate(s) to one or both of the other plots.

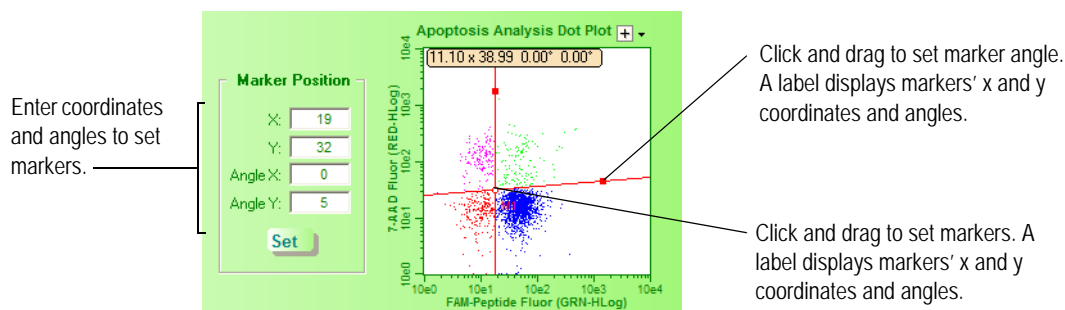


- NOTE: For two-color data, the gated data automatically appears in the SR-Peptide (or FAM-Peptide) vs 7-AAD analysis dot plot. It is not necessary to apply the gate to the plot.

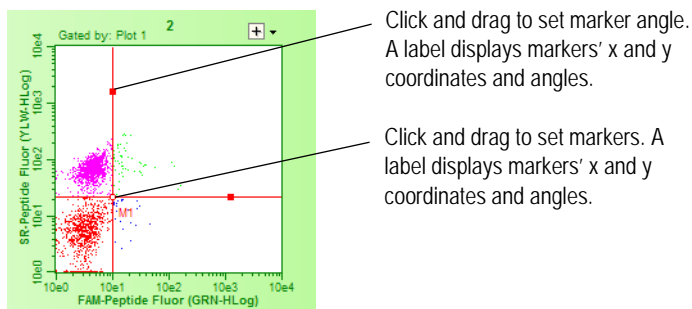
Quadrant Markers

The quadrant markers on the SR-Peptide (YLW) [or FAM-Peptide (GRN)] vs 7-AAD (RED) dot plot provide statistics on the selected populations. If you are analyzing three-color data, you can also view FAM-Peptide vs SR-Peptide. The Analysis Results are automatically updated each time you move the markers. For two-color data, only quadrant markers are available for the analysis plot. For three-color data, you may use quadrant markers, as well as elliptical, octagonal, or rectangular gates in either analysis plot (2 or 3), then you can apply the gate to the other analysis plot.

- 7 To set the quadrant markers, position the cursor over the handle at the intersection, then click and drag to the desired location.
If you are analyzing two-color data and viewing two plots, you may also set the markers by entering the coordinates in the Marker Position fields and clicking **Set**.
- 8 If necessary, you can adjust the angle of the markers $\pm 44^\circ$ from their original location. Click and drag the handle (solid square) towards the end of the marker and tilt the marker to the desired angle.
For two-color data, you may also angle the markers by entering the degrees in the Marker Position Angle fields and clicking **Set**.



FAM Peptide vs 7-AAD quadrant markers differentiating early/mid- and late-apoptotic cells from non-apoptotic cells.



FAM-Peptide vs SR-Peptide, three-color data with quadrant markers differentiating mid- and late-apoptotic cells from non-apoptotic cells.

- 9 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.

- 10 You can apply gate and/or markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 11 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
Results for two-color data are automatically exported to a CSV file that is given the same name as the FCS file. See “Selecting Statistics for the Spreadsheet File” on page 9-17 for more information on exporting three-color data to a spreadsheet file.
■ NOTE: Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 12 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

Caspase Results

Caspase results appear immediately after the acquisition of the sample is complete. Statistics for each quadrant are displayed. Results include the count, cells/mL, % Total (all data in the dot plot) or % Gated (if a gate was enabled), and the mean fluorescence intensities for YLW and RED. Additionally, the SR-Peptide (or FAM-Peptide)-positive (upper-right and lower-right quadrants) and 7-AAD-positive (upper-left and upper-right quadrants) are included.

For three-color data, if gates, rather than quadrant markers, were set in plot 2 and/or 3, statistics for events within the gate(s) are displayed.

Analysis Results					
	Count	Cells/mL	% of Total	YLW MFI	RED MFI
Live (LL)	1908	2.67e05	95.40%	7.65	11.02
Mid Apoptotic (LR)	47	6.58e03	2.35%	68.45	14.73
Late Apoptotic/Dead (UL)	2	2.80e02	0.10%	12.20	32.75
Necrotic (UR)	43	6.02e03	2.15%	116.86	168.76
Caspase +	90	1.26e04	4.50%	91.57	88.32
7-AAD +	45	6.30e03	2.25%	112.20	162.71

Statistics for each quadrant, as well as SR-Peptide-positive (UR and LR quadrants) and 7-AAD-positive (UL and UR quadrants) are displayed.

Results for each quadrant are displayed as a count, cells/mL, and percentage of the total or gated data in the dot plot. Additionally, the mean fluorescence intensities for YLW and RED are also displayed. If a gate was set, the values represent data within the gate.

If a gate was set, the number of cells within the gate, as well as the percentage of cells inside and outside the gate are displayed.

The summary of each quadrant for SR-Peptide (or FAM-Peptide) vs 7-AAD is outlined below.

Quadrant	Staining	Population	Color
lower left	SR-Peptide (or FAM Peptide)–dim/negative, 7-AAD–negative	live cells	red
lower right	SR-Peptide (or FAM Peptide)–positive, 7-AAD–negative	mid-apoptotic cells	blue
upper right	SR-Peptide (or FAM Peptide)–positive, 7-AAD–positive	late apoptotic and dead cells	green
upper left	SR-Peptide (or FAM Peptide)–negative, 7-AAD–positive	necrotic cells	pink

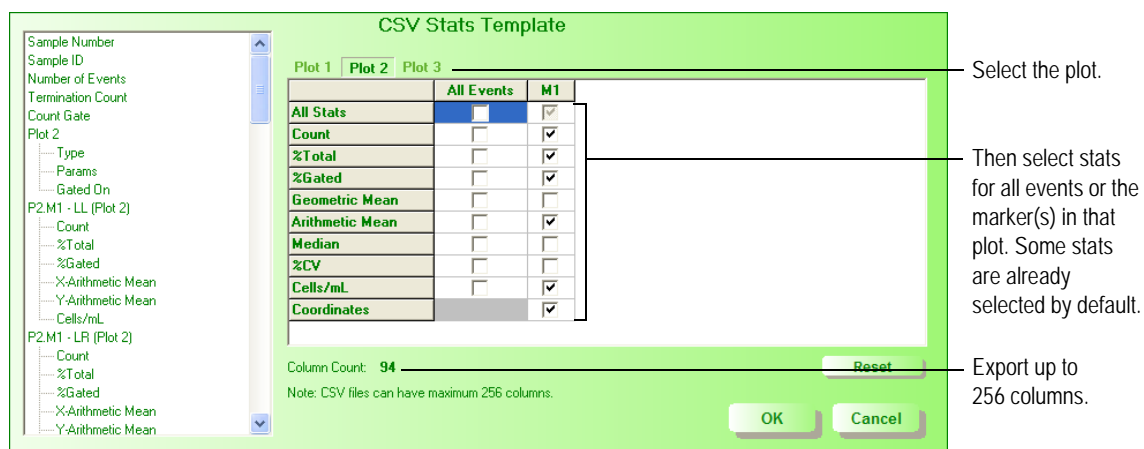
The summary of each quadrant for SR-Peptide vs FAM-Peptide is outlined below.

Quadrant	Staining	Population	Color
lower left	SR-Peptide–negative, FAM-Peptide–negative	live cells	red
lower right	SR-Peptide–positive, FAM-Peptide–negative	apoptotic and/or dead cells	blue
upper right	SR-Peptide–positive, FAM Peptide–positive	apoptotic and/or dead cells	green
upper left	SR-Peptide–negative, FAM Peptide–positive	apoptotic and/or dead cells	pink

Selecting Statistics for the Spreadsheet File

The statistics for two-color data (SR Peptide with 7-AAD or FAM with 7-AAD) are automatically exported to a spreadsheet file. Some statistics for three-color data (SR **and** FAM with 7-AAD) are automatically exported; however you may select additional statistics to export as well.

- 1 Open the plot menu from any plot and select **CSV Stats Template**. The CSV Stats Template dialog box appears allowing you to modify or select additional statistics to export for three-color data files.



The left side of the screen displays a list of instrument settings and sample information that is always exported to the spreadsheet file. The right side of the screen allows you to select the specific statistics that will be exported for each plot. Some statistics are selected by default. You can select additional statistics and/or deselect the default statistics.

When you click **OK**, the statistic you selected will be remembered the next time you open the CSV Stats Template.

- Click to select the statistic for export. Stats are shown for each marker in the plot or total events in the plot if no markers are set.
- Click **Reset** to ignore any changes made and revert to the stats that were selected when you opened the CSV Stats Template.

- **NOTE:** If you exceed 256 columns, a warning message appears requesting that you review and remove stats until there are no more than 256 columns of stats for export.

Exporting Caspase Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel. All statistics, as well as sample information and instrument settings, for two-color data will be exported. For three-color data, some statistics are exported with the sample information and instrument settings. To select additional statistics or deselect the default statistics, refer to “Selecting Statistics for the Spreadsheet File” on page 9-17.

- **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting Caspase Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.
The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Batch Printing

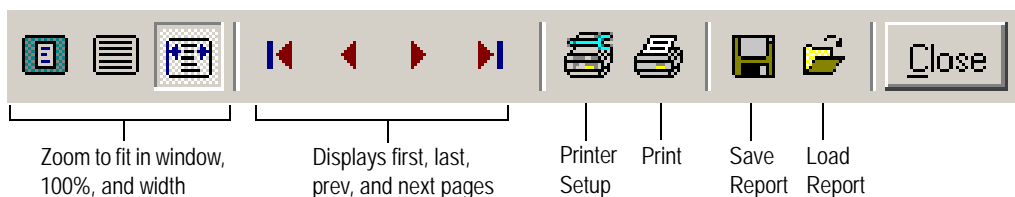
If you wish to print data from multiple samples, highlight the samples in the Sample Analysis List control panel, then click Print.

Note that when printing in batch mode:

- Print preview is not available.
- The number of pages to print shown in the print dialog box refers to the number of pages each sample will print on (typically 2 per sample) and not to the total number of pages that will be printed.
- You can select to print only the 1st page or 2nd page for each sample.

Print Preview Icons

Move the cursor over the icon to display the description.



Caspase Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

The Sample Information panel displays the following data:

- Original Sample Data:**
 - Sample #: 3
 - Sample ID: T03
 - Events Acquired: 3462
 - A progress bar shows the acquisition progress.
- Cell Count:**
 - Forward Scatter:
 - Total Count: 3462
 - Cells/ μ L: 411.0
- Flow Information:**
 - Flow Rate: 0.59 μ L / s
 - Volume: 8.42 μ L
 - Duration: 00:14 mn:s
- Sample List Navigation:**
 - Previous
 - Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 2000 for two-color assays and 3000 for three-color assays.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells and cells/ μ L that have exceeded the FSC threshold.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

The Unit Control panel displays the following data:

- Detection:**
 - Laser: ☒
 - FSC Gain: x 8 (100%)
 - SSC: 360 V
 - GRN: 440 V
 - YLW: 466 V
 - RED: 585 V
- Pump Status:**
 - End of Stroke: ☒
 - Waste: ☒
 - Pump Reset: ☒
 - Position: 0 Steps
 - Actual Speed: 0 Steps/s
- Pump Action:**
 - Pump on: ☒
 - Waste: ☒
 - Pump Reset: ☒
 - Purge: ☒
 - Sample: ☒
 - Speed: 0 Steps/s
- Threshold parameters:**
 - Offset: 0.12 V
 - Threshold: 50 units
 - Baseline Values:**
 - GRN: 0
 - SSC: 0
 - RED: 0
 - FSC: 0
 - YLW: 0
- Sample List Navigation:**
 - Previous
 - Next

Detection

Displays laser status, FSC gain, and the SSC (if installed), GRN, YLW, and RED voltage settings. Only the parameters for the selected reagent combinations appear.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold settings for FSC and the baseline values for GRN, RED, YLW, SSC, and FSC.

- **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field. Do not change the voltages from this panel. Use the sliders to adjust the voltages.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava Caspase Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save Caspase spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save Caspase FCS file to another directory or give it a new name.
Caspase Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.

Problem	Possible Cause	Solutions
Unexpected events appearing in a two-color dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in gating dot plot but not in analysis dot plot.	Gate excludes events.	Ensure gate is set to include population of interest.
Poor resolution between Caspase Reagent–positive and Caspase Reagent–negative populations.	<ol style="list-style-type: none"> 1. Voltage too low to detect fluorescence signal. 2. Incomplete staining with Caspase Reagent. 3. Incomplete induction of apoptosis in test suspension. 4. Uninduced population reacting with Caspase Reagent because cells have been stressed. 5. Apoptotic bodies included in analysis. 6. Caspase Reagent expired or not stored properly. 7. Background noise too high. 8. Fluorescence background too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescence signal. 2. Refer to package insert for proper staining instructions. 3. Check induction control. 4. Stabilize unstained cells by resuspending in 10–20% FBS for 30 min and restain. 5. Adjust FSC threshold or set gate to remove debris. 6. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light. 7. Adjust settings to increase FSC threshold to remove debris. 8. Washing cells may remove residual reagent.

Problem	Possible Cause	Solutions
Poor resolution between 7-AAD ⁺ and Caspase Reagent–positive populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with 7-AAD. 3. Too much Caspase Reagent in staining tube. 4. Fluorescence background too high. 5. Fluorescence signal too high causing Caspase Reagent to overlap into other fluorescence parameter. 6. RED voltage too low to optimally detect 7-AAD signal. 7. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check 7-AAD expiration date. 3. Washing cells may remove residual reagent. 4. Washing cells may remove residual reagent. 5. Adjust settings to reduce voltage corresponding to parameter used to detect Caspase Reagent. 6. Adjust settings to increase RED voltage. 7. Adjust settings to increase FSC threshold to remove debris.
Events appear off scale in dot plots.	GRN, YLW, and /or RED voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease GRN, YLW, and/or RED voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample.

CHAPTER 10

guava TUNEL Assay

Introduction

Apoptosis, or programmed cell death, is an important regulatory pathway of cell growth and proliferation. A distinctive marker along the apoptotic pathway to cell death is the degradation of chromatin structure and nuclear DNA by activated nucleases. Chromatin is degraded into fragments of 50 to 300 kb, and subsequently into smaller 200 bp pieces of DNA. The fragmentation of DNA during apoptosis generates exposed 3'-hydroxyl ends in the nuclear DNA.

In the guava TUNEL Assay, terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of BrdU residues into the fragmenting nuclear DNA at the 3'-hydroxyl ends by nicked end labeling. TRITC-conjugated anti-BrdU antibody binds to the incorporated BrdU residues, labeling the apoptotic cells.

Two populations of cells can be distinguished:

- Non-apoptotic cells – TRITC-negative (or TUNEL-negative)
- Apoptotic cells – TRITC-positive (or TUNEL-positive)

The guava TUNEL Assay quantifies cells at mid- to late-stage apoptosis, when DNA degradation is occurring. By discrimination of the relative percentage of positively stained cells, the extent of apoptosis can be determined in the cell population of interest.

To run the assay, follow the instructions in the *Guava PCA-96 TUNEL Reagent* package insert to stain the cells. Acquire samples on the guava easyCyte HT System using guavaSoft Software. Data are displayed in a single-parameter histogram. You can set two or three markers to analyze non-apoptotic and apoptotic cells.

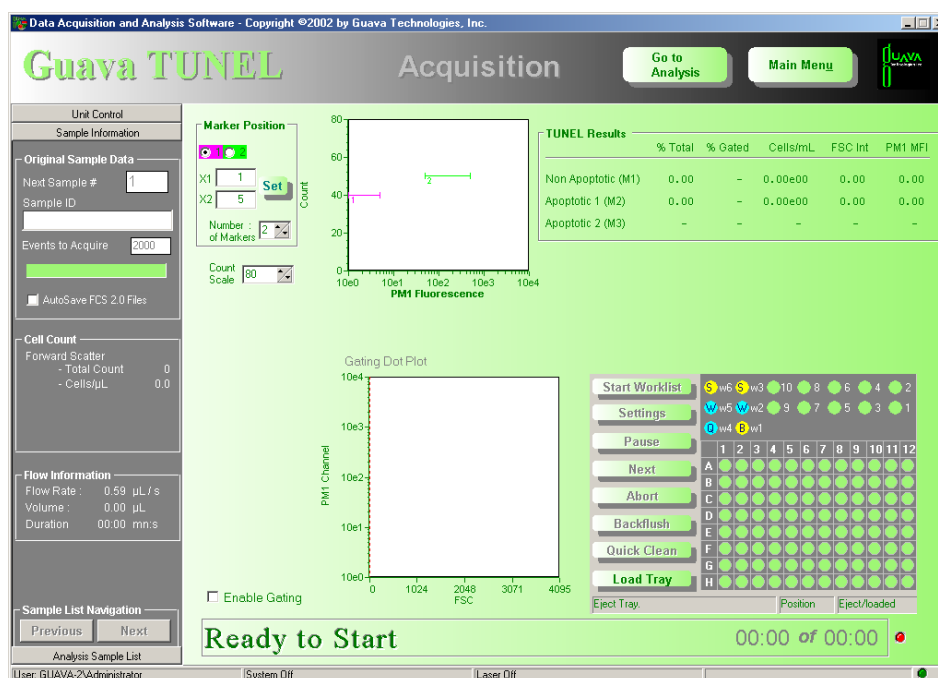
Statistics for each population within the histogram include percentage of total, percentage of gated, cells/mL, FSC intensity, and PM1 mean fluorescence intensity.

The guava TUNEL data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you selected to save FCS 2.0 files. In addition to the saved data file, all results, instrument settings, and the acquisition summary information are exported to a spreadsheet file.

Running the guava TUNEL Assay

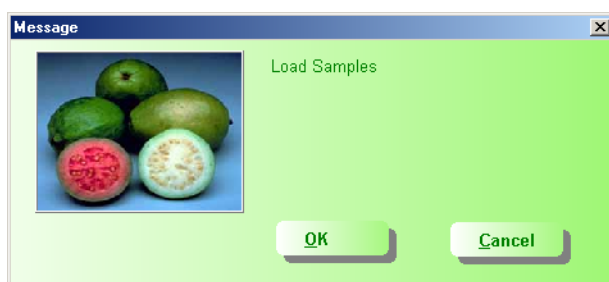
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “TUNEL Software Module Control Panels” on page 10-13.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.
- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.
- 3 Click **TUNEL** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

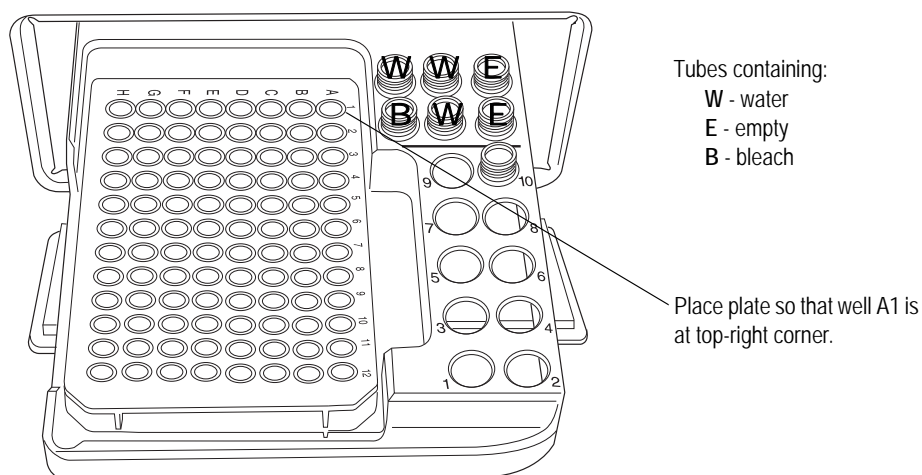


- 4 Prepare samples for analysis in a microplate or 0.5-mL tubes. Refer to the *Guava PCA-96 TUNEL Reagent* package insert for information.
- 5 Click **Start Worklist**.
The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

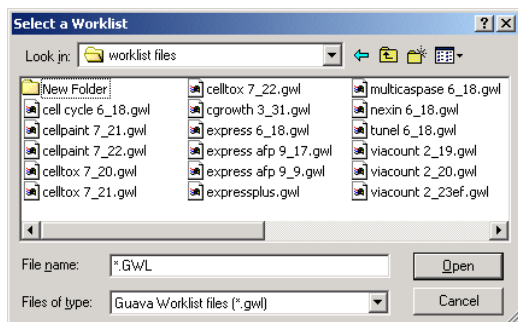


- 6 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

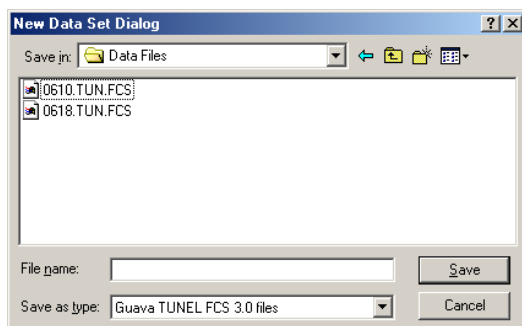
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select a worklist file and click Open.

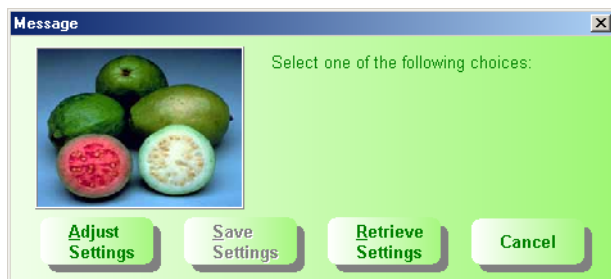
- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.

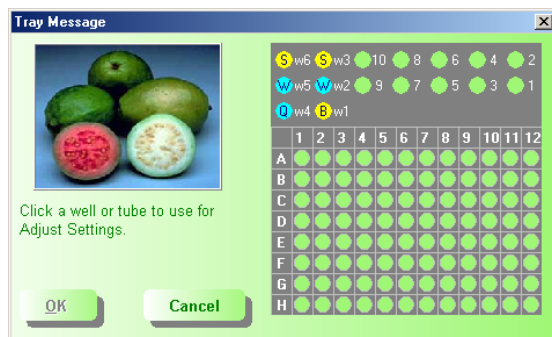


Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.
- To adjust instrument settings, click **Adjust Settings**.
 - To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



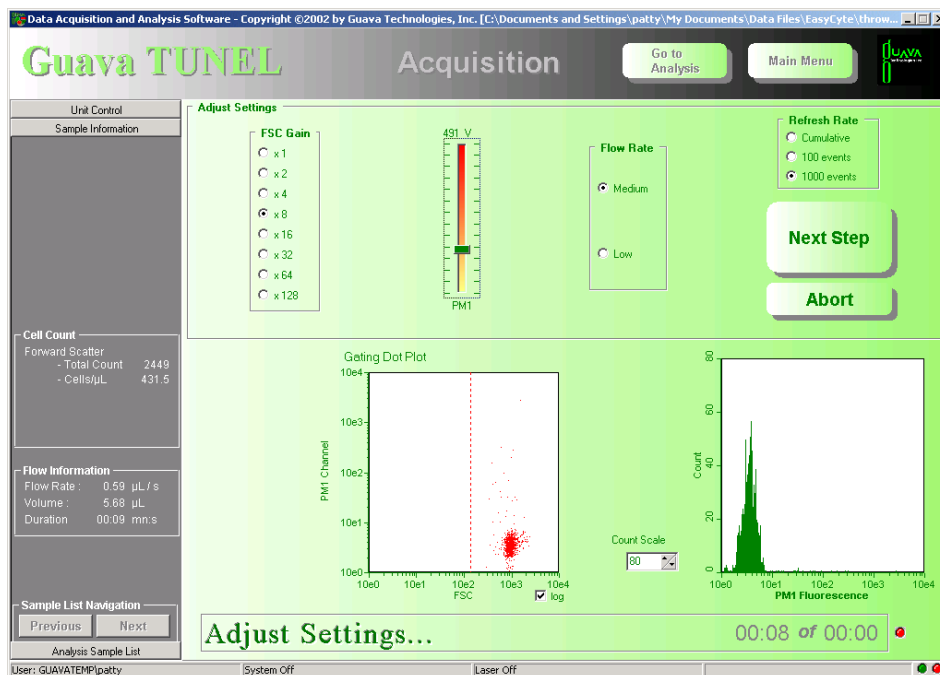
- 11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends using a stained negative (non-apoptotic or uninduced) control sample. Click to select the well/tube used to adjust settings, then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

- 12 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is below 500 cells/ μ L.
- **NOTE:** If the value is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with Rinsing Buffer to lower the concentration to <500 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.
- 13 If you need to fine tune the settings, you can make the following adjustments once events start to appear on the screen:
- Click the log check box to display a log scale.
 - Set the **Refresh Rate** to the number of events you want to display.
 - Set the **Flow Rate** to Low (0.24 μ L/s) or Medium (0.59 μ L/s).
 - Use the **FSC Gain** setting to reduce or amplify the FSC signal so that the cells are visible and on scale.
 - To adjust the FSC threshold, click and drag the vertical marker up or down the FSC axis of the FSC vs PM1 dot plot until the desired amount of debris is eliminated below the marker.
- **NOTE:** When setting the threshold, allow for a possible shift to the left in the apoptotic cell population. Any debris that gets acquired can be excluded with the optional dot plot gate during analysis.
 - Adjust the voltage (using the PM1 slider or the arrow keys on the keyboard) so that the negative population is positioned in the first decade of the PM1 histogram. Adjust the voltage by starting from a lower voltage setting and gradually increasing the voltage.

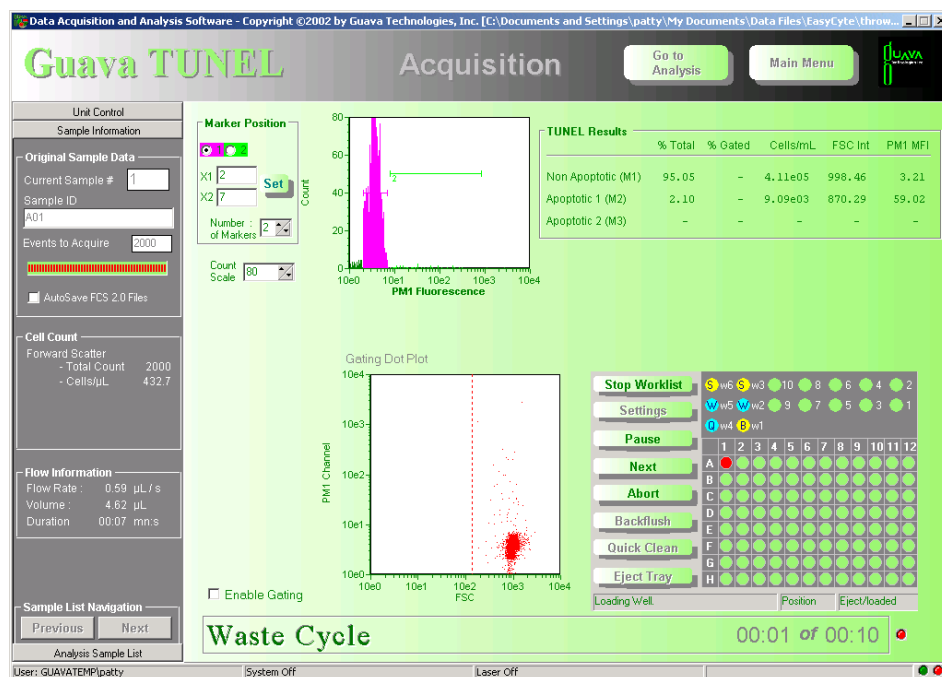
- If necessary, use the Count Scale field to the left of the histogram to adjust the y-axis scale.



- 14** When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.
- If necessary, you can repeat the adjust settings step to ensure that other samples such as the positive control are on scale and appropriately positioned by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

15 Click **Resume**.

The system acquires the first sample and displays the results. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 2 minutes.

- 16** EMD Millipore recommends setting a gate and adjusting the markers prior to acquiring the remaining samples. Click **Pause** during the acquisition of the first sample. The system will pause when the acquisition is complete. Once the system is paused, adjust the gate and/or marker(s), then click **Resume**. The remaining samples are automatically acquired.
- Refer to “Optional Dot Plot Gate” on page 10-9 and “Histogram Markers” on page 10-10 for information on setting a gate and markers.

The system automatically performs a Quick Clean at the end of the assay.

guava TUNEL Analysis

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

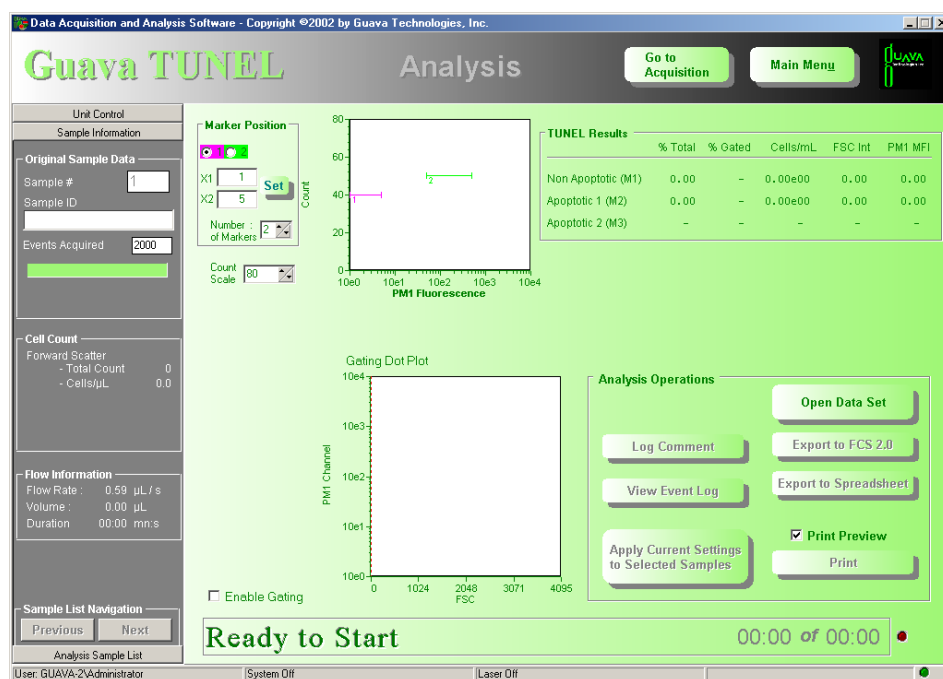
You can save changes made to the sample ID, gate, or markers within Analysis by overwriting the existing file or saving a new file.

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

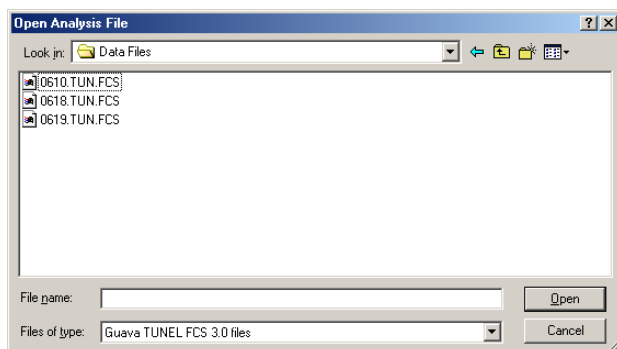
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log.

However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

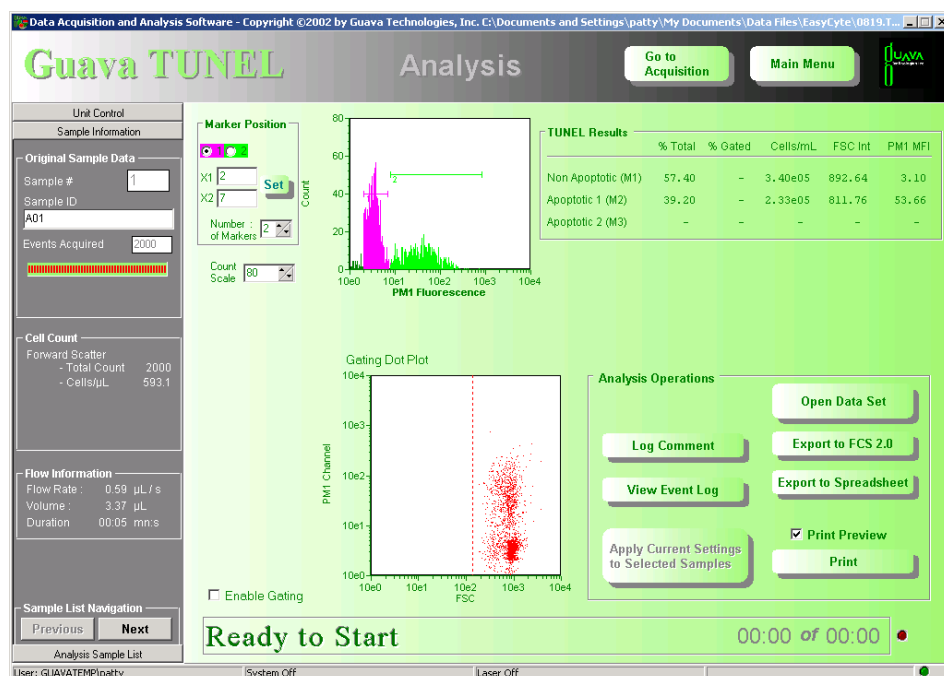
- 1 Click **TUNEL** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- 3 Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker setting appears as it was when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



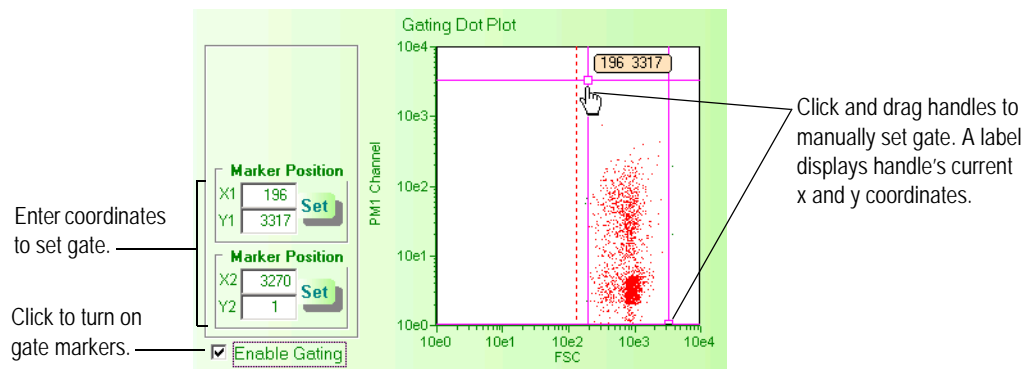
Optional Dot Plot Gate

The dot plot gate is optional. It allows you to remove excess debris or unwanted events from the FSC vs PM1 dot plot. The TUNEL Results are updated each time you adjust the gate.

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

You may also set the gate by entering the coordinates in the Marker Position fields and clicking **Set**. The gated data appears in the PM1 Fluorescence histogram.

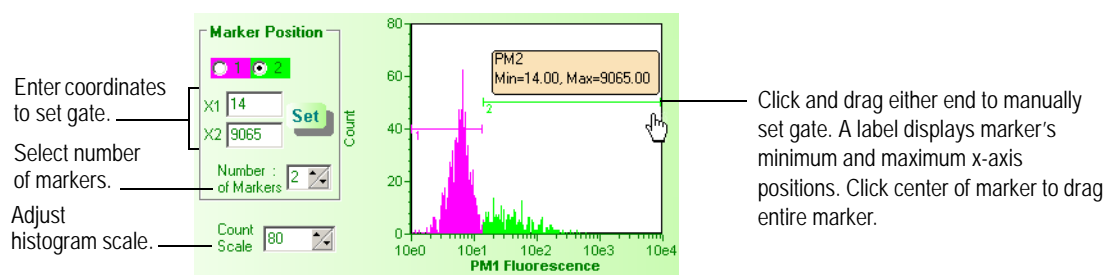
- **NOTE:** If using a negative control to set the gate, be sure to extend the gate high enough up the y-axis (as shown) so that PM1-positive events in subsequent samples are included in the gate.



Histogram Markers

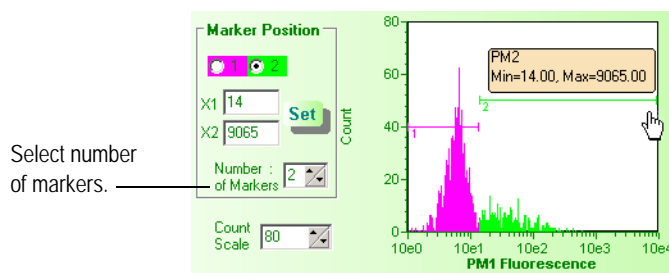
The histogram markers allow you to get statistics on any population within the PM1 parameter. You can set two or three markers to identify and generate statistics on the non-apoptotic population and up to two apoptotic populations. The TUNEL Results are automatically updated each time you move the markers.

- To set the PM1 histogram markers, click and drag either side of the marker to size it. Click the horizontal line to drag the entire marker. You cannot overlap markers 1 and 2. Events that fall within marker 1 appear in pink, events that fall within marker 2 appear in light green. You may also set the marker by entering the coordinates in the Marker Position fields and clicking **Set**.



PM1 histogram markers 1 and 2 selecting non-apoptotic (pink) and apoptotic (light green) cells, respectively.

- 7 To set a third marker, click the Number of Markers up arrow. Adjust the marker appropriately. You can extend marker 3 to any position.



PM1 histogram with marker 3 activated and selecting apoptotic cells (blue), if applicable.

- 8 Click **Next** under Sample List Navigation in the Sample information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 9 You can apply gate and/or markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 10 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
 - **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 11 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

TUNEL Results

TUNEL Results appear immediately after the acquisition of the sample is complete. Results include the statistics within each marker—percentage of total, percentage of gated, cells/mL, FSC intensity, and the PM1 mean fluorescence intensity. % Gated values appear only if a gate was set.

TUNEL Results	% Total	% Gated	Cells/mL	FSC Int	PM1 MFI
Non Apoptotic (M1)	58.85	73.98	2.37e05	979.42	5.73
Apoptotic 1 (M2)	20.60	25.90	8.29e04	977.40	62.06
Apoptotic 2 (M3)	0.05	0.06	2.01e02	1295.00	603.00

Statistics for each marker set are displayed. If a gate was set, the values represent data within the gate. Results include % Total, % Gated (results appear only if a gate is enabled), cells/mL, FSC intensity, and PM1 mean fluorescence intensity.

Exporting TUNEL Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting TUNEL Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.
The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

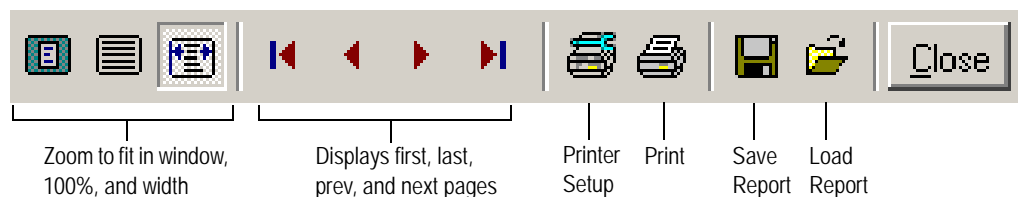
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

Move the cursor over the icon to display the description.



TUNEL Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

Sample Information

Original Sample Data

Current Sample # 1

Sample ID A01

Events to Acquire 2000

AutoSave FCS 2.0 Files

Cell Count

Forward Scatter

- Total Count 2000

- Cells/μL 432.7

Flow Information

Flow Rate : 0.59 μL / s

Volume : 4.62 μL

Duration 00:07 mn:s

Sample List Navigation

Previous Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 2000.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells and cells/μL that have exceeded the FSC threshold.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

The Unit Control panel is divided into several sections:

- Detection:** Includes indicators for Laser (red dot), PM Power (yellow dot), and Setting (yellow dot). The PM 1 voltage is displayed as 300 V. The FSC Gain is set to x 1.
- Pump Status:** Includes indicators for End of Stroke (red dot), Waste (red dot), and Pump Reset (red dot). The Position is 10 Steps and the Actual Speed is 0 Steps/s.
- Pump Action:** Includes indicators for Pump on (red dot), Waste (yellow dot), Pump Reset (yellow dot), Purge (yellow dot), and Sample (green dot). The Speed is 0 Steps/s.
- Threshold parameters:** Includes Offset and Threshold settings for FSC and PM 1. FSC Offset is 1.12 V and Threshold is 728 mV. PM 1 Offset is 0.45 V and Threshold is 0 mV.
- Sample List Navigation:** Includes Previous and Next buttons.

Detection

Displays laser status and the PM1 voltage setting and the FSC gain.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the FSC and PM1 offset and threshold settings.

■ **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field. Do not change the PM1 voltage from this panel. Use the PM1 slider to adjust the PM1 voltage.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava TUNEL Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save TUNEL spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save TUNEL FCS file to another directory or give it a new name.
TUNEL Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Poor resolution between TRITC ⁺ and TRITC ⁻ populations.	<ol style="list-style-type: none"> 1. Fluorescence background too high. 2. PM1 voltage too low to detect PM1 signal. 3. Incomplete induction of apoptosis in test suspension. 4. Reagent expired or not stored properly. 	<ol style="list-style-type: none"> 1. Washing cells may remove residual reagent. Wash with Rinsing Buffer or PBS. 2. Adjust settings to increase PM1 signal. 3. Check induction control. 4. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light.

Problem	Possible Cause	Solutions
Apoptotic population shows dim TRITC signal.	Sample was not stained completely.	<ol style="list-style-type: none"> 1. Increase the incubation time during the DNA labeling reaction. 2. Increase the amount of TdT enzyme. Refer to the <i>Guava TUNEL Kit</i> package insert. 3. Ensure reagent is not expired or was not exposed to excessive light.
Staining artifacts appear in dot plot or histogram.	Cells were not properly resuspended in the DNA Labeling Mix or Anti-BrdU Staining Mix.	<ol style="list-style-type: none"> 1. Aspirate carefully to avoid disturbing pellet. 2. Wash sides of tube when resuspending cells.

CHAPTER 11

guava MitoPotential Assay

Introduction

The guava MitoPotential Assay is a fluorescence-based assay designed to determine mitochondrial activity in apoptosis and other cellular processes. Loss of mitochondrial inner transmembrane potential is often observed to be associated with the early stage of apoptosis. Collapse of this potential is believed to coincide with the opening of the mitochondrial permeability transition pores, leading to the release of cytochrome C into the cytosol, which then triggers the downstream events in the apoptosis cascade.

The Guava MitoPotential Kit contains JC-1, a cationic dye used to evaluate mitochondrial membrane potential changes. JC-1 fluoresces either green or orange depending upon membrane potential. When the mitochondria are polarized, the dye accumulates in the mitochondria, forming J-aggregates, which fluoresce orange (~590 nm). When the mitochondria lose their membrane potential and become depolarized, the concentration of dye decreases, the J-aggregates dissociate and now JC-1 fluoresces green (~530 nm).

A viability stain, 7-AAD, is also included in the assay to monitor cell membrane permeability changes commonly observed later in apoptosis and in cell death.

To run the MitoPotential Assay, follow the instructions in the Guava MitoPotential Kit package insert to prepare your samples. Acquire the samples on the guava easyCyte HT System using guavaSoft Software. Data are displayed in three dot plots. A gating dot plot, which shows forward scatter (FSC) vs fluorescence of either JC-1 Green (PM3), 7-AAD Red (PM2), or JC-1 Orange (PM1), allows you to set a counting gate and eliminate unwanted events and debris from the analysis. Plot 2, which displays JC-1 Green (PM3) vs JC-1 Orange (PM1), allows you to set quadrant markers to distinguish polarized cells from depolarized cells. Plot 3, which displays 7-AAD Red (PM2) versus JC-1 Orange (PM1), allows you to set a viability marker to distinguish late apoptotic/dead cells from early apoptotic/live cells.

Statistics for the analysis dot plots include the cell count, cells/mL, and mean signal intensity for the depolarized cells and apoptotic cells. Additionally, the data are expressed as a percentage of the total data within the plot and as a percentage of data within the gate.

The MitoPotential data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you selected to

save FCS 2.0 files. In addition to the saved data file, all results, instrument settings, and the acquisition summary information is exported to a spreadsheet file.

Running the guava MitoPotential Assay

For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “MitoPotential Software Module Control Panels” on page 11-14.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.

- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.

- 3 Click **MitoPotential** from the main menu.

If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.

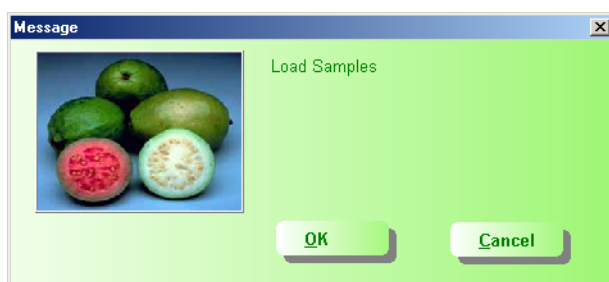
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.



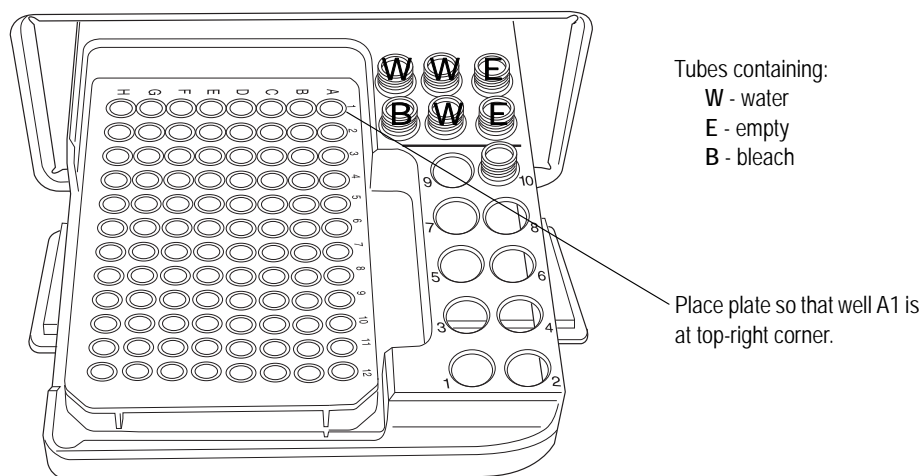
- 4 Prepare samples for analysis in a microplate or 0.5-mL tubes.

- 5 Click **Start Worklist** on the guava MitoPotential screen. The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

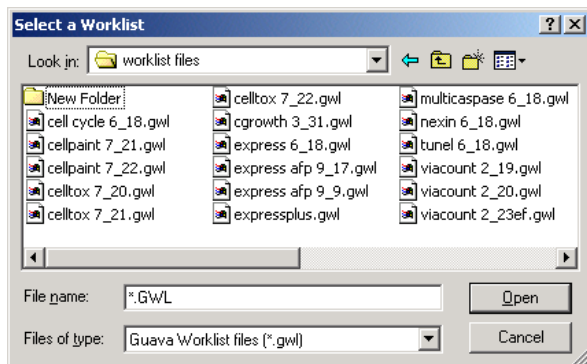


- Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

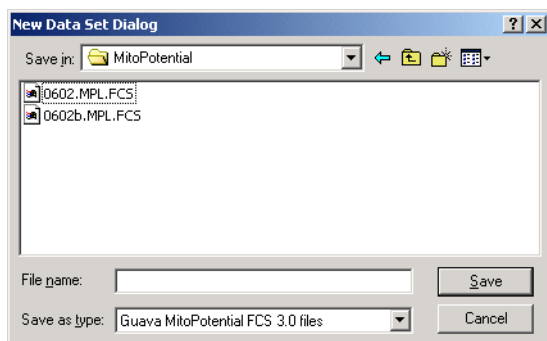
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select worklist file and click Open.

- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

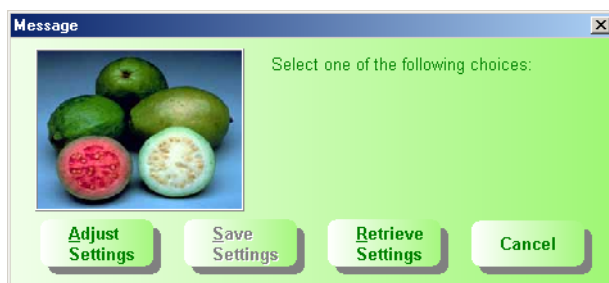
- **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



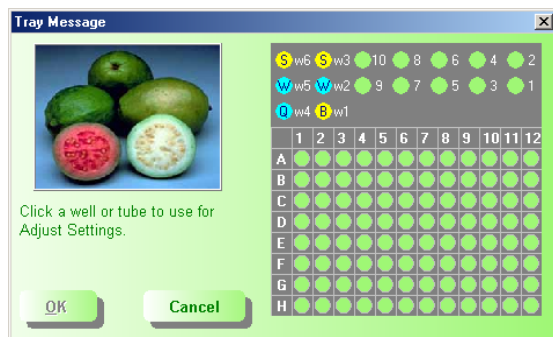
Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.

- To adjust instrument settings, click **Adjust Settings**.
- To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



- 11 A dialog box appears prompting you to select the sample for adjusting settings. Select a sample that is 20–70% induced. Click to select the well/tube used to adjust settings, then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

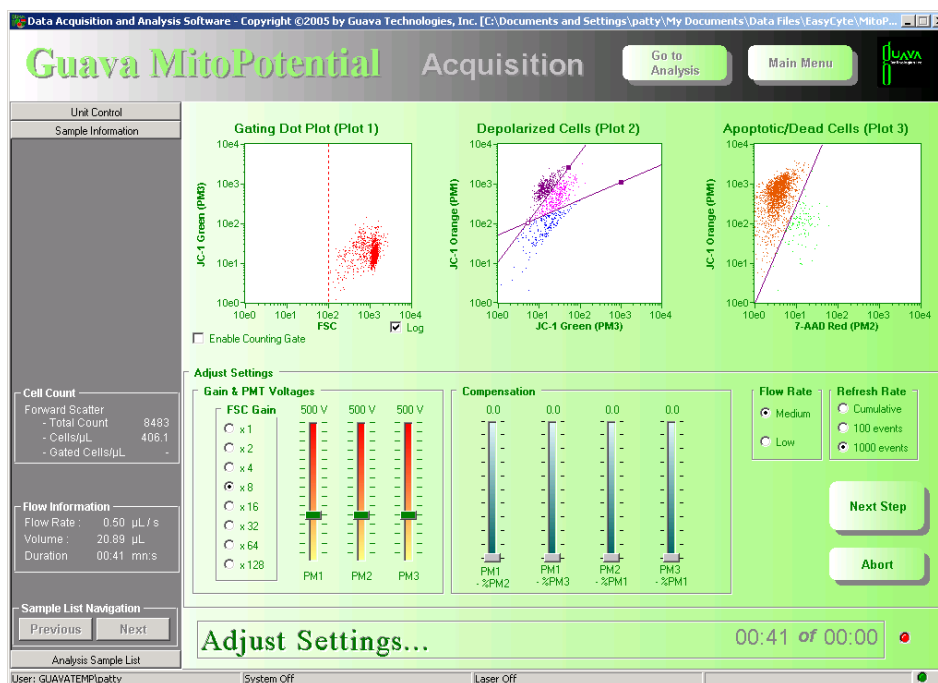
- 12 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is less than or equal to 500.

- **NOTE:** If the value is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with dye solution in medium to lower the concentration to <500 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

- 13 If you need to fine tune the settings, you can make the following adjustments once events start to appear on the screen:

- **NOTE:** You may change the y-axis label of the Gating Dot Plot (Plot 1) by right-clicking the label and selecting the new parameter from the pop-up menu.
- Set the **Refresh Rate** to the maximum number of events you want to display.
- Set the **Flow Rate** to Low (0.24 μ L/s), or Medium (0.59 μ L/s). The recommended flow rate is Medium.
- Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the cells are visible and on scale.
- To adjust the FSC threshold, click and drag the threshold marker (dotted red line) up or down the axis of Plot 1 until the desired amount of debris or other unwanted events are eliminated below the threshold.
- **NOTE:** When setting the threshold, place it at least 2 to 3 mm to the left of the cell population to ensure that you are not excluding cells.
- Adjust the voltages (using the PM1, PM2, and/or PM3 sliders, or the arrow keys on the keyboard). Adjust PM1 so that the polarized cells (upper-left quadrant) are between 10e2 and 10e3. Adjust PM3 so that the polarized cells are at approximately 10e1. If you need to increase a voltage setting, start from a lower setting and gradually increase the voltage. If you need to decrease a voltage

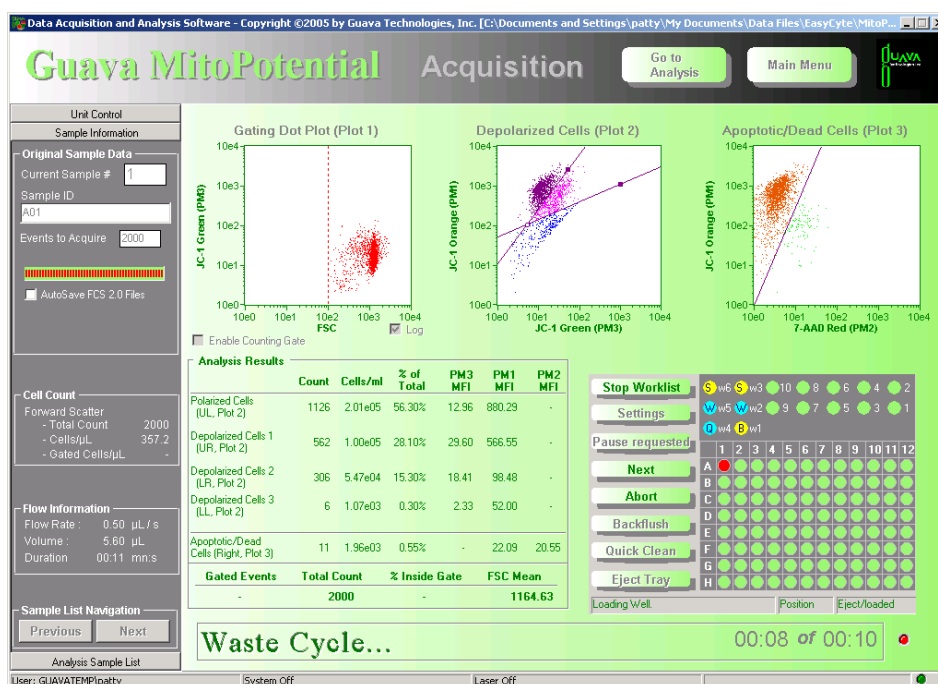
setting, do so gradually, allowing a few seconds for the PMT to equilibrate to the new voltage.



- You may need to adjust PM2-%PM1 compensation to eliminate the JC-1 Orange that may overlap into the Red (PM2) channel for some cell lines. To adjust the compensation, use the slider or click on the slider and use the up arrow key to slowly increase compensation.
- 14** You may set gates and markers prior to acquiring the samples. Refer to “Dot Plot Gate” and “Quadrant Markers” on page 11-10 for information. If you wish to apply a counting gate, click Enable Counting Gate below Plot 1, then set the region. You can select and set the count gate from the Adjust Settings screen only.
- 15** When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen. If necessary, you can repeat the adjust settings step to ensure that other samples (such as another positive control) are on scale, appropriately positioned, and compensated, by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

16 Click **Resume**.

The system acquires the first sample and automatically displays the results. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 2 minutes.

The system automatically performs a Quick Clean at the end of the assay.

guava MitoPotential Analysis

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

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

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

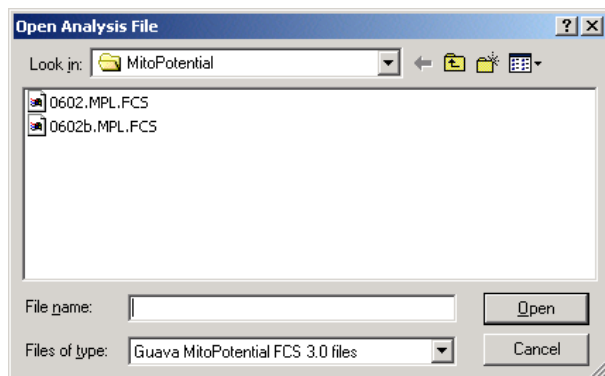
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis settings (gates and markers) from the analysis

screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

- 1 Click **MitoPotential** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.

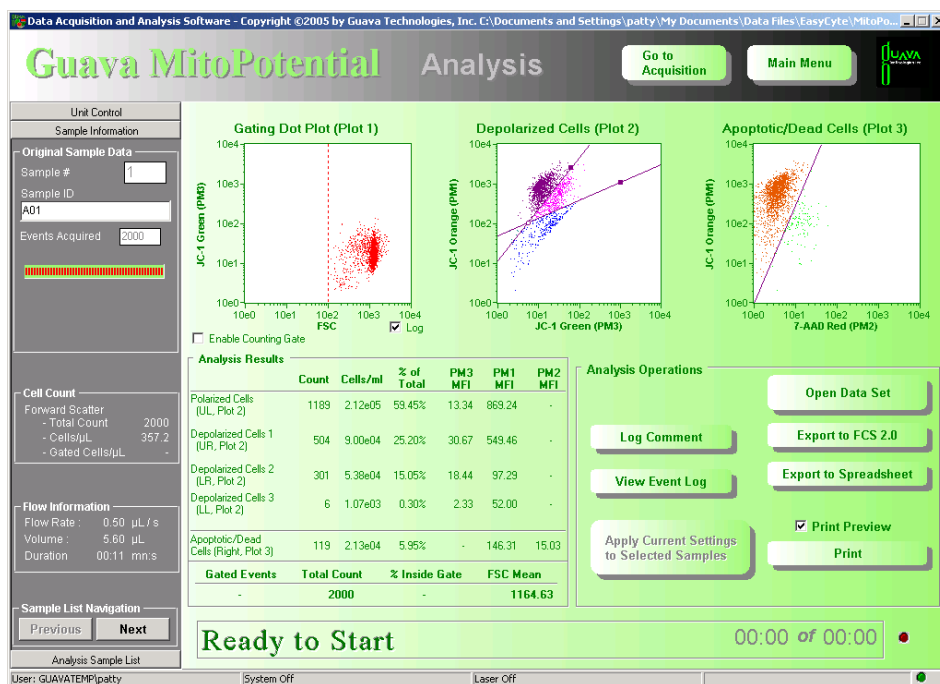


- 3 Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker settings appear as they were when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.

Ensure that the gates and markers are set appropriately for negative and positive controls. Adjust if necessary.



Gates

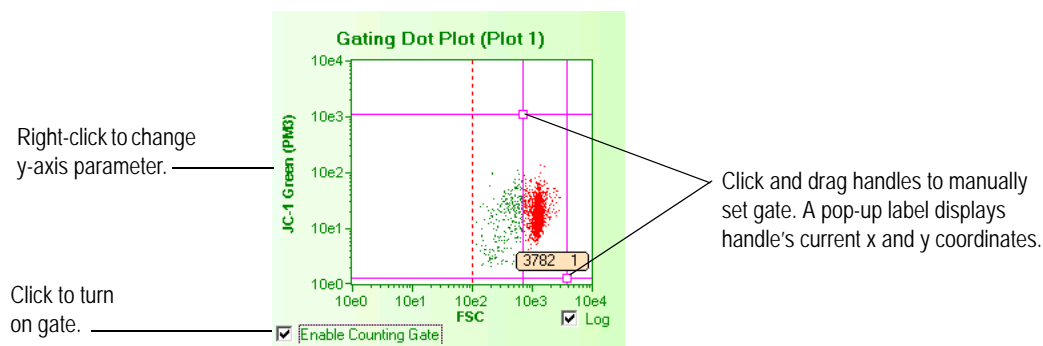
If you did not set a counting gate prior to acquisition, you can set a gate in Plot 1 during analysis. The gate is applied to the data displayed in Plots 2 and 3.

Dot Plot Gate

The dot plot gate allows you to exclude debris and unwanted events from the analysis.

- 4 To change the y-axis, right-click the axis label and select the new parameter from the pop-up menu.
- 5 Click Enable Counting Gate below Plot 1 to turn on the gate.

- 6 To set the gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle and appear in red are included in the gate.
- **NOTE:** If using the negative control to set the gate, be sure to extend the gate high enough up the y-axis so that PM-positive events in subsequent samples are included in the gate.



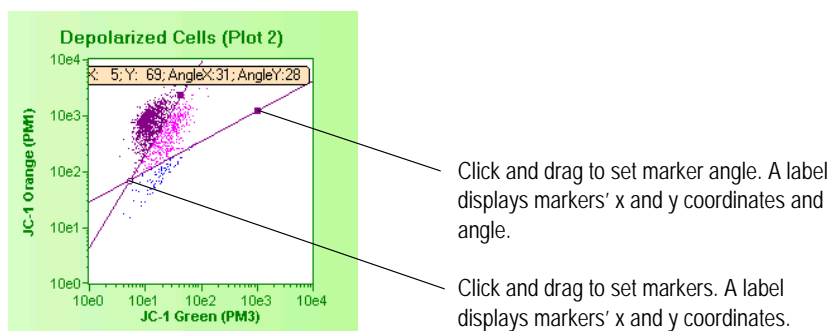
Dot plot gate to remove debris.

Markers for Statistics

Use Plot 2 and Plot 3 to set quadrant markers and a viability marker, respectively, to generate statistics on all the data acquired or the data within the analysis gate.

Quadrant Markers

- 7 To set quadrant markers, position the cursor over the handle at the intersection, then click and drag to the desired location
- If necessary, you can adjust the angle of the markers $\pm 44^\circ$ from their original locations. Click and drag the handle (solid square) towards the end of the marker and tilt it to the desired location.

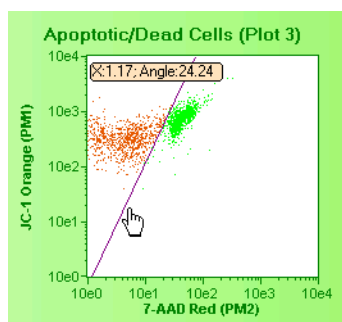


Quadrant markers set on induced sample.

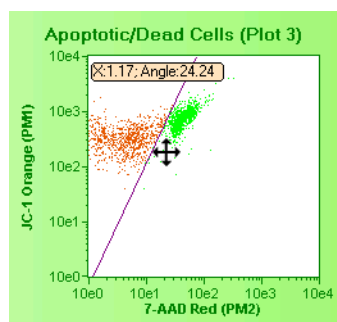
Viability Marker

You can adjust the location and angle of the viability marker in Plot 3. The viable cells appear in burnt orange to the left of the marker. The dead cells appear in lime green to the right of the marker.

- To adjust the angle of the marker, click and drag the top of the line to tilt it to the desired location.
- To move the entire marker to the left or right, click and drag the bottom of the line to shift it to the desired location.



Click and drag bottom of line to move location.



Click and drag top of line to adjust angle.

Viability marker adjusted to separate viable cells (left) from apoptotic and dead cells (right).

- 8 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 9 You can apply gate and/or markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 10 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
 - **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 11 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

guava MitoPotential Results

The statistics provide you with the count, cell concentration, % and mean signal intensities for all data in the plot (% of Total) or for data within the gate (% of Gated).

Results Table

Analysis Results						
	Count	Cells/ml	% of Total	PM3 MFI	PM1 MFI	PM2 MFI
Polarized Cells (UL, Plot 2)	67	1.33e04	3.35%	23.03	685.06	-
Depolarized Cells 1 (UR, Plot 2)	170	3.38e04	8.50%	42.89	377.75	-
Depolarized Cells 2 (LR, Plot 2)	1745	3.46e05	87.25%	16.21	81.29	-
Depolarized Cells 3 (LL, Plot 2)	18	3.57e03	0.90%	6.94	150.83	-
Apoptotic/Dead Cells (Right, Plot 3)	69	1.37e04	3.45%	-	104.51	18.99
Gated Events	Total Count	% Inside Gate		FSC Mean		
-	2000	-		1159.03		

The quadrant statistics provide the count, cells/mL, and PM1 and PM3 mean signal intensities for the populations within each quadrant of Plot 2. Additionally, the % of Total or Gated shows the percentage of the data displayed in that plot or the subset of data within the gate.

Apoptotic/Dead Cells provides similar statistics for the dead cell data in Plot 3.

The Gated Events shows the number of events in the count gate (if one was set), the total count (all events acquired), and the gated events as a percent of all events, and the FSC mean intensity.

MitoPotential quadrant statistics.

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

Quadrant	Staining	Color
lower left	depolarized cells 3 (PM3 dim, PM1 dim)	teal
lower right	depolarized cells 2 (PM3 bright, PM1 dim)	blue
upper right	depolarized cells 1 (PM3 bright, PM1 bright)	pink
upper left	polarized cells (PM3 dim, PM1 bright)	purple

The summary of the data on either side of the viability marker is outlined in the table below:

Marker	Staining	Color
left	viable cells (PM1 positive, PM2 negative)	burnt orange
right	dead cells (PM1 positive, PM2 positive)	lime green

Exporting MitoPotential Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting MitoPotential Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.
The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

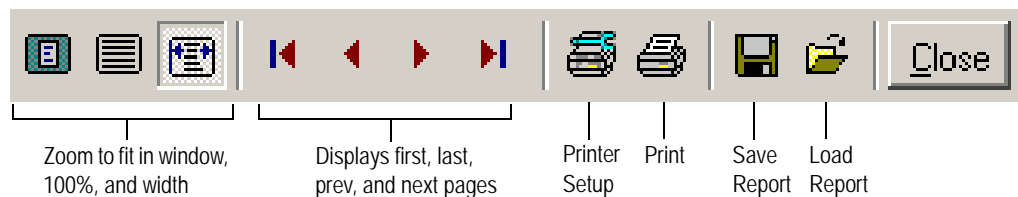
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

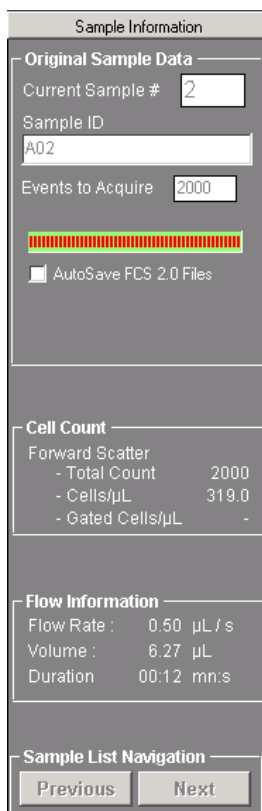
Move the cursor over the icon to display the description.



MitoPotential Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information



The screenshot shows the 'Sample Information' control panel. It is divided into several sections: 'Original Sample Data' with fields for 'Current Sample #' (2), 'Sample ID' (A02), and 'Events to Acquire' (2000), a progress bar, and an 'AutoSave FCS 2.0 Files' checkbox; 'Cell Count' with a table showing 'Forward Scatter' data; 'Flow Information' with fields for 'Flow Rate', 'Volume', and 'Duration'; and 'Sample List Navigation' with 'Previous' and 'Next' buttons.

Cell Count	
Forward Scatter	
- Total Count	2000
- Cells/ μ L	319.0
- Gated Cells/ μ L	-

Flow Information	
Flow Rate :	0.50 μ L / s
Volume :	6.27 μ L
Duration	00:12 mn:s

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 2000.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells and cells/ μ L that have exceeded the FSC threshold.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

Unit Control

Detection

Laser ☒

Setting

PM 1 500 V

PM 2 500 V

PM 3 500 V

FSC Gain: x 8

Pump Status

End of Stroke ☒

Waste ☒

Pump Reset ☒

Position 10 Steps

Actual Speed 0 Steps/s

Pump Action

Pump on ☒

Waste ☒

Pump Reset ☒

Purge ☒

Sample ☒

Speed 0 Steps/s

Threshold parameters

Offset Threshold

FSC 0.00 V 10 units

Sample List Navigation

Previous Next

Detection

Displays the laser status, the PM1, PM2, and PM3 voltage settings, and the FSC gain.

■ **NOTE:** Do not change the voltages from this panel. Use the sliders in Adjust Settings to adjust the PM1, PM2, and PM3 voltages.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold settings for the threshold parameter.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava MitoPotential Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save MitoPotential spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save MitoPotential FCS file to another directory or give it a new name.
MitoPotential Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Cell Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying PM1, PM2, or PM3.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but there are no events in plots displaying PM1, PM2, and/or PM3.	<ol style="list-style-type: none"> 1. Sample was not stained. 2. Cell lysis. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, restain sample from original suspension. 2. Check buffers used to process cells.
Events appear in Plot 1 but not in Plot 2 or Plot 3.	Plot 1 gate excludes events, and gate is applied to Plot 2 and Plot 3.	Set Plot 1 gate to include population of interest.

Problem	Possible Cause	Solutions
Events appear off scale in dot plots or histograms.	FSC gain or PM1, PM2, and / or PM3 voltages set incorrectly, or samples staining brightly.	Adjust gain setting or voltage settings so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Voltages too low to detect fluorescent signals. 2. Voltages too high to detect fluorescent signals. 3. Incomplete staining with JC1 and/or 7-AAD. 4. Reagents over-exposed to light, stored improperly, or expired. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Adjust settings to decrease fluorescent signal. Adjust compensation settings. 3. Ensure positive control is staining adequately. Otherwise, titer reagents to determine optimal concentration. 4. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.

Problem	Possible Cause	Solutions
Poor resolution between positive populations in plots displaying PM1, PM2, and/or PM3.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s), cell concentration too high, or staining time too short. 2. Too much reagent in staining tube. 3. Voltage too high causing signal to bleed into other parameters. 4. Voltage too low to optimally detect positive signal. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. Ensure sample is not too concentrated. Ensure staining time was appropriate (see package insert). 2. Titer both dyes to determine their optimum concentration. Repeat experiment with optimized reagents. 3. Adjust settings to reduce voltage. Adjust compensation settings. 4. Adjust settings to increase voltage. Adjust compensation settings. 5. Adjust settings to increase FSC threshold to remove debris.
Cell concentration decreases during acquisition.	<ol style="list-style-type: none"> 1. Cells are settling. 2. Adherent cells are reattaching. 	<ol style="list-style-type: none"> 1. Be sure that samples are mixed prior to acquisition. 2. Use low-binding, 96-well plates. If that is not sufficient to prevent reattachment, lower the FBS in the medium to 5%.

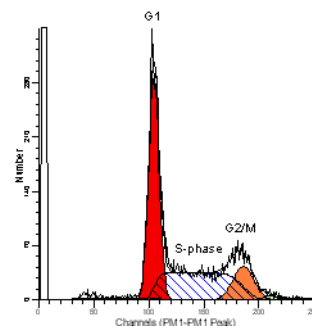
CHAPTER 12

guava Cell Cycle Assay

Introduction

Cell cycle describes the stages of replication and division of chromosomes within the nucleus, which occurs prior to a cell dividing. Cancer cells develop when the normal mechanisms for regulating the cell cycle are disrupted. It is important to identify the genetic basis for this disruption and to develop therapies to preferentially target those cells with abnormalities. One of the most rapid ways of screening potentially therapeutic drugs, or the effects of specific genes on the cell cycle, is to measure changes in cell cycle kinetics under varying conditions.

For cells to divide they must first duplicate their DNA. By labeling nuclear DNA with propidium iodide (PI) you can discriminate cells at different stages of the cell cycle (see figure). Resting cells (G0/G1 phase) contain two copies of each chromosome. As cells begin cycling, they synthesize chromosomal DNA (S phase). Fluorescence intensity from intercalating PI increases, until all chromosomal DNA has doubled (G2/M phase). At this stage, the G2/M cells fluoresce with twice the PI intensity of the G0/G1 population. The G2/M cells eventually divide into two cells.



Although a third-party “curve-fitting” software package such as ModFit™ can more accurately identify the three phases of the cell cycle and calculate the relevant statistics, you may use guavaSoft Software to identify the populations and estimate the number of cells within each phase.

To run the assay, stain the cells according to the *Guava Cell Cycle Reagent* package insert. Acquire samples on the guava easyCyte HT System using guavaSoft Software. Data are displayed in a single-parameter histogram. You can set up to four markers to analyze the various populations.

Statistics for each population within the histogram include percentage of positive, PM2 mean and median, % PM2 histogram CV, and count.

The guava Cell Cycle data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you selected to save FCS 2.0 files. In addition to the saved data file, all results, instrument settings, and the acquisition summary information are exported to a spreadsheet file.

Running the guava Cell Cycle Assay

For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “Cell Cycle Software Module Control Panels” on page 12-13.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.

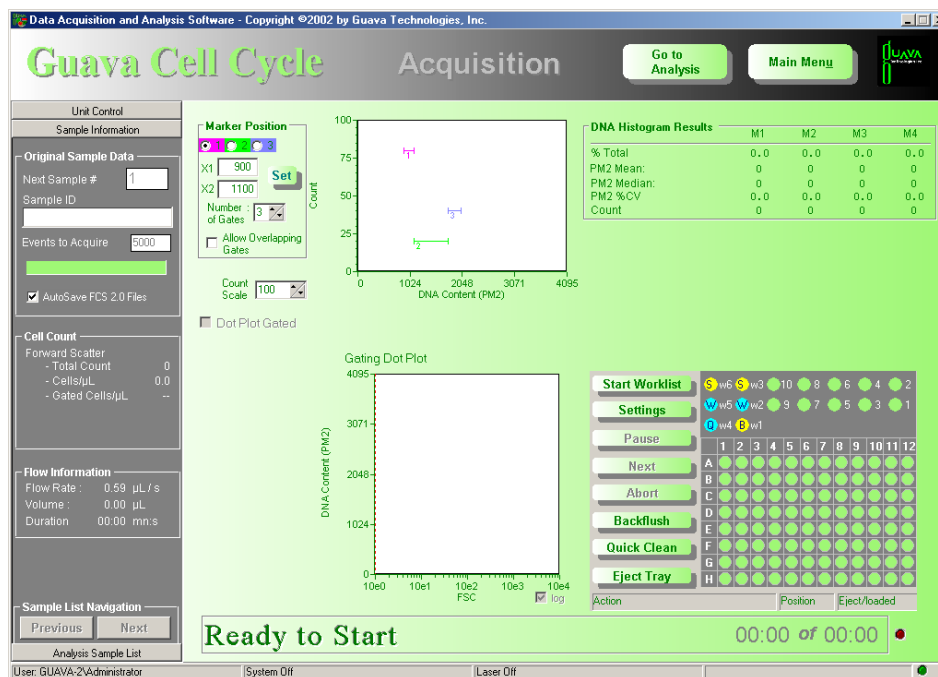
■ **NOTE:** EMD Millipore recommends programming the worklist for a Quick Clean at least every 24 samples.

- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.

- 3 Click **Cell Cycle** from the main menu.

If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.

Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

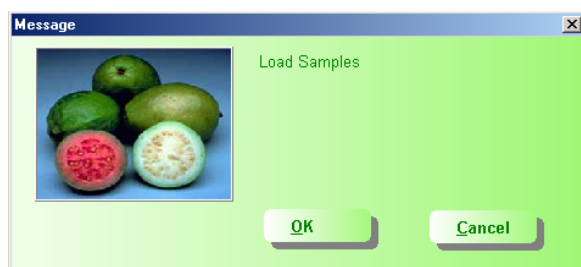


- 4 Prepare samples for analysis in a microplate or 0.5-mL tubes. Refer to the *Guava Cell Cycle Reagent* package insert for information.

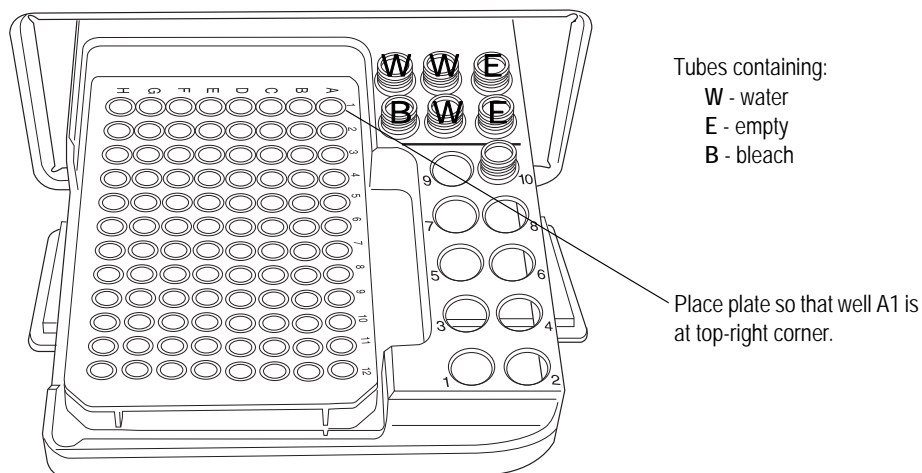
- 5 Click **Start Worklist**.

The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

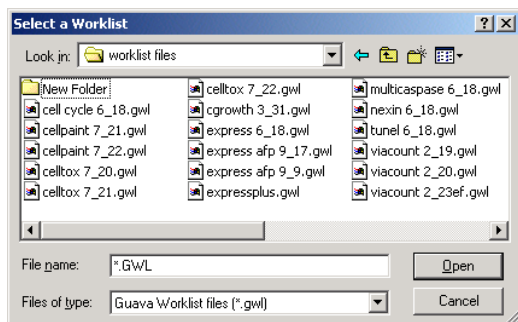


- 6 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

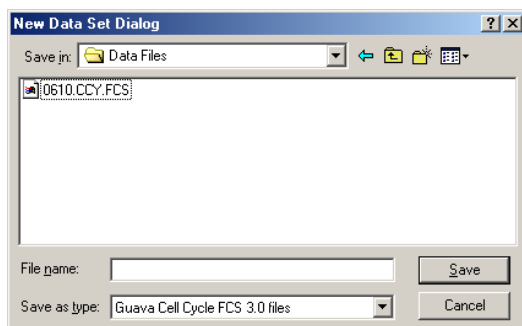
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select a worklist file and click Open.

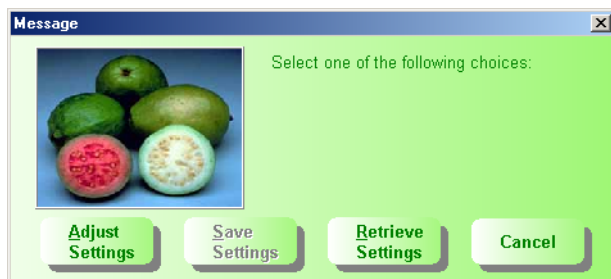
- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.

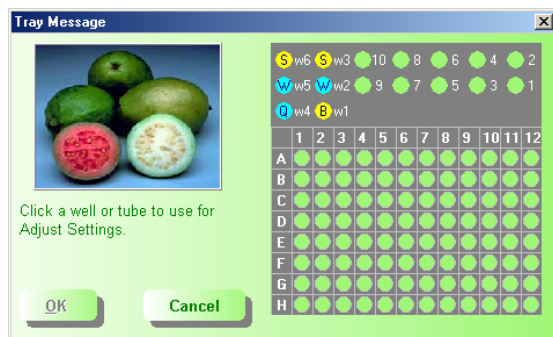


Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.
- To adjust instrument settings, click **Adjust Settings**.
 - To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



- 11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends using a stained negative (untreated) control sample. Click to select the well/tube used to adjust settings, then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.

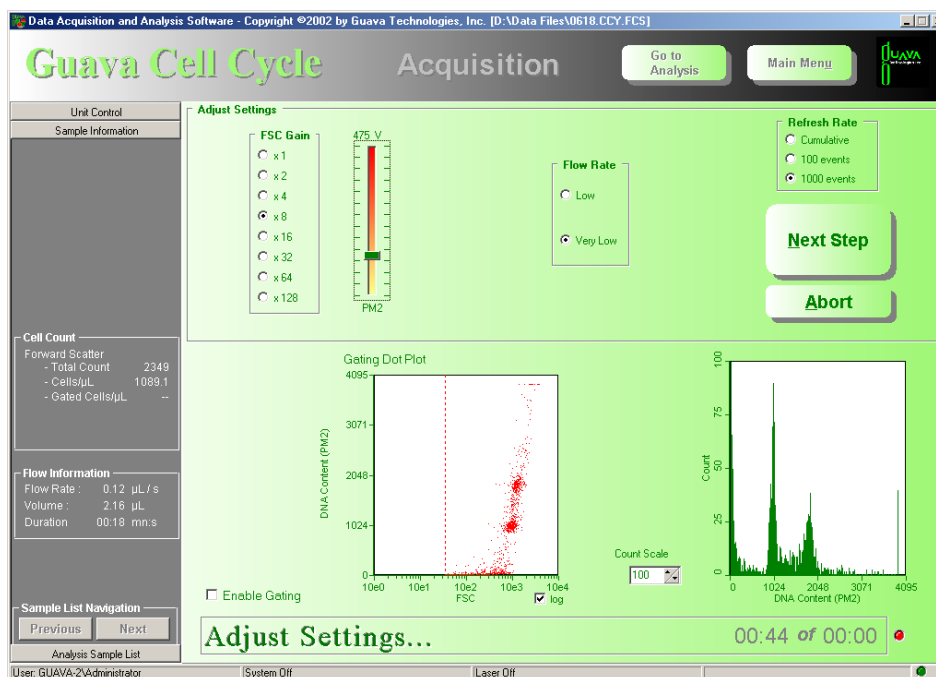
- 12 Check the Cells/ μ L value in the Sample Information control panel and ensure that it is between 25 and 1200 cells/ μ L.

- **NOTE:** If the value is greater than 1200, click **Abort**, then click **Eject Tray**. Dilute the sample with Cell Cycle Reagent to lower the concentration to <1200 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

- 13 If you need to fine tune the settings, you can make the following adjustments once events start to appear on the screen:
 - Set the **Refresh Rate** to the number of events you want to display.
 - Set the **Flow Rate** to Very Low (0.12 μ L/s) or Low (0.24 μ L/s).
 - Use the **FSC Gain** setting to reduce or amplify the FSC signal so that the cells are visible and on scale.
 - To adjust the FSC threshold, click and drag the vertical marker up or down the FSC axis of the FSC vs PM2 dot plot until the desired amount of debris is eliminated below the marker.
 - Adjust the voltage (using the PM2 slider or the arrow keys on the keyboard) so that the G0/G1 population is positioned at approximately 1024 on the PM2 histogram. Adjust the voltage by starting from a lower voltage setting and gradually increasing the voltage.
 - If necessary, use the Count Scale field to the left of the histogram to adjust the y-axis scale.

- **NOTE:** If you want the gate enabled during acquisition, click Enable Gating. During acquisition this gate can be used for counting. All events above the FSC threshold are saved to the file whether they are in the gate or not. However, the number of Events to Acquire is applied to events that fall within the gate. You cannot *enable* or *change* the gate once you click **Next Step**. You can enable it to exclude debris when you open the saved data file during analysis.

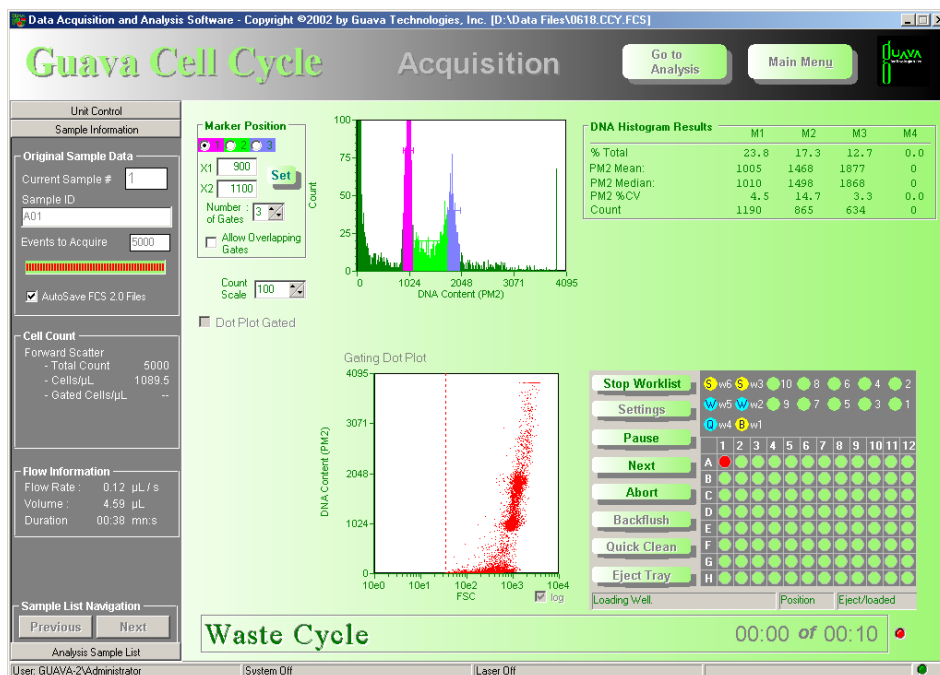
- **NOTE:** If you wish to display the data in a FSC linear scale, click to remove the check mark from the Log check box.



- 14 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.
If necessary, you can repeat the adjust settings step to ensure that other samples such as the positive control are on scale and appropriately positioned by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

15 Click **Resume**.

The system acquires the first sample and displays the results. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 10 minutes.

- 16** EMD Millipore recommends setting a gate and adjusting the markers prior to acquiring the remaining samples. Click **Pause** during the acquisition of the first sample. The system will pause when the acquisition is complete. Once the system is paused, adjust the marker, then click **Resume**. The remaining samples are automatically acquired.
- Refer to “Optional Dot Plot Gate” on page 12-9 and “Histogram Markers” on page 12-10 for information on setting a gate and markers.

The system automatically performs a Quick Clean at the end of the assay.

guava Cell Cycle Analysis

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

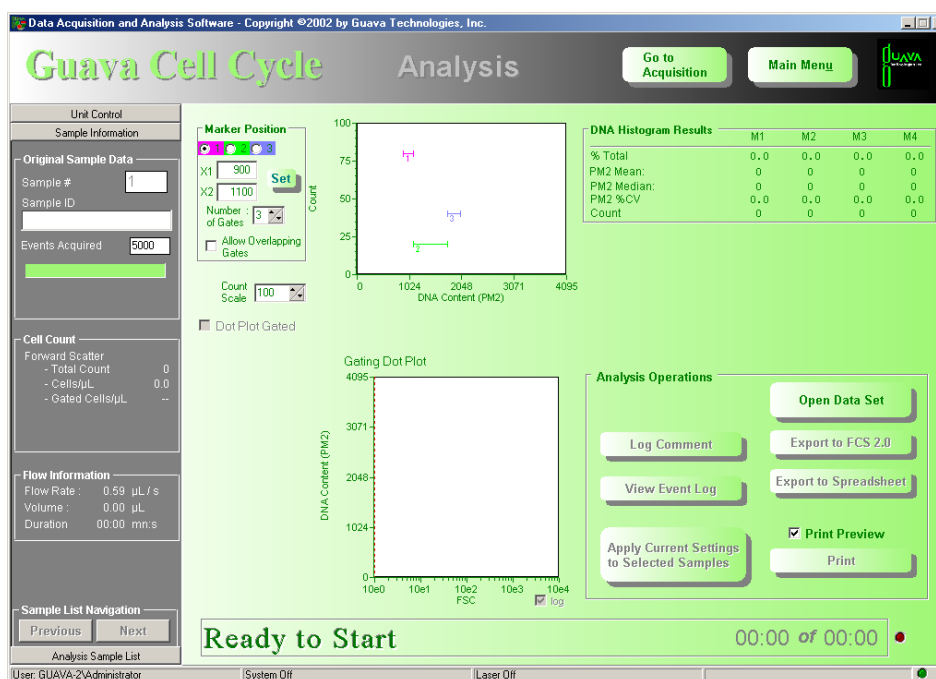
You can save changes made to the sample ID, gate, or markers within Analysis by overwriting the existing file or saving a new file.

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

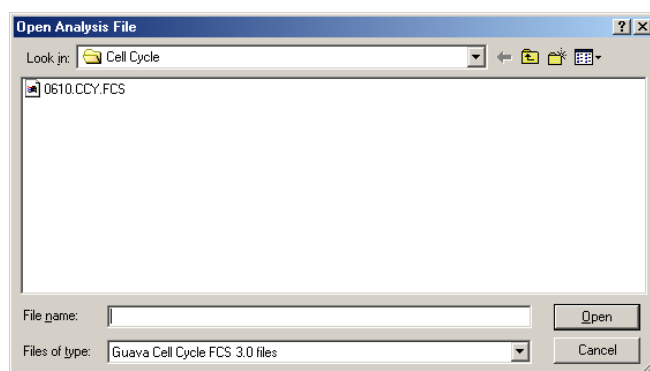
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log.

However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

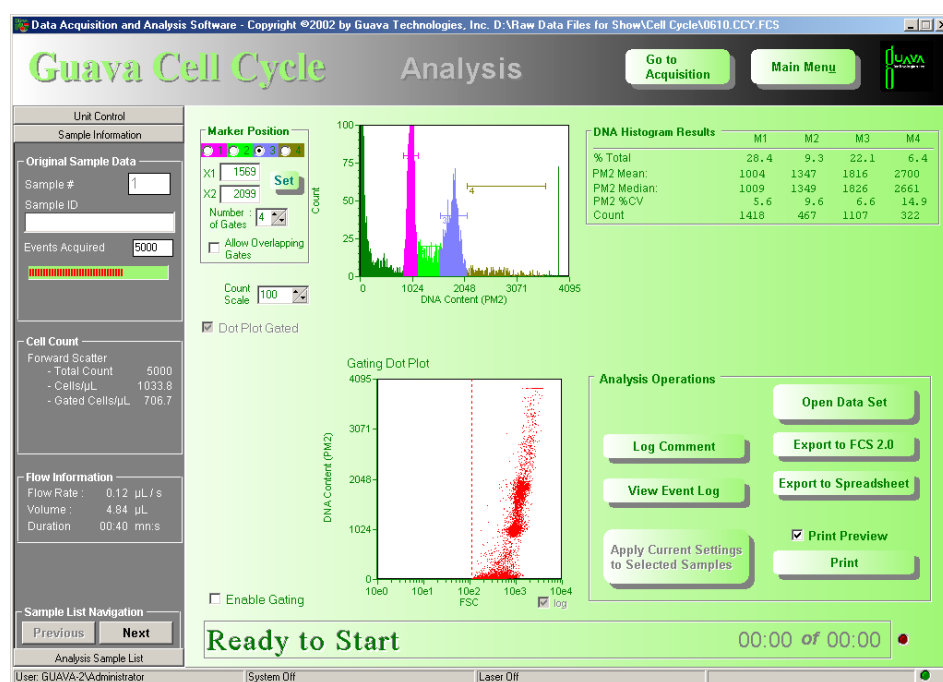
- 1 Click **Cell Cycle** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- 3 Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker setting appears as it was when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.

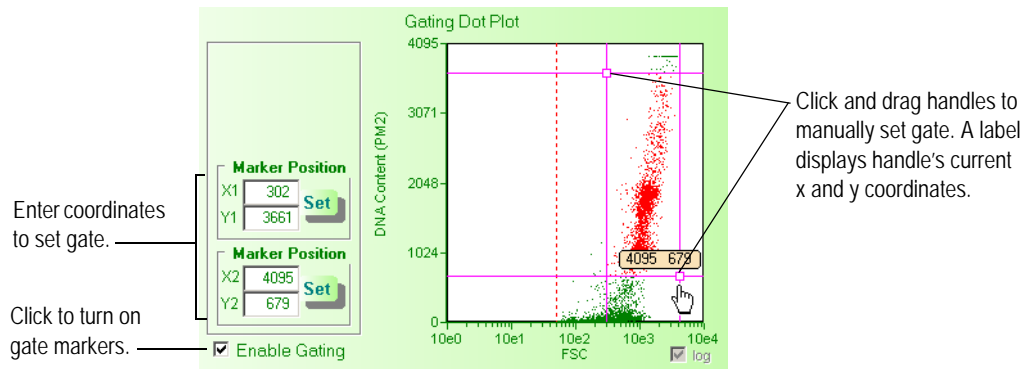


Optional Dot Plot Gate

The dot plot gate is optional. You can enable it during the adjust settings step or during analysis after the data set has been saved. During analysis, it allows you to remove excess debris from the FSC vs PM2 dot plot. The Cell Cycle Results are updated each time you adjust the gate.

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

You may also set the gate by entering the coordinates in the Marker Position fields and clicking **Set**.



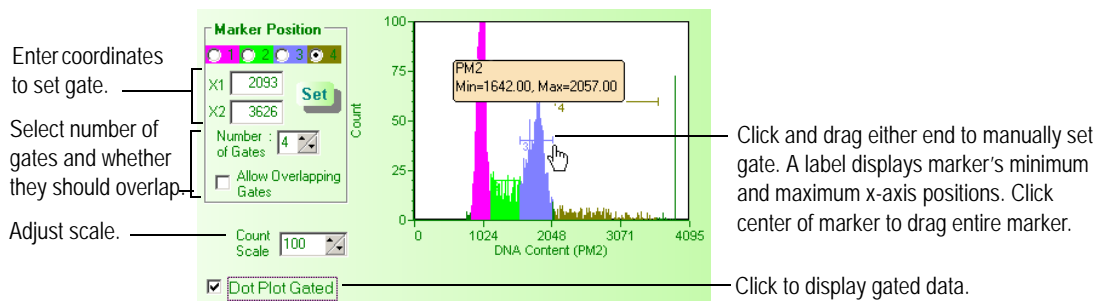
Histogram Markers

The histogram markers allows you to get statistics on any population within the PM2 parameter. You can set up to four markers to identify and generate statistics on the G0/G1, S-phase, and G2/M populations. The PM2 Results are automatically updated each time you move the markers. To analyze the data within the dot plot gate, click Enable Gating to the left of the dot plot, then click Dot Plot Gated.

- To set the PM2 histogram markers, click and drag either side of the marker to size it. Click the horizontal line to drag the entire marker. Events that fall within marker 1 appear in pink, events that fall within marker 2 appear in light green, events that fall within marker 3 appear blue, events that fall within marker 4 appear brown. Because the default is non-overlapping markers, when you move the left side of marker 2, the right side of marker 1 will move automatically.

You may also set the markers by entering the coordinates in the X1 and X2 fields and clicking **Set**.

You may overlap the markers by clicking Allow Overlapping Gates. Be aware that events falling within two markers will be counted twice. Marker 4 can overlap even if Allow Overlapping Gates is not selected.



PM2 histogram markers 1, 2, and 3 selecting diploid G0/G1 cell (pink), S phase (light green), and G2/M cells (blue).

- 7 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 8 You can apply gate and/or markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 9 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
 - **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 10 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

Cell Cycle Results

Cell Cycle results appear immediately after the acquisition of the sample is complete. The results provide an estimate of the number of cells within each phase. For more accurate results, analyze data using a third-party, curve-fitting software program. To analyze data using a third-party application, export the data to FCS 2.0 files. See “Exporting Cell Cycle Results to an FCS 2.0 File” on page 12-12.

Cell cycle results include the percentage, mean, median, %CV, and count for the data within each marker.

DNA Histogram Results				
	M1	M2	M3	M4
% Total	27.9	10.8	21.3	0.0
PM2 Mean:	1002	1359	1828	0
PM2 Median:	1007	1360	1833	0
PM2 %CV	5.4	10.8	6.2	0.0
Count	1395	539	1065	0

Statistics for the data within each marker include percentage, mean, median, % CV, and count. If a gate was set, the values represent the gated data.

Exporting Cell Cycle Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting Cell Cycle Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

■ **NOTE:** The AutoSave FCS files 2.0 checkbox in the Sample Information control panel is checked by default for the Cell Cycle Assay. Therefore, FCS 2.0 files are automatically saved.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**. You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**. The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

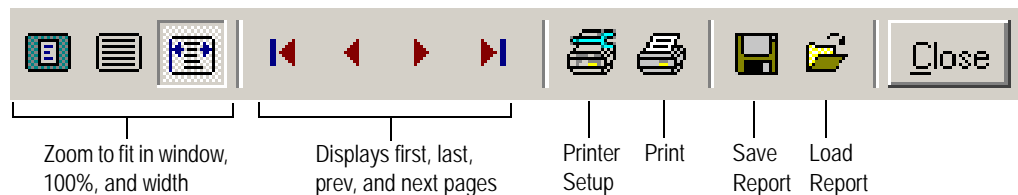
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**. The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

Move the cursor over the icon to display the description.



Cell Cycle Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

Sample Information

Original Sample Data

Current Sample # 1

Sample ID A01

Events to Acquire 5000

☐ AutoSave FCS 2.0 Files

Cell Count

Forward Scatter

- Total Count 5000
- Cells/μL 1089.5
- Gated Cells/μL --

Flow Information

Flow Rate : 0.12 μL / s

Volume : 4.59 μL

Duration 00:38 mn:s

Sample List Navigation

Previous Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 5000. If you enable the gate during the adjust settings step, the number of events applies to events that fall within the gate.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the total number of cells and cells/μL that have exceeded the FSC threshold, as well as the cells/μL in the gate.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

Detection

Displays the PM2 voltage setting and FSC gain.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the FSC offset and threshold and the PM2 offset.

■ **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field. Do not change the PM2 voltage from this panel. Use the PM2 slider to adjust the PM2 voltage.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava Cell Cycle Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save Cell Cycle spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save Cell Cycle FCS file to another directory or give it a new name.
Cell Cycle Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Debris appearing in the PM2 histogram.	FSC threshold set too low.	Increase the FSC threshold to remove as much debris as possible.
Poor resolution among G0/G1, S, and G2/M phases.	Poor sample quality or protocol not properly followed.	Ensure cells were healthy and growing prior to fixing and staining. Ensure proper protocol was followed and correct amount of PI was used.

CHAPTER 13

guava CellPaint Assay

Introduction

Monoclonal antibodies produced by hybridomas have become the preferred source of antibodies for much immunological research. They are specific for single epitopes, highly pure, and available in large amounts. Hybridoma cells are developed and used to produce monoclonal antibodies (mAbs) specific to an antigen of interest. An important step in the production of monoclonal antibodies is to screen the hybridoma supernatants for the presence of the antibody. By painting the antigen-positive control cells with the dye from the Guava EasyCyte CellPaint Kit prior to mixing with the antigen-negative control cells, you can reduce the number of tubes or wells used for screening. Other applications of the Guava EasyCyte CellPaint Kit include monitoring the effects of in vitro stimulation on heterogeneous mixtures of cells in the same tube and tracking painted cells after injection into animals in ex vivo samples.

To run the assay, follow the instructions in the *Guava EasyCyte CellPaint Kit* package insert to stain the cells. Acquire samples on the guava easyCyte HT System using guavaSoft Software. Data are displayed in two dot plots—a gating dot plot and an analysis dot plot. The gating dot plot, which shows forward scatter (FSC) versus either green (PM3) or orange (PM1) fluorescence, can be used to eliminate events such as debris or dead cells from the analysis. The analysis dot plot, which displays green (PM3) fluorescence versus orange (PM1) fluorescence, allows you to set quadrant markers for immediate on-screen results. Statistics for each quadrant in the two-color dot plot include the count, cells/mL, percentage of cells, and mean fluorescence intensity (MFI).

The CellPaint data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired or recalled later for analysis. In addition to the saved data file, all results and the acquisition information are exported to a spreadsheet file.

Running the guava CellPaint Assay

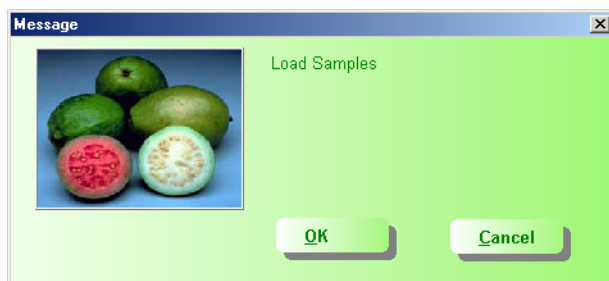
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “CellPaint Software Module Control Panels” on page 13-15.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.
- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.
- 3 Click **CellPaint** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

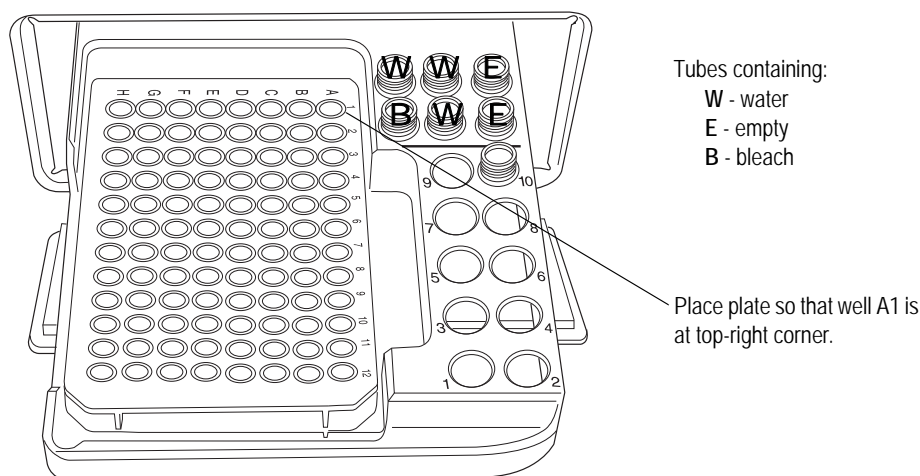


- 4 Prepare samples for analysis in a microplate or tubes. Refer to the *Guava EasyCyte CellPaint Kit* package insert for information.
- 5 Click **Start Worklist**.
The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

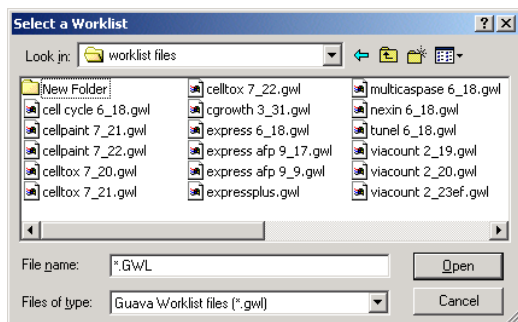


- 6 Place the microplate, any sample tubes, and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner.
Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

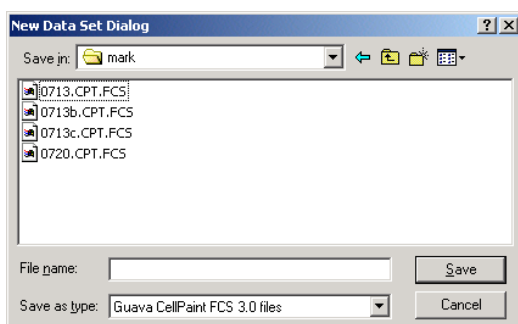
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select a worklist file and click Open.

- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

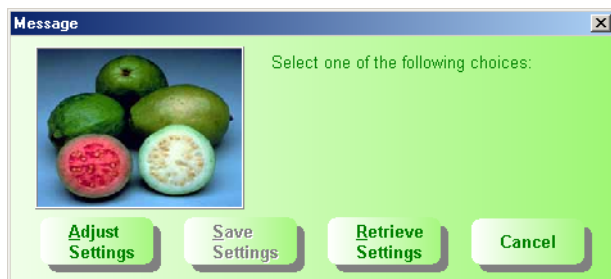
■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



Enter a file name for the data set and click Save.

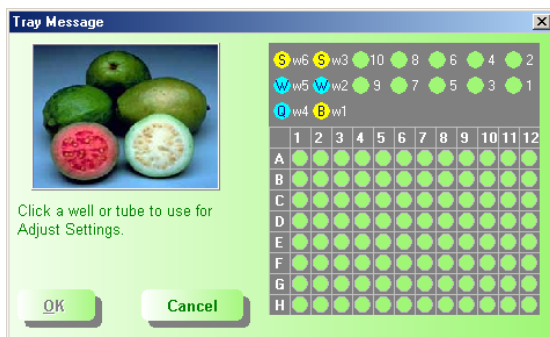
- 10 Select an instrument settings option.

- To adjust instrument settings, click **Adjust Settings**.
- To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.

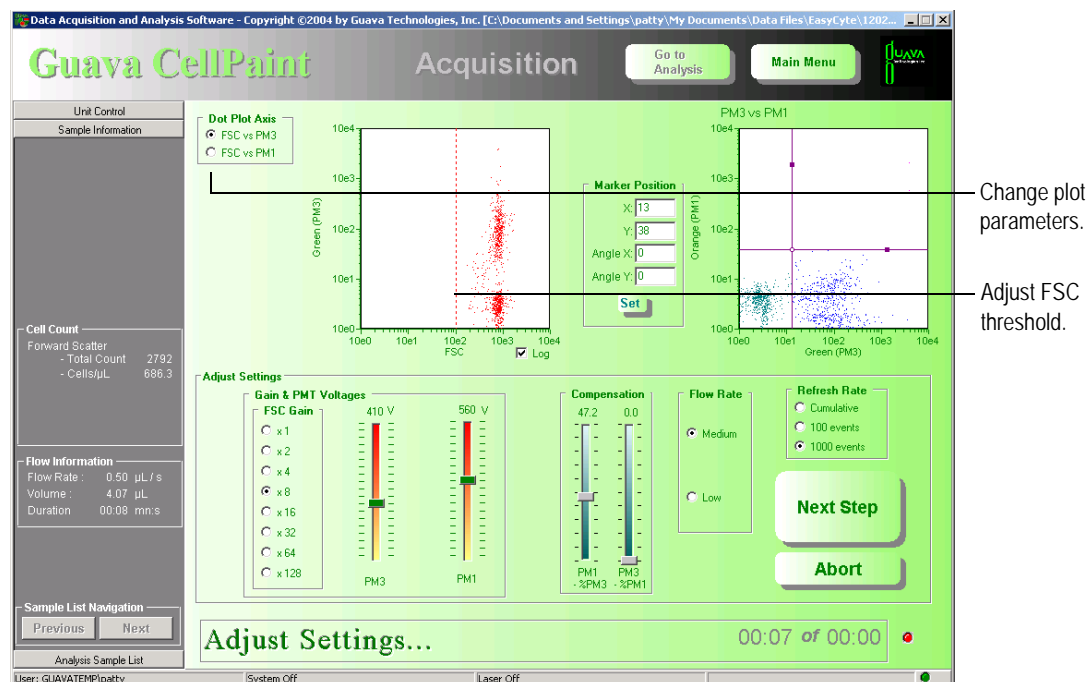


- 11 A dialog box appears prompting you to select the sample for adjusting settings. Click to select the well/tube used to adjust settings, then click **OK**. Use a positive control sample for adjusting the settings. The positive control should consist of a mixture of painted and unpainted cells stained with a monoclonal antibody

(and orange fluorescent secondary) known to recognize an antigen expressed on the painted cells.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.



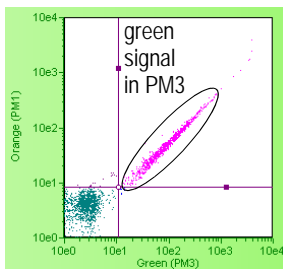
- 12 Open the Sample Information control panel and check the Cells/μL field. For accurate counting, the concentration should be between 10 and 500 cells/μL.

■ **NOTE:** If the concentration is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with staining buffer or fixative solution to lower the concentration to <500 cells/μL. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

- 13 To fine tune the settings, you can make the following adjustments once events start to appear on the screen:
 - Select the applicable dot plot axes—FSC vs PM3 or FSC vs PM1.
 - Set the **Refresh Rate** to the number of events you want to display.

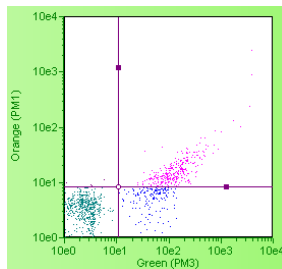
- Set the **Flow Rate** to Low (0.24 $\mu\text{L/s}$) or Medium (0.59 $\mu\text{L/s}$). The recommended flow rate is Medium.
- Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the cells are visible and on scale.
- **NOTE:** If you wish to display the data in a FSC linear scale, click to remove the check mark from the Log check box. This is the only chance you will have to select the FSC scale.
- To adjust the FSC threshold, click and drag the threshold marker up or down the FSC axis of the FSC vs PM3 (or FSC vs PM1) dot plot until the desired amount of debris is eliminated below the threshold.
- **NOTE:** When setting the threshold, leave enough debris to ensure that you are not excluding cells. Any debris that gets acquired can be excluded during analysis with the optional dot plot gate.
- Adjust the voltages (using the PM3 and PM1 sliders or the arrow keys on the keyboard) so that the unpainted, mAb-negative (double-negative) population is positioned in the lower-left corner of the Green (PM3) vs Orange (PM1) plot. These cells should be evenly distributed between $10e0$ and $10e1$ on both scales. The painted, mAb-positive cells should not be saturated (that is, they should be below 4000 on the PM3 and PM1 scales). Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.
- To adjust compensation, click **Next Step, Settings, Adjust Settings**, then select the location of the negative control sample. The negative control sample should consist of a mixture of painted and unpainted cells stained with an isotype control antibody and orange fluorescent secondary.
The example on page 13-6 shows a mixture of cells that do not react with the mAb-PE conjugate. With no compensation, the PM3/green signal overlaps into the PM1 channel (Figure 1).
Use the PM1 compensation slider to adjust the compensation so that the bottom of the PM3/Green-positive population just touches the x-axis (Figure 3). The higher the compensation value the more the unwanted, overlapping signal is removed from the PM1 channel. *Do not* overcompensate (Figure 4).

Figure 1



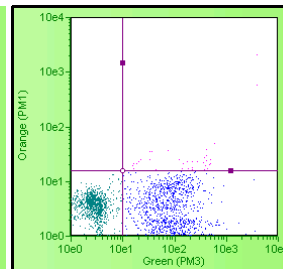
PM1 - no compensation

Figure 2



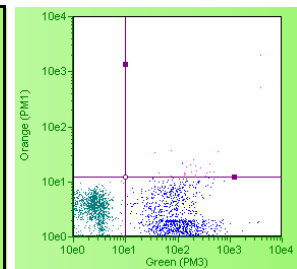
PM1 - undercompensated

Figure 3



PM1 - correct compensation

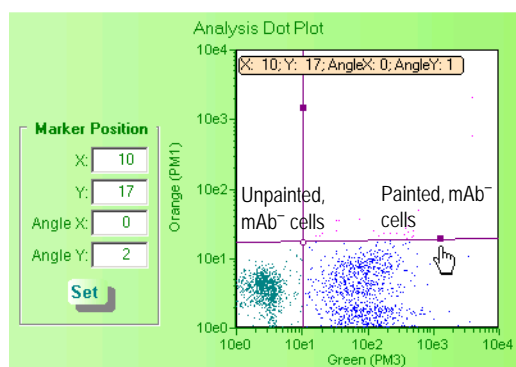
Figure 4



PM1 - overcompensated

- Adjust the quadrant markers so that the painted, mAb-negative cells are in the lower-right quadrant and the two populations of cells—the unpainted, mAb-negative cells (lower left) and the painted, mAb-positive cells (lower right) are

separated. Refer to “Quadrant Markers” on page 13-11, if necessary.



Adjust the quadrant markers for the negative control sample.

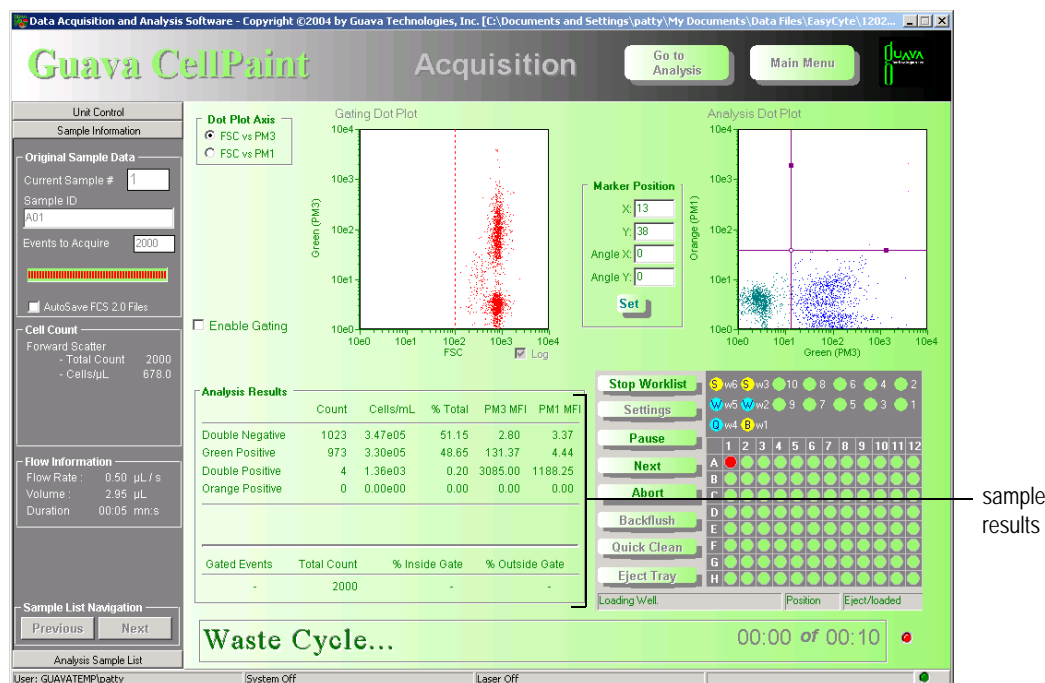
- **NOTE:** Although it may not be necessary to adjust the PM3 compensation for samples stained with PE-conjugated mAbs because the signal from PE does not significantly overlap into the PM3 channel, you may check the PM3 compensation by selecting a sample in which the unpainted cells are stained with PE. If you do make additional compensation adjustments, you may need to readjust the quadrant markers.

14 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.

If necessary, you can repeat the adjust settings step to ensure that other samples are on scale and appropriately positioned by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

15 Click **Resume**.

The system acquires the first sample and displays the results. The remaining samples are automatically acquired. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- If the sample is too dilute or too concentrated, a message appears indicating that the counting accuracy may be compromised. Refer to the *Guava EasyCyte CellPaint Kit* package insert for the recommended cell concentration and proper dilution instructions.
- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 2 minutes.

16 If you need to adjust the markers or enable the gate prior to acquiring the remaining samples, click **Pause** during the acquisition of the first sample. The system will pause when the acquisition is complete. Once the system is paused, adjust the marker or the gate, then click **Resume**.

Refer to “Quadrant Markers” on page 13-11 for information on setting markers.

The system automatically performs a Quick Clean at the end of the assay.

guava CellPaint Analysis

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

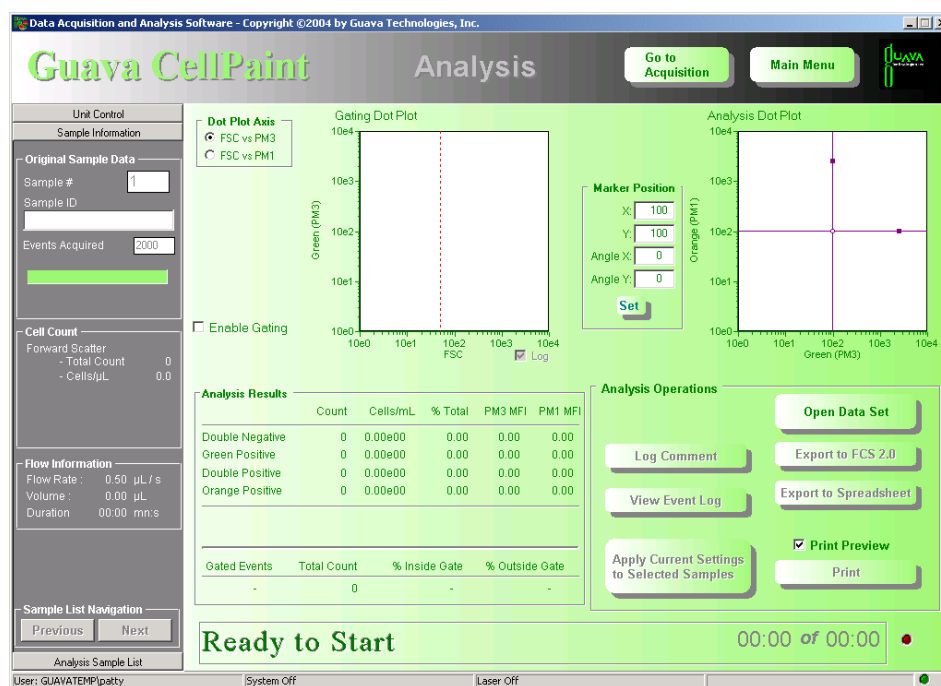
You can save changes made to the sample ID, gate, or markers within Analysis by overwriting the existing file or saving a new file.

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

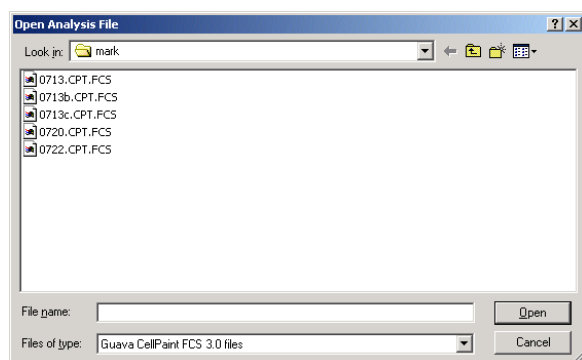
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

This version of the CellPaint Software Module within guavaSoft Software, version 2.7 allows you to open and perform off-line analysis of files acquired using the CellPaint Software Module version within CytoSoft 5.3.

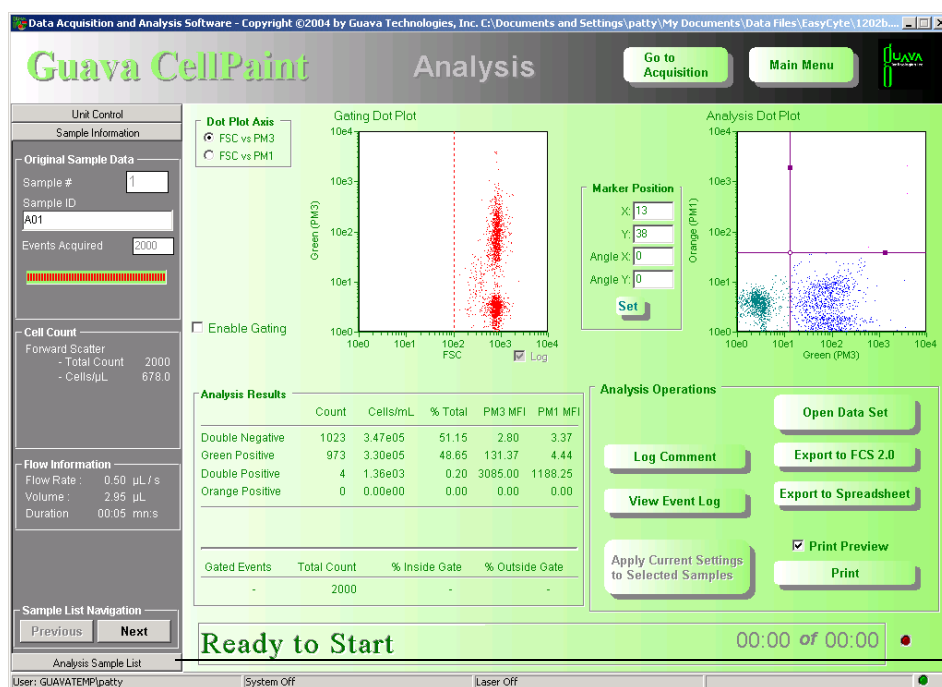
- 1 Click **CellPaint** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- 3 Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker settings appear as they were when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



Analysis Sample List control panel

Optional Dot Plot Gate

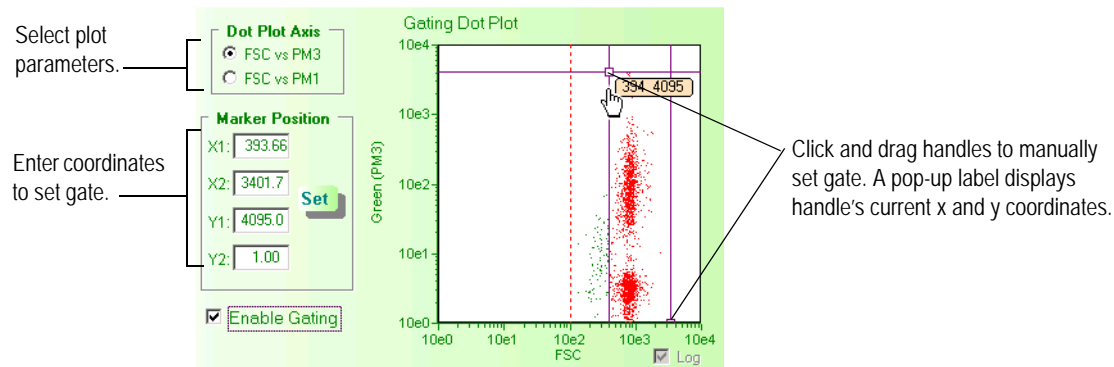
The dot plot gate is optional. It allows you to remove excess debris from the FSC vs Green (PM3)/Orange (PM1) dot plot. The Analysis Results are updated each time you adjust the gate.

- 4 Select the plot parameters by clicking the appropriate button under Dot Plot Axis to the left of the plot. Click Enable Gating to turn on the gate.
- 5 To set a gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center

rectangle and appear in red are included in the gate.

You may also set the gate by entering the coordinates in the Marker Position fields and clicking **Set**.

- **NOTE:** Be sure to extend the gate high enough up the y-axis (as shown) so that PM3-brighter events in subsequent samples are included in the gate.



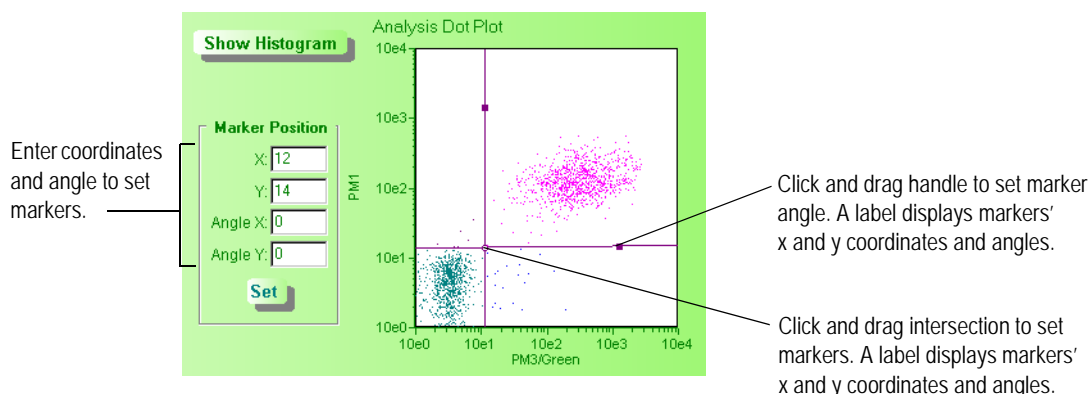
Gate set on positive control sample

Quadrant Markers

Adjust the quadrant markers on the Green (PM3) vs Orange (PM1) dot plot to generate statistics on the selected populations. The Analysis Results are automatically updated each time you move the markers.

- 6 To set the markers, position the cursor over the handle at the intersection, then click and drag to the desired location.
You may also set the markers by entering the coordinates in the Marker Position fields and clicking **Set**.
- 7 If necessary, you can adjust the angle of the markers $\pm 44^\circ$ from their original location. Click and drag the handle (solid square) towards the end of the marker and tilt the marker to the desired angle.

You may also angle the markers by entering the degrees in the Marker Position Angle fields and clicking **Set**.



Quadrant markers with painted, mAb-positive cells in the upper-right quadrant and unpainted, mAb-negative cells in the lower-left quadrant

- 8 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 9 You can apply markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Then, click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 10 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
 - **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting files.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
 - **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 11 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

CellPaint Results

The results appear immediately after the acquisition of the sample is complete. Statistics for each quadrant are displayed. Results include the count, cells/mL, % Total (all data in the dot plot) or % Gated (if a gate was enabled), and the mean fluorescence intensities for PM3 and PM1.

Quadrant Marker Statistics

Analysis Results					
	Count	Cells/mL	% Gated	PM3 MFI	PM1 MFI
Double Negative	958	3.25e05	50.10	2.58	3.31
Green Positive	929	3.15e05	48.59	131.41	3.91
Double Positive	25	8.48e03	1.31	699.04	211.00
Orange Positive	0	0.00e00	0.00	0.00	0.00
Gated Events	Total Count	% Inside Gate	% Outside Gate		
1912	2000	95.60	4.40		

Results for each quadrant are displayed as a count, cells/mL, and percentage of the total or gated data in the dot plot. Additionally, the mean fluorescence intensities for PM3 and PM1 are displayed.

If a gate was set, the number of cells within the gate, as well as the percentage of cells inside and outside the gate are displayed.

CellPaint quadrant marker statistics.

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

Quadrant	Staining	Population	Color
lower left	Green (PM3) negative, Orange (PM1) negative	negative (unpainted) control cells, mAb negative	teal
lower right	Green (PM3) positive, Orange (PM1) negative	positive (painted) control cells, mAb negative	blue
upper right	Green (PM3) positive, Orange (PM1) positive	positive (painted) control cells, mAb positive	pink
upper left	Green (PM3) negative, Orange (PM1) positive	negative (unpainted) control cells, mAb positive	purple

Exporting CellPaint Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting CellPaint Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**. The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

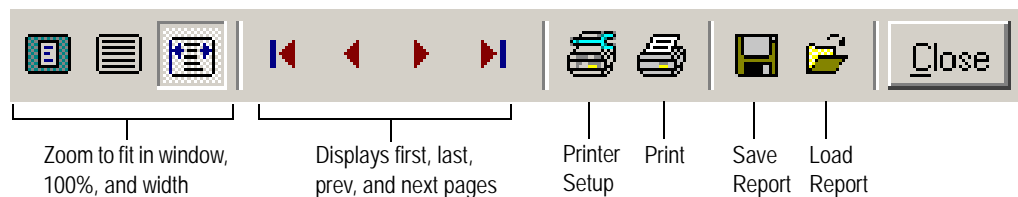
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

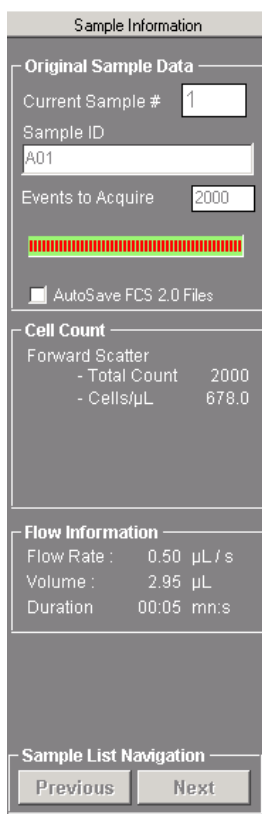
Move the cursor over the icon to display the description.



CellPaint Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information



The screenshot shows the 'Sample Information' control panel. It has a title bar 'Sample Information'. Below it is a section 'Original Sample Data' containing three input fields: 'Current Sample #' with the value '1', 'Sample ID' with the value 'A01', and 'Events to Acquire' with the value '2000'. Below these fields is a progress bar with red and green segments. At the bottom of this section is a checkbox labeled 'AutoSave FCS 2.0 Files'. The next section is 'Cell Count', which displays 'Forward Scatter' with two sub-values: '- Total Count' as '2000' and '- Cells/μL' as '678.0'. The third section is 'Flow Information', displaying 'Flow Rate : 0.50 μL / s', 'Volume : 2.95 μL', and 'Duration 00:05 mn:s'. The final section is 'Sample List Navigation', which contains two buttons: 'Previous' and 'Next'.

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 2000.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the number of cells (total count) detected by the FSC detector, as well as the concentration (cells/μL).

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

The screenshot shows the 'Unit Control' software window. It has a title bar 'Unit Control' and a menu bar. The main area is divided into five sections:

- Detection:** Includes a 'Laser' status indicator (red circle), 'Setting' for PM 3 (362 V) and PM 1 (560 V), and 'FSC Gain: x 8'.
- Pump Status:** Includes status indicators for 'End of Stroke', 'Waste', and 'Pump Reset' (all red). It also shows 'Position' (10 Steps) and 'Actual Speed' (0 Steps/s).
- Pump Action:** Includes status indicators for 'Pump on', 'Waste', 'Pump Reset', 'Purge', and 'Sample' (all green).
- Threshold parameters:** Includes 'FSC' with 'Offset' (1.41 V) and 'Threshold' (393 units).
- Sample List Navigation:** Includes 'Previous' and 'Next' buttons.

Detection

Displays laser status and the PM3 and PM1 voltage settings and the FSC gain.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the FSC offset and threshold settings.

■ **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field. Do not change the voltages from this panel. Use the sliders to adjust the PM3 and PM1 voltages.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava CellPaint Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save CellPaint spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save CellPaint FCS file to another directory or give it a new name.
CellPaint Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube not properly loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in PM3 vs PM1 dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM3 dot plot but not in PM3 vs PM1 dot plot.	FSC vs PM3 gate excludes events.	Ensure FSC vs PM3 gate is set to include population of interest.

Problem	Possible Cause	Solutions
Poor resolution between Green (PM3)-positive and double-negative populations.	<ol style="list-style-type: none"> 1. PM3 voltage too low to detect signal. 2. Incomplete staining with Guava CFSE reagent. 3. Debris included in analysis. 4. Guava CFSE expired, or not stored properly. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase Green (PM3) signal. Repeat compensation adjustment. 2. Refer to package insert for proper staining instructions. 3. Adjust FSC threshold or set gate to remove debris. 4. Refer to package insert for proper storage instructions. Do not use expired reagent. Do not expose reagent to light. 5. Adjust settings to increase FSC threshold to remove debris.
Poor resolution between Green (PM3)-positive and double-positive populations.	<ol style="list-style-type: none"> 1. Incomplete staining with orange fluorescent antibody probes. 2. Too much Guava CFSE reagent used. 3. Fluorescence background too high. 4. Green (PM3) signal too high, causing Guava CFSE reagent to bleed into Orange (PM1). 5. PM1 voltage too low to optimally detect orange signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date of probes and/or titer reagent to optimize staining. 2. Repeat experiment, painting cells with a lower reagent concentration. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce PM3 voltage. Repeat compensation adjustment. 5. Adjust settings to increase PM1 voltage. Repeat compensation adjustment. 6. Adjust settings to increase FSC threshold to remove debris.
Events appear off scale in dot plots.	PM3 and /or PM1 voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM3 and/or PM1 voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample. Repeat compensation adjustment.

CHAPTER 14

guava CellToxicity Assay

Introduction

One of the functions of the immune system is to recognize and destroy targets, such as tumor cells or cells infected with microbes (bacteria or viruses). This is accomplished by effector cells, such as T cells, NK cells, and macrophages or granulocytes, which employ cell-mediated cytotoxicity (CMC), natural killer (NK) activity, and antibody-dependent cellular cytotoxicity (ADCC), respectively. CMC and NK activities are commonly monitored to assess the potency of immune cell stimulation by small molecules, antibodies, or other regulatory molecules; whereas ADCC activity is used to assess the potency of antibodies for therapy.

The Guava EasyCyte CellToxicity Kit contains a cell painting dye that differentiates effector or cytotoxic cells from target cells by staining the target cells. Painted target cells are subsequently incubated with unpainted effector cells at various ratios to allow effector cells to function. The dye is retained in the target cell's cytoplasm even after the cell is killed. 7-AAD, a viability stain, is then added to determine the number of painted target cells that have been killed.

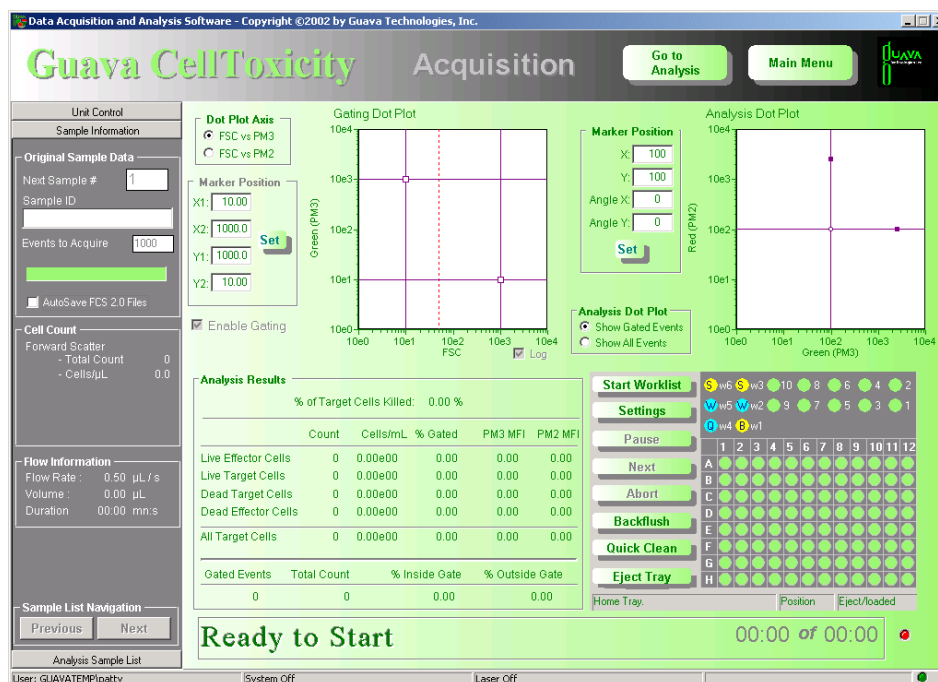
To run the assay, follow the instructions in the *Guava EasyCyte CellToxicity Kit* package insert to stain the cells. Acquire samples on the guava easyCyte HT System using guavaSoft Software. Data are displayed in two dot plots—a gating dot plot and an analysis dot plot. The gating dot plot, which shows forward scatter (FSC) versus fluorescence of either Green (PM3) or Red (PM2), allows you to set a counting gate to acquire the same number of target cells across multiple samples regardless of the effector to target ratio. The gate can also be used to eliminate unwanted events such as debris from the analysis. The analysis dot plot, which displays Green (PM3) versus Red (PM2), allows you to set quadrant markers for immediate on-screen results. Statistics for the two-color dot plot include the percent of dead target cells as well as individual quadrant statistics, which include the percentage of cells, cell concentration, and mean fluorescence intensity (MFI).

The CellToxicity data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired or recalled later for analysis. In addition to the saved data file, all results and the acquisition information are exported to a spreadsheet file.

Running the guava CellToxicity Assay

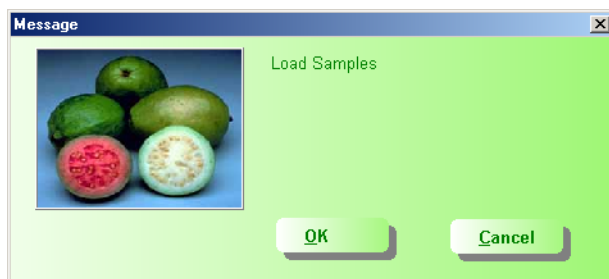
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “CellToxicity Software Module Control Panels” on page 14-14.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.
- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.
- 3 Click **CellToxicity** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

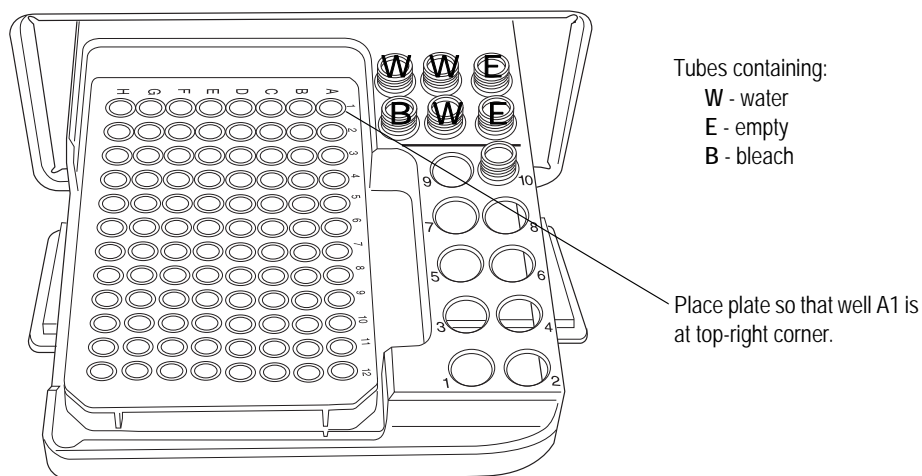


- 4 Prepare samples for analysis in a microplate or tubes. Refer to the *Guava EasyCyte CellToxicity Kit* package insert for information.
- 5 Click **Start Worklist**.
The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

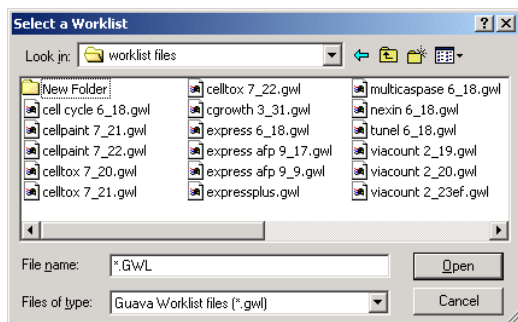


- 6 Place the microplate, any sample tubes, and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner.
Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

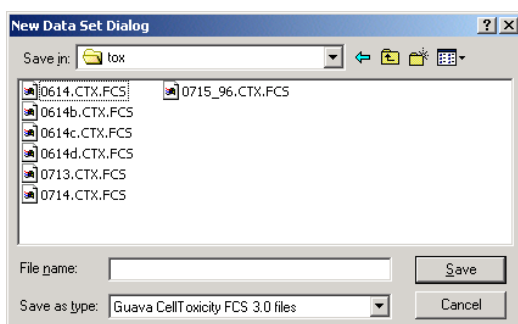
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select a worklist file and click Open.

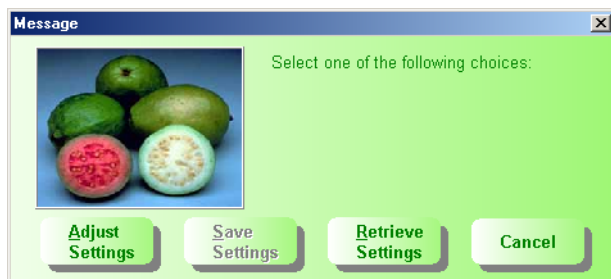
- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

■ **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



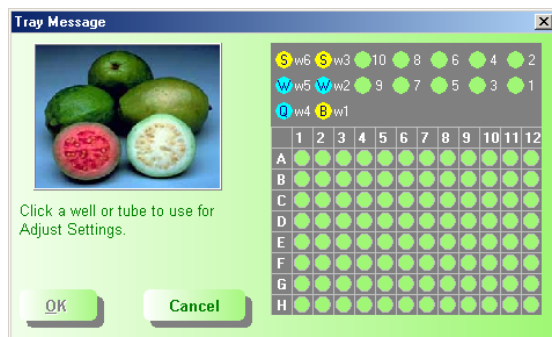
Enter a file name for the data set and click Save.

- 10 Select an instrument settings option.
- To adjust instrument settings, click **Adjust Settings**.
 - To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.

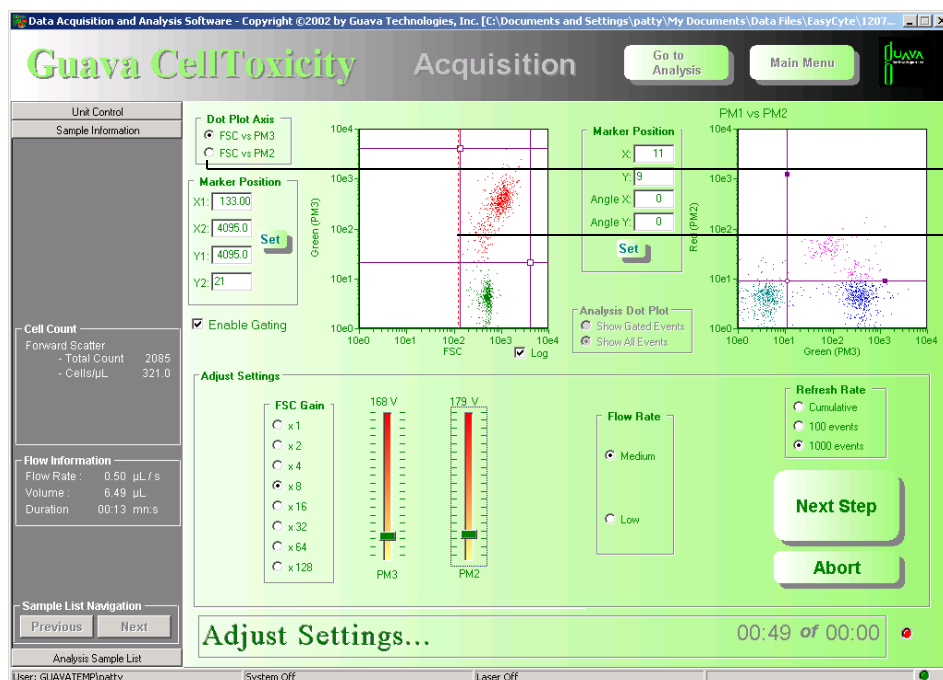


- 11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends running a sample containing both target and effector cells,

where you expect that about 50% of the target cells are killed. Click to select the well/ tube used to adjust settings, then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.



Change plot parameters.
Adjust FSC threshold.

12 Open the Sample Information control panel and check the Cells/μL field. For accurate counting, the concentration should be between 10 and 500 cells/μL.

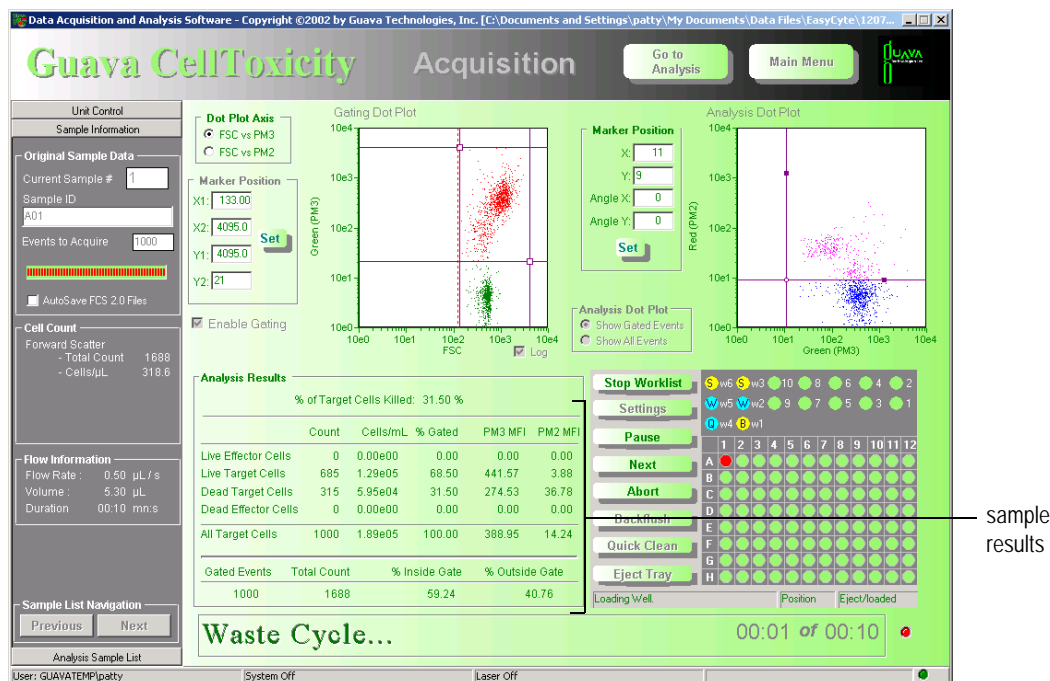
■ **NOTE:** If the concentration is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with Guava® CellToxicity 7-AAD Reagent diluted 1:5 with serum-containing medium to lower the concentration to <500 cells/μL. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

13 To fine tune the settings, you can make the following adjustments once events start to appear on the screen:

- Select the applicable dot plot axes—FSC vs PM3 or FSC vs PM2.
- Set the **Refresh Rate** to the number of events you want to display.

- Set the **Flow Rate** to Low (0.24 $\mu\text{L/s}$) or Medium (0.59 $\mu\text{L/s}$). The recommended flow rate is Medium.
 - Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the effector cells are positioned between 500 and 10e3, and the target cells are at 2000 or less.
 - **NOTE:** If you wish to display the data in a FSC linear scale, click to remove the check mark from the Log check box. This is the only chance you will have to select the FSC scale.
 - To adjust the FSC threshold, click and drag the threshold marker up or down the FSC axis of the FSC vs Green (PM3) [or FSC vs Red (PM2)] dot plot until the desired amount of debris is eliminated below the threshold.
 - **NOTE:** When setting the threshold, leave enough debris to ensure that you are not excluding any small effector or dead target cells.
 - Adjust the voltages (using the PM3 and PM2 sliders or the arrow keys on the keyboard) so that the unmarked, double-negative population is positioned in the lower-left corner of the Green (PM3) vs Red (PM2) plot. These cells should be evenly distributed between 10e0 and 10e1 on both scales. The marked, live (PM2-negative) and dead (PM2-positive) target cells should not be saturated (that is, they should be below 4000 on the PM3 and PM2 scales). Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.
 - On the Gating Dot Plot with the parameters set to FSC vs Green (PM3), adjust the gate to include target cells only. Be sure to include the dead target cells, which often have a lower FSC signal than the live target cells. The gate is used as a counting gate. Events that are included in the gate are counted toward the number of Events to Acquire. For example, if the Events to Acquire is set to 1000, acquisition is complete when 1000 events have passed through the gate. Refer to “Dot Plot Gate” on page 14-10.
 - **NOTE:** If you turn off the counting gate, you may need to increase the number of Events to Acquire to ensure acquiring a sufficient number of target cells. EMD Millipore recommends acquiring at least 1000 target cells. At a 1:1 effector-to-target ratio, you would need to acquire 2000 total events; whereas, at a 10:1 effector-to-target ratio, you would need to acquire 10,000 total events.
 - Adjust the quadrants to separate the four populations—live effectors (lower left), dead effectors (upper left), live targets (lower right), dead targets (upper right). Refer to “Quadrant Markers” on page 14-11.
- 14** When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.
- If necessary, you can repeat the adjust settings step to ensure that other samples are on scale and appropriately positioned by clicking **Settings**, then **Adjust** or **Retrieve Settings**, then indicating the location of the sample(s).

- 15 Select **Show All Events** to the left of the Analysis Dot Plot, then click **Resume**. The system acquires the first sample and displays the results. The remaining samples are automatically acquired. The tube/well currently being acquired is highlighted in red.



■ ACQUISITION NOTES

- If the sample is too dilute or too concentrated, a message appears indicating that the counting accuracy may be compromised. Refer to the *Guava EasyCyte CellToxicity Kit* package insert for the recommended cell concentration and proper dilution instructions.
- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- At high effector to target ratios (25:1 or greater, depending on the total cell concentration), with a counting gate enabled, acquisition will be very slow because of the large number of effector cells. Although acquisition may time-out before 1000 target events are acquired, a robust analysis can still be performed.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 4 minutes.

- 16 If you need to adjust the markers prior to acquiring the remaining samples, click **Pause** during the acquisition of the first sample. The system will pause when the acquisition is complete. Once the system is paused, adjust the markers, then click **Resume**. Refer to “Quadrant Markers” on page 14-11 for information.

The system automatically performs a Quick Clean at the end of the assay.

guava CellToxicity Analysis

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

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

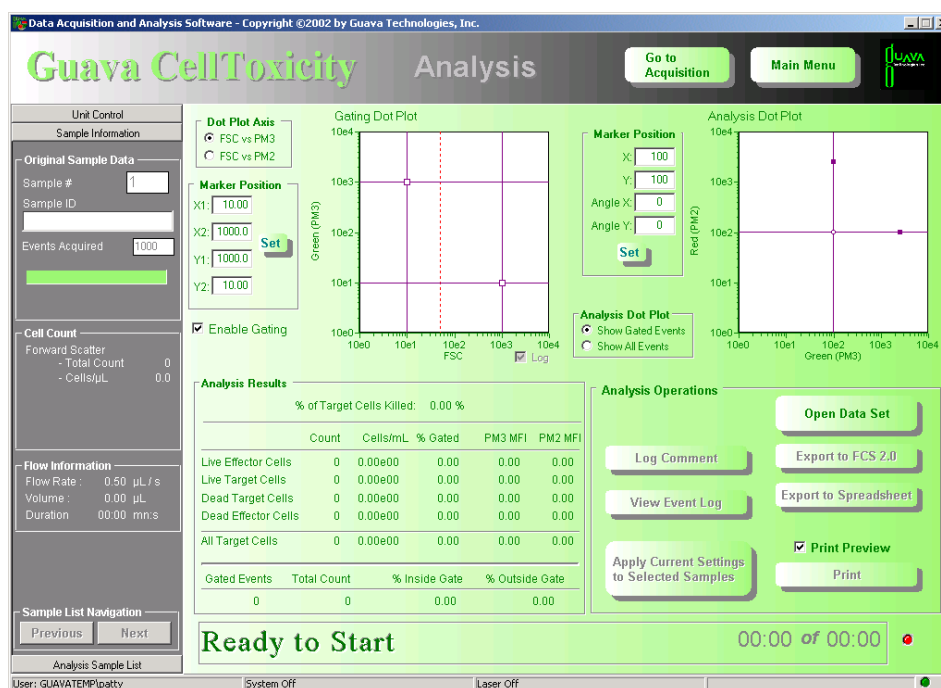
■ **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log.

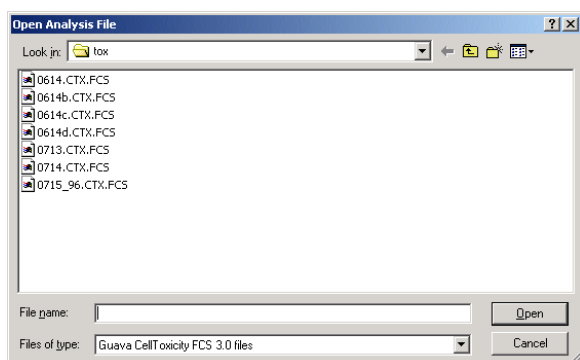
However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

This version of the CellToxicity Software Module within guavaSoft Software, version 2.7 allows you to open and perform off-line analysis of files acquired using the CellToxicity Software Module version within CytoSoft 5.3.

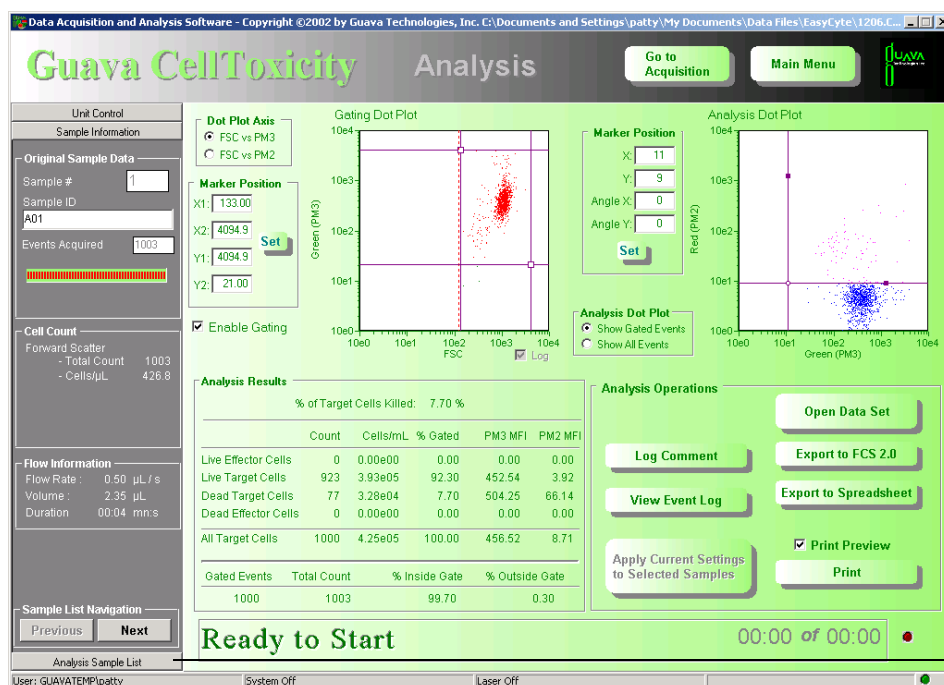
- 1 Click **CellToxicity** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker settings appear as they were when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



Analysis Sample List control panel

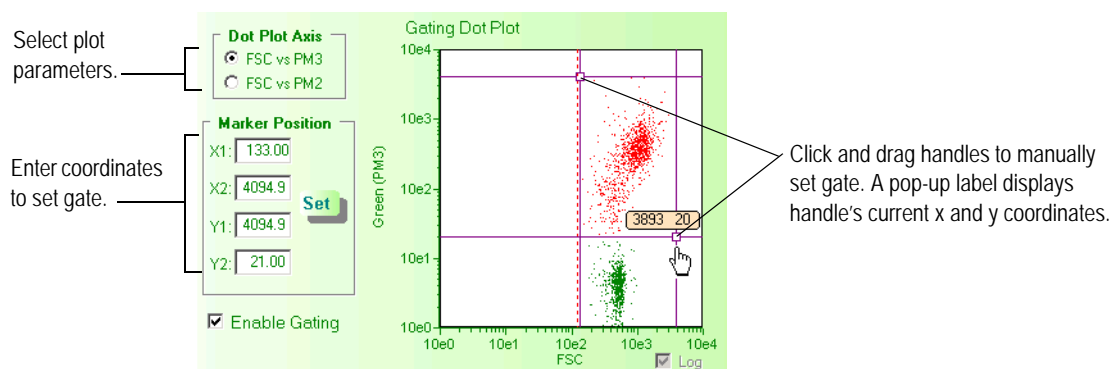
Dot Plot Gate

During acquisition, the dot plot gate is used as a counting gate. Events that are included in the gate are counted toward the number of Events to Acquire. During analysis, you may disable the gate or adjust it. However, once you adjust the gate, it is no longer considered a counting gate. In other words the number of events in the gate will not match the number of events acquired.

- 4 Select the plot parameters (FSC vs PM3) by clicking the appropriate button under Dot Plot Axis to the left of the plot. Click Enable Gating to turn on the gate.

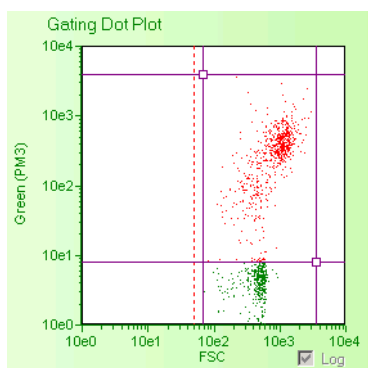
■ **NOTE:** EMD Millipore recommends not using parameters FSC vs PM2 for setting the gate on target cells. It is too difficult to exclude effector cells from the live and dead target cells. However, you may set the gate to include effector and target cells, and therefore generate statistics on all four populations.

- 5 Set the gate on the target cell population. Be sure to include the dead target cells, which have lower FSC and PM3 signal intensities than the live target cells. To set a gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle and appear in red are included in the gate. You may also set the gate by entering the coordinates in the Marker Position fields and clicking **Set**.

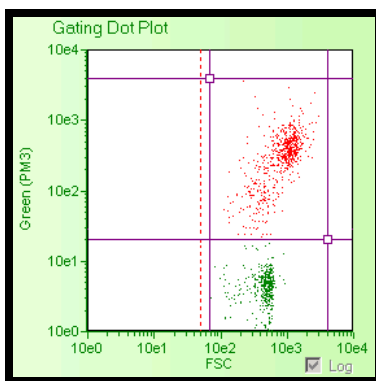


Gate set on live and dead target cells

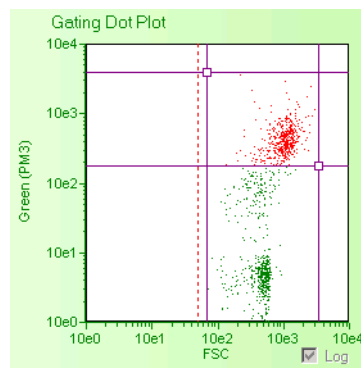
Examples of good and bad gates:



Gate too large



Gate optimal

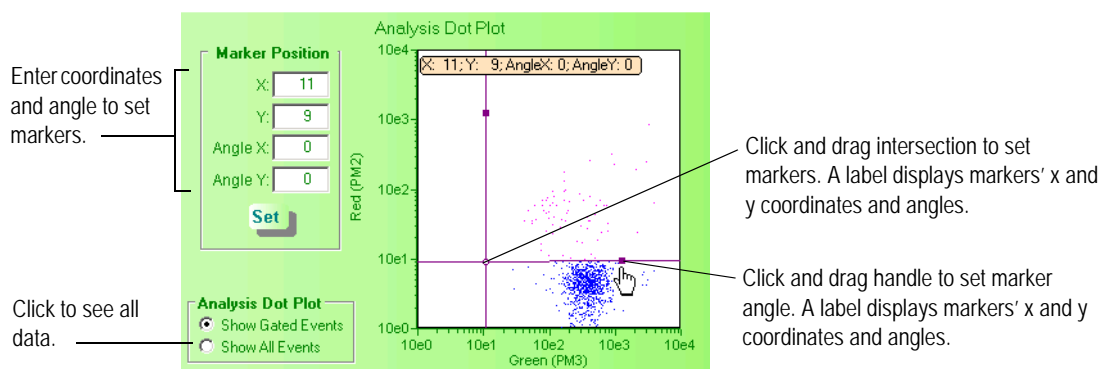


Gate too small

Quadrant Markers

Adjust the quadrant markers on the Green (PM3) vs Red (PM2) dot plot to generate statistics on the selected populations. The Analysis Results are automatically updated each time you move the markers.

- 6 To set the markers, position the cursor over the handle at the intersection, then click and drag to the desired location. Select **Show All Events** to see all the data. This option affects only what is displayed in the Analysis Dot Plot. It does not affect the statistics. You may also set the markers by entering the coordinates in the Marker Position fields and clicking **Set**.
- 7 If necessary, you can adjust the angle of the markers $\pm 44^\circ$ from their original location. Click and drag the handle (solid square) towards the end of the marker and tilt the marker to the desired angle. You may also angle the markers by entering the degrees in the Marker Position Angle fields and clicking **Set**.



Quadrant markers separating dead effectors (UL), dead targets (UR), live effectors (LL), and live targets (LR)

- 8 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 9 You can apply markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Then, click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 10 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog

box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.

■ **NOTE:** You system administrator may have configured guavaSoft Software to disable overwriting files.

Results are automatically exported to a CSV file that is given the same name as the FCS file.

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

- 11 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

CellToxicity Results

The results appear immediately after the acquisition of the sample is complete. The percent of target cells killed and statistics for each quadrant are displayed. Results include the count, cells/mL, % Total (all data in the dot plot) or % Gated (if a gate was enabled), and the mean fluorescence intensities for PM3 and PM2. Additionally, statistics for all target cells are displayed.

Quadrant Marker Statistics

Analysis Results					
% of Target Cells Killed: 21.95 %					
	Count	Cells/mL	% Gated	PM3 MFI	PM2 MFI
Live Effector Cells	0	0.00e00	0.00	0.00	0.00
Live Target Cells	480	1.51e05	77.17	427.31	5.28
Dead Target Cells	135	4.24e04	21.70	189.61	49.55
Dead Effector Cells	7	2.20e03	1.13	28.57	43.86
All Target Cells	615	1.93e05	98.87	375.13	15.00
Gated Events	Total Count	% Inside Gate		% Outside Gate	
622	1073	57.97		42.03	

Statistics include the percentage of target cells killed.

Results for each quadrant are displayed as a count, cells/mL, and percentage of the total or gated data in the dot plot. Additionally, the mean fluorescence intensities for PM3 and PM2 are displayed.

If a gate was set, the number of cells within the gate, as well as the percentage of cells inside and outside the gate are displayed.

CellToxicity quadrant marker statistics.

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

Quadrant	Staining	Population	Color
lower left	Green (PM3) negative, Red (PM2) negative	live effector cells	teal
lower right	Green (PM3) positive, Red (PM2) negative	live target cells	blue
upper right	Green (PM3) positive, Red (PM2) positive	dead target cells	pink
upper left	Green (PM3) negative, Red (PM2) positive	dead effector cells	purple

Exporting CellToxicity Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting CellToxicity Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.
The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

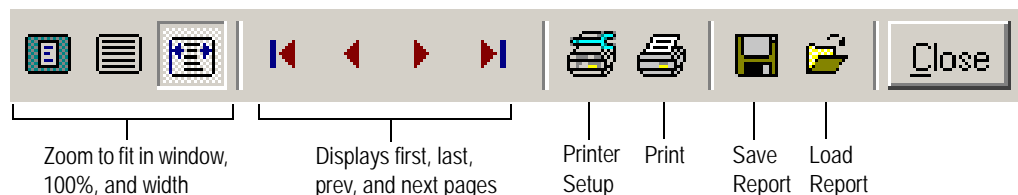
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analyze Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click Print Preview before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

Move the cursor over the icon to display the description.



CellToxicity Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

The screenshot shows the 'Sample Information' control panel. It has a title bar 'Sample Information'. Below it are four sections: 'Original Sample Data' with input fields for 'Current Sample #' (1), 'Sample ID' (A01), and 'Events to Acquire' (1000), plus a progress bar and an 'AutoSave FCS 2.0 Files' checkbox; 'Cell Count' showing 'Forward Scatter' with 'Total Count' (1015) and 'Cells/μL' (559.1); 'Flow Information' showing 'Flow Rate' (0.50 μL / s), 'Volume' (1.82 μL), and 'Duration' (00:03 mn:s); and 'Sample List Navigation' with 'Previous' and 'Next' buttons.

Original Sample Data	
Current Sample #	1
Sample ID	A01
Events to Acquire	1000
Progress Bar: [Red bar showing progress]	
<input type="checkbox"/> AutoSave FCS 2.0 Files	

Cell Count	
Forward Scatter	
- Total Count	1015
- Cells/μL	559.1

Flow Information	
Flow Rate :	0.50 μL / s
Volume :	1.82 μL
Duration	00:03 mn:s

Sample List Navigation	
Previous	Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 1000.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the number of cells (total count) detected by the FSC detector, as well as the concentration (cells/μL).

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

The screenshot shows the 'Unit Control' software window. It has a title bar 'Unit Control' and a menu bar. The main area is divided into five sections:

- Detection:** Includes a 'Laser' status indicator (red circle), 'Setting' for PM 3 (10 V) and PM 2 (10 V), and 'FSC Gain: x 8'.
- Pump Status:** Includes status indicators for 'End of Stroke', 'Waste', and 'Pump Reset' (all red). It also shows 'Position' (10 Steps) and 'Actual Speed' (0 Steps/s).
- Pump Action:** Includes status indicators for 'Pump on', 'Waste', 'Pump Reset', 'Purge', and 'Sample' (all green).
- Threshold parameters:** Includes 'Offset' (0.58 V) and 'Threshold' (50 units) for FSC.
- Sample List Navigation:** Includes 'Previous' and 'Next' buttons.

Detection

Displays laser status and the PM3 and PM2 voltage settings and the FSC gain.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold settings for each parameter.

■ **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field. Do not change the voltages from this panel. Use the sliders to adjust the PM3 and PM2 voltages.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava CellToxicity Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save CellToxicity spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save CellToxicity FCS file to another directory or give it a new name.
CellToxicity Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not properly loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in PM3 vs PM2 dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM3 dot plot but not in PM3 vs PM2 dot plot.	FSC vs PM3 gate excludes events.	Ensure FSC vs PM3 gate is set to include population of interest.

Problem	Possible Cause	Solutions
Poor resolution between target and effector cell populations.	<ol style="list-style-type: none"> 1. PM3 voltage too low to detect PM3 signal. 2. Incomplete staining with Guava CFSE reagent. 3. Debris included in analysis. 4. Guava CFSE reagent expired or not stored properly. 5. Background noise too high. 6. Some effector cells appear to be picking up Guava CFSE dye and can't be separated from target cells using a gate. 	<ol style="list-style-type: none"> 1. Adjust settings to increase PM3 signal. Adjust compensation settings. 2. Refer to package insert for proper staining instructions. 3. Adjust FSC threshold or set gate to remove debris. 4. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light. 5. Adjust settings to increase FSC threshold to remove debris. 6. Use quadrant markers to separate CFSE-positive effector cells from dead target cells.
Poor resolution between dead and live target cell populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with 7-AAD. 3. Too much Guava CFSE reagent used. 4. PM3 signal too high causing Guava CFSE reagent to bleed into PM2. 5. PM2 voltage too low to optimally detect 7-AAD signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check 7-AAD expiration date. 3. Repeat experiment, painting target cells with a lower reagent concentration. 4. Adjust settings to reduce PM3 voltage. Adjust compensation settings. 5. Adjust settings to increase PM2 voltage. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris.
Events appear off scale in dot plots.	PM3 and /or PM2 voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM3 and/or PM2 voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.

Problem	Possible Cause	Solutions
All samples contain target cells that are 100% killed.	FSC vs PM2 dot plot axis selected with gate enabled.	Select FSC vs PM3 dot plot and adjust gate if necessary to include all target cells.

CHAPTER 15

guava CellGrowth Assay

Introduction

One of the functions of the immune system is to respond to mitogenic stimuli. When this occurs, resting lymphocytes, which are exposed to a foreign antigen or chemical stimulus, are triggered to proliferate and differentiate into an active state with distinct regulatory or effector functions. Such cellular proliferation has traditionally been measured in vitro by incorporating ^3H -thymidine. This approach not only generates long-lived radioactive waste, but because of radiation-induced cell cycle arrest and apoptosis, it may also underestimate the number of cells capable of proliferating. Alternative colorimetric and flow cytometric assays have been developed, which circumvent the need for radionuclides.

The guava CellGrowth Assay uses two dyes—a cell permeant painting dye and a cell impermeant DNA binding dye. The cell painting dye diffuses freely into cells where intracellular esterases cleave off the acetate groups, converting it to a fluorescent, membrane impermeant dye. The painting dye is equally distributed among daughter cells because of covalent crosslinking to proteins through its succinimidyl groups. The stain is long lived allowing resolution of up to five cycles of cell division. The DNA binding dye, propidium iodide (PI), is then added at the end of the culture time to distinguish live from dead cells.

To run the assay, follow the instructions in the *Guava EasyCyte CellGrowth Kit* package insert to stain the cells. Acquire samples on the guava easyCyte HT System using guavaSoft Software. Data are displayed in two dot plots—a gating dot plot and an analysis dot plot. The gating dot plot, which shows forward scatter (FSC) versus Green (PM3) [or Red (PM2)] fluorescence, allows you to set a counting gate to acquire a specific number of resting and proliferating cells. The gate can also be used to eliminate unwanted events such as debris from the analysis. The analysis dot plot, which displays Green (PM3) versus Red (PM2), allows you to set quadrant markers for immediate on-screen results. Statistics for the two-color dot plot include the percent of live proliferating cells, and the individual quadrant statistics, which include the count, cell concentration, %Gated (or %Total), and mean fluorescence intensity (MFI) for live and dead resting cells, live proliferating cells, and dead stimulated cells. A Green (PM3) histogram is also available, allowing you to set two or three markers to analyze resting and up to two populations of proliferating cells.

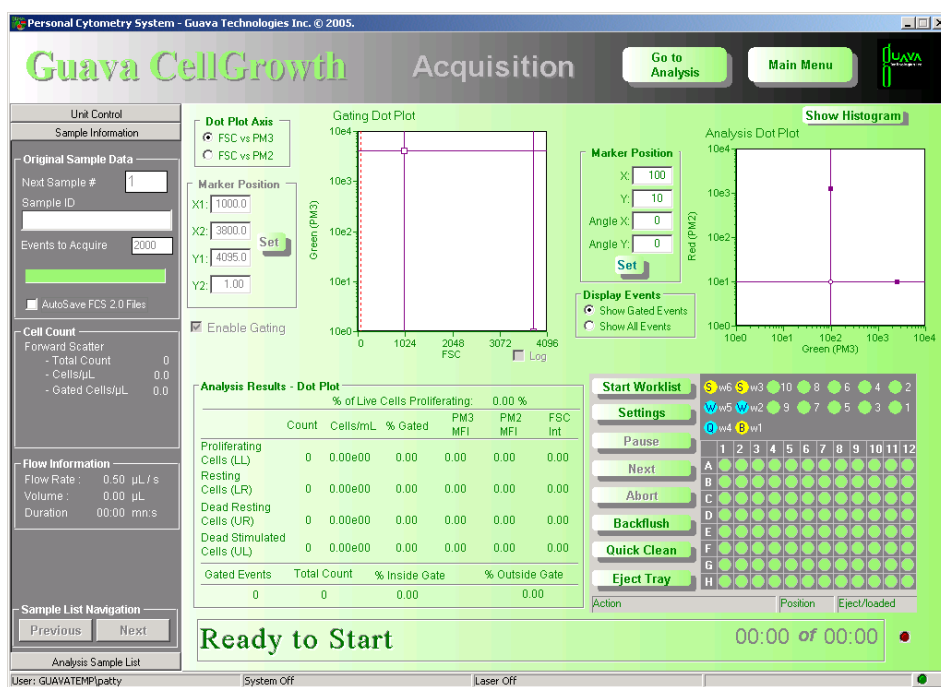
The CellGrowth data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after the sample is acquired or recalled later for

analysis. In addition to the saved data file, all results and the acquisition information are exported to a spreadsheet file.

Running the guava CellGrowth Assay

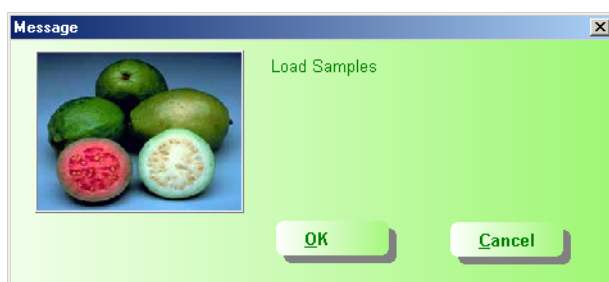
For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “CellGrowth Software Module Control Panels” on page 15-16.

- 1 Use WorkEdit Software to create a worklist file for the run. See “Creating a Worklist” on page 1-29 for information.
- 2 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop. Be sure to close WorkEdit Software before starting guavaSoft Software.
- 3 Click **CellGrowth** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.

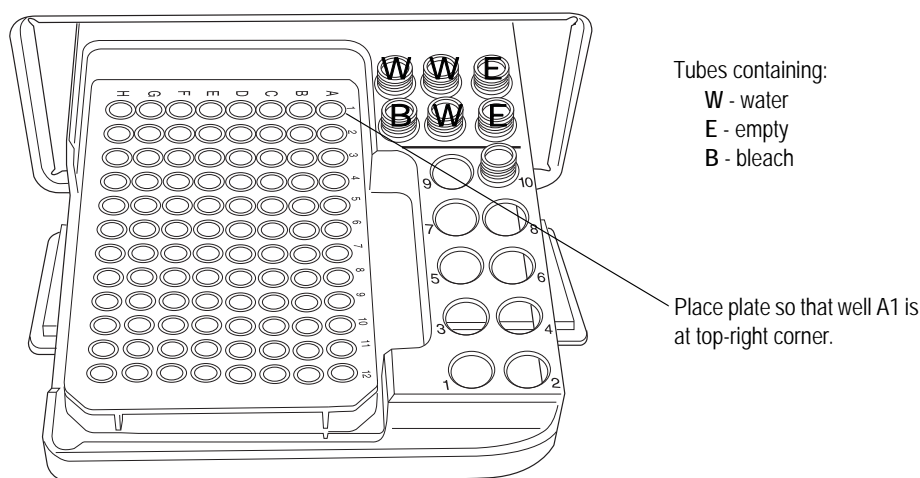


- 4 Prepare samples for analysis in a microplate or tubes. Refer to the *Guava EasyCyte CellGrowth Kit* package insert for information.
- 5 Click **Start Worklist**.
The sample tray ejects. A dialog box appears prompting you to load samples.

- ◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.

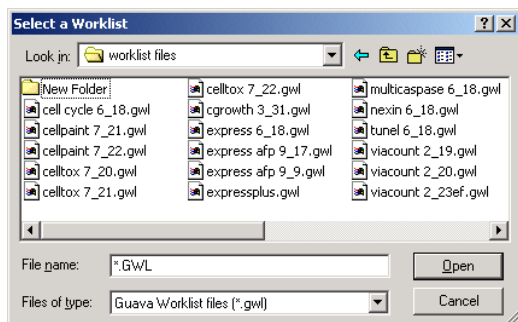


- 6 Place the microplate, any sample tubes, and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner.
Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).
 - Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).



- 7 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.
- ◆ **WARNING:** Keep the area clear as the tray loads.
 - ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

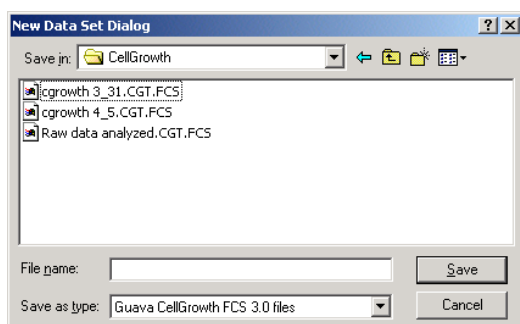
- 8 A dialog box appears prompting you to select the worklist file. Select the worklist file for the current run and click **Open**.



Select a worklist file and click Open.

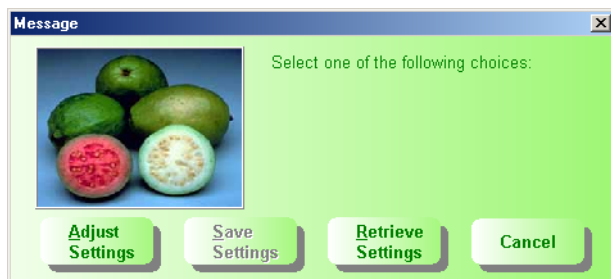
- 9 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

- **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.



Enter a file name for the data set and click Save.

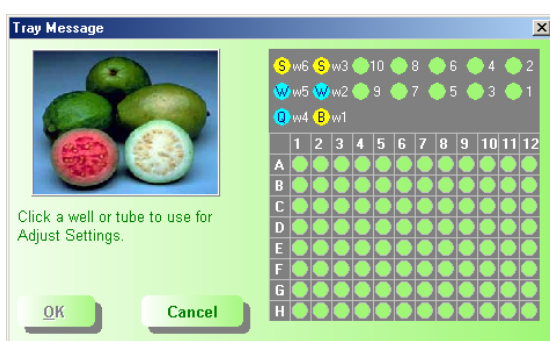
- 10 Select an instrument settings option.
- To adjust instrument settings, click **Adjust Settings**.
 - To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



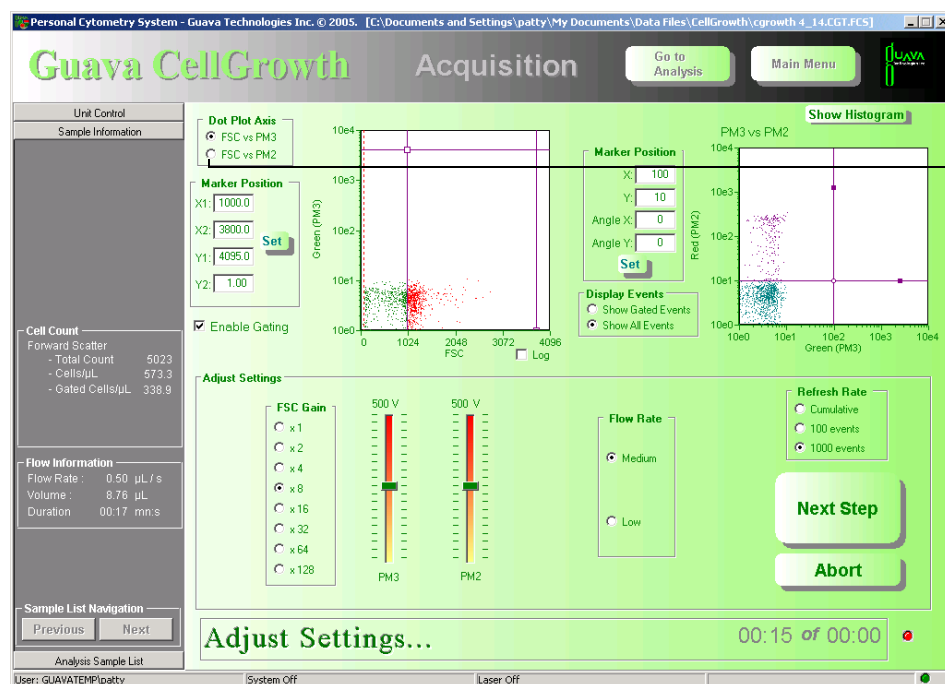
11 A dialog box appears prompting you to select the sample for adjusting settings. EMD Millipore recommends performing the adjust settings step three times with the following samples:

- first using resting (unstimulated), unpainted cells, PI-stained
- to set cells within the first decade on the PM3 and PM2 axes
- next using resting (unstimulated), painted, PI-stained cells
- to ensure that the green fluorescence is on scale—below 4000 and not saturating
- and finally using proliferating (stimulated), painted, PI-stained cells
- to ensure that the quadrant markers are set properly to distinguish live resting, live proliferating, dead resting, and dead stimulated

Click to select the well/tube for the first adjust settings sample (unstimulated/unpainted), then click **OK**.



The Adjust Settings screen appears, allowing you to adjust the threshold and optimize the display of the data.



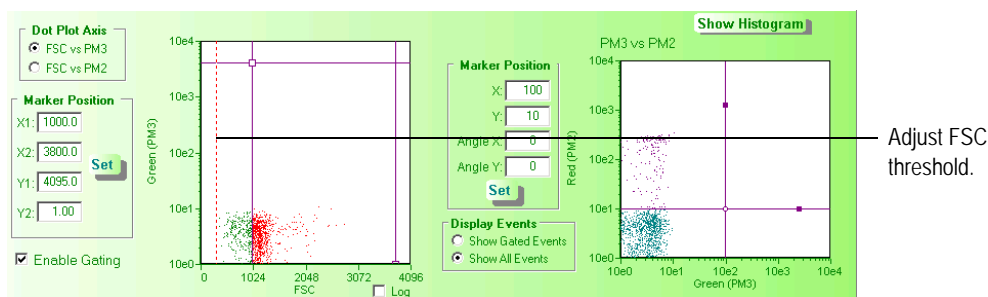
Change plot parameters.

- 12 Open the Sample Information control panel and check the Cells/ μ L field. For accurate counting, the concentration should be between 10 and 500 cells/ μ L.

■ **NOTE:** If the concentration is greater than 500, click **Abort**, then click **Eject Tray**. Dilute the sample with Guava® CellGrowth PI Reagent diluted 1:40 with serum-containing medium to lower the concentration to <500 cells/ μ L. Click **Load Tray**, then **Settings**, then **Adjust** or **Retrieve Settings**.

- 13 To fine tune the settings, you can make the following adjustments once events start to appear on the screen:

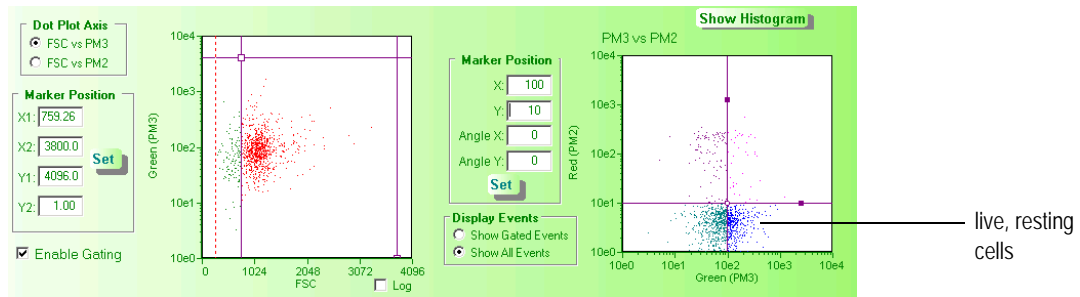
- Select the applicable dot plot axes—FSC vs PM3 or FSC vs PM2.
- Set the **Refresh Rate** to the number of events you want to display.
- Set the **Flow Rate** to Low (0.24 μ L/s) or Medium (0.59 μ L/s). The recommended flow rate is Medium.
- Use the **FSC Gain** settings to reduce or amplify the FSC signal so that the unstimulated, unpainted cells are positioned at about 1024 on the FSC axis.



unstimulated, unpainted cells

- **NOTE:** If you wish to display the data in a FSC log scale, click the Log check box. This is the only chance you will have to select the FSC scale.
- To adjust the FSC threshold, click and drag the threshold marker up the FSC axis of the FSC vs Green (PM3) [or FSC vs Red (PM2)] dot plot until it is approximately 1/3 the distance from the y axis to the left side of the resting cell population.
- **NOTE:** When setting the threshold, leave enough debris to ensure that you are not excluding any cells.
- Adjust the voltages (using the PM3 and PM2 sliders or the arrow keys on the keyboard) so that the unstimulated, unpainted population is positioned in the lower-left corner of the Green (PM3) vs Red (PM2) plot. These cells should be evenly distributed between 10e0 and 10e1 on both scales. Adjust the PMTs by starting from a lower voltage setting and gradually increasing the voltage.

Click **Next Step** to advance to the data acquisition screen. Click **Settings**, select the well/tube for the second adjust settings sample (unstimulated/painted), then click **OK**.

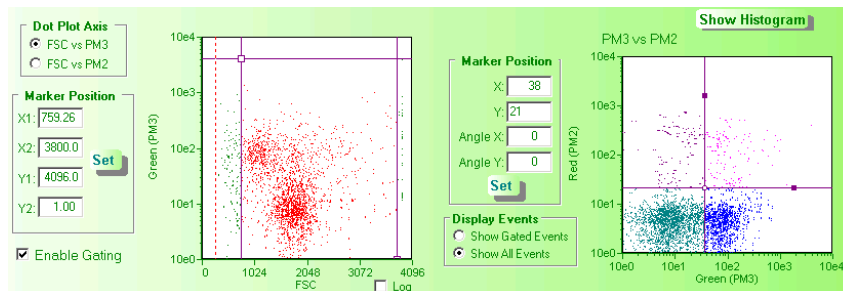


unstimulated, painted cells

- On the Gating Dot Plot with the parameters set to FSC vs Green (PM3), adjust the gate to include the unstimulated, painted cells. The gate is used as a counting gate. Events that are included in the gate are counted toward the number of Events to Acquire. For example, if the Events to Acquire is set to 2000, acquisition is complete when 2000 events have passed through the gate. Ensure the live, resting cells are on scale in the PM3 parameter. If not, adjust the PM3 voltage.
- NOTE: It may help to view FSC vs PM2 when setting the left side of the gate. Red (PM2) displays the PI-stained debris and small dead cells, which you may want to exclude.
- NOTE: If you turn off the counting gate, you may need to increase the number of Events to Acquire to ensure acquiring a sufficient number of resting and proliferating cells.

Refer to “Dot Plot Gate” on page 15-10 for more information on setting the gate.

Click **Next Step** to advance to the data acquisition screen. Click **Settings**, select the well/tube for the third adjust settings sample (stimulated/painted), then click **OK**.



stimulated, painted cells

- Adjust the right side of the gate, if necessary to include all proliferating cells, which display a higher FSC value than the resting cells. Do not include saturated cells in the gate.
- Adjust the quadrants to separate the four populations—live proliferating (lower left), dead stimulated (upper left), live resting (lower right), dead resting (upper right). Refer to “Quadrant Markers” on page 15-11.

14 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.

15 Click **Resume**.

The system acquires the first sample and displays the results. The remaining samples are automatically acquired. The tube/well currently being acquired is highlighted in red.



sample results

■ ACQUISITION NOTES

- If the sample is too dilute or too concentrated, a message appears indicating that the counting accuracy may be compromised. Refer to the *Guava EasyCyte CellGrowth Kit* package insert for the recommended cell concentration and proper dilution instructions.
- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 4 minutes.

16 If you need to adjust the markers prior to acquiring the remaining samples, click **Pause** during the acquisition of the first sample. The system will pause when the acquisition is complete. Once the system is paused, adjust the markers, then click **Resume**. Refer to “Quadrant Markers” on page 15-11 for information.

The system automatically performs a Quick Clean at the end of the assay.

guava CellGrowth Analysis

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

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

- **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

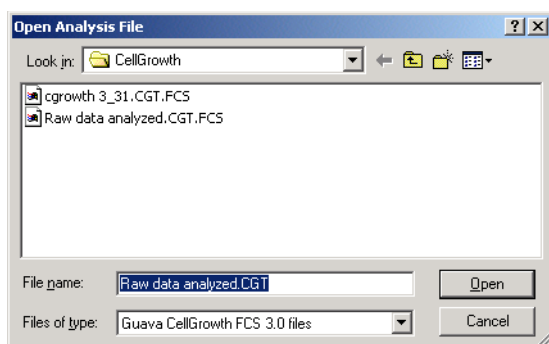
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also log comments or view the event log.

However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

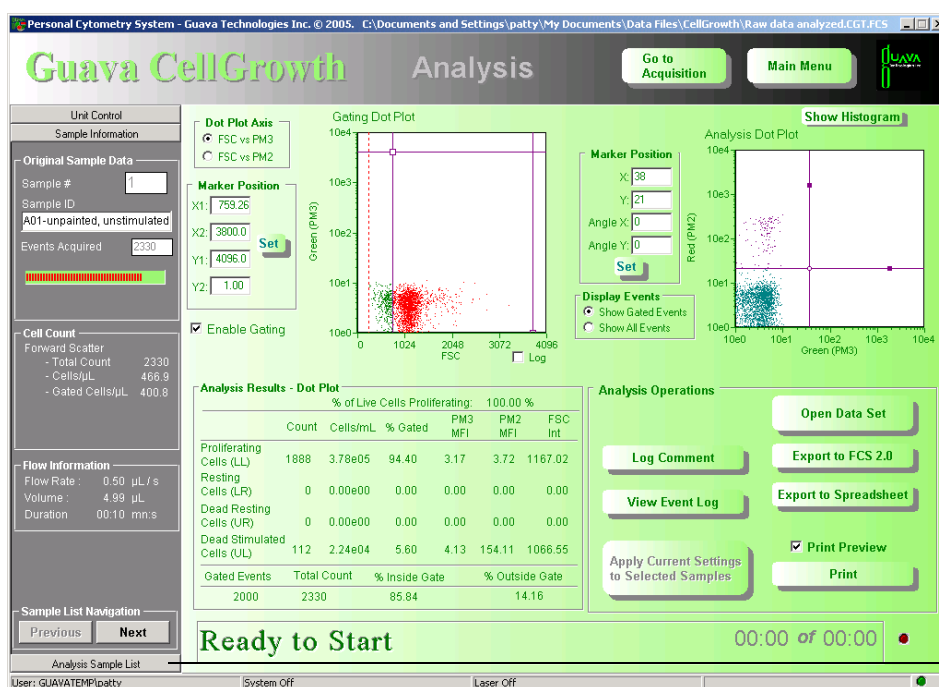
- 1 Click **CellGrowth** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- 3 Click **Open Data Set**. Select an FCS file for analysis and click **Open**.



The data and results for the first sample in the data set appear. The marker settings appear as they were when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



Analysis Sample List control panel

Dot Plot Gate

During acquisition, the dot plot gate is used as a counting gate. Events that are included in the gate are counted toward the number of Events to Acquire. During analysis, you may disable the gate or adjust it. However, once you adjust the gate, it is no longer considered a counting gate. In other words the number of events in the gate will not match the number of events acquired.

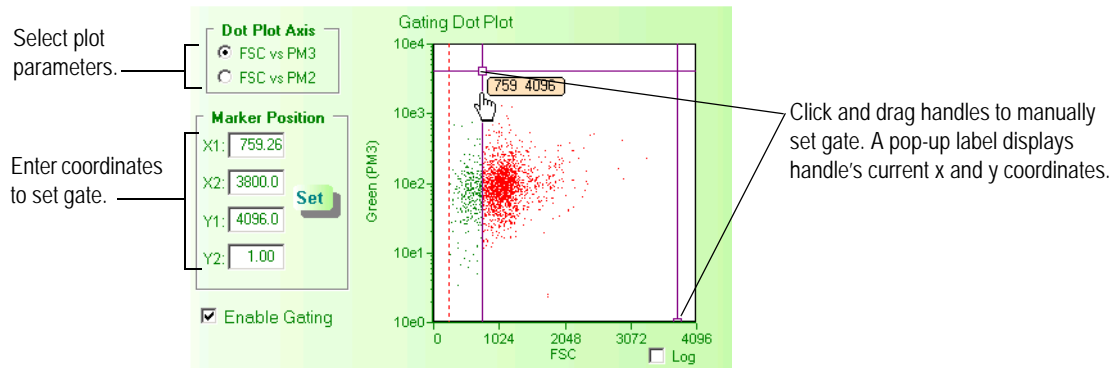
- 4 Select the plot parameters (FSC vs PM3) by clicking the appropriate button under Dot Plot Axis to the left of the plot. Click Enable Gating to turn on the gate.

- **NOTE:** You may want to view FSC vs PM2 when setting the gate to help you eliminate debris and dead cells. You may use the gate to include only live cells and use the histogram to determine the proliferating statistics.

- 5 Set the gate on the unstimulated, painted sample. Then check the gate on the painted, stimulated sample to ensure it holds for that sample as well. Set the left side of the gate to exclude debris. Set the right side of the gate to include the proliferating cells, which have a higher FSC. Be sure to *exclude* the cells saturated to the far right, which may be cell aggregates.

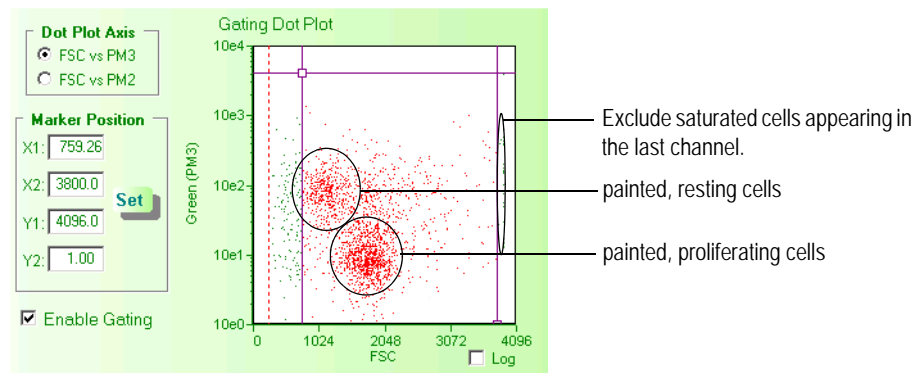
To set a gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-right handle. Events that fall within the center rectangle and appear in red are included in the gate.

You may also set the gate by entering the coordinates in the Marker Position fields and clicking **Set**.



Set gate on unstimulated, painted cells.

Check the gate on the stimulated, painted cells to ensure all proliferating cells are included in the gate.



Check gate on stimulated, painted cells.

Quadrant Markers

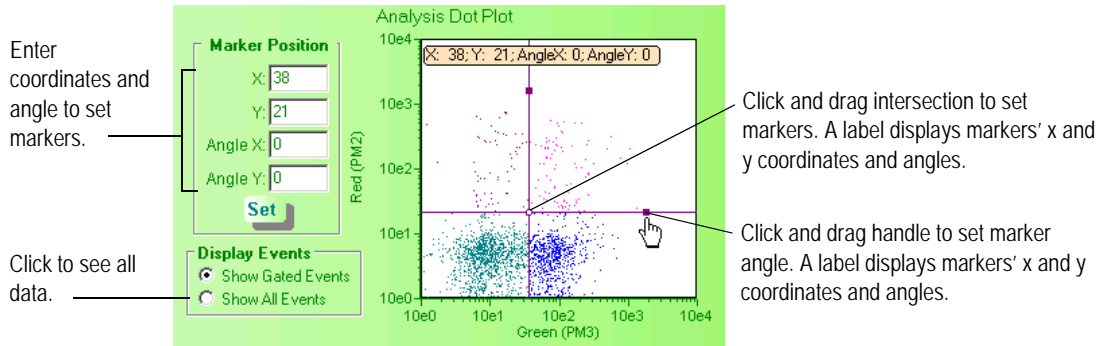
Adjust the quadrant markers on the Green (PM3) vs Red (PM2) dot plot to generate statistics on the selected populations. The Analysis Results are automatically updated each time you move the markers.

- 6 To set the markers, position the cursor over the handle at the intersection, then click and drag to the desired location. Select Show All Events if you want to see all the data. This option affects only what is displayed in the Analysis Dot Plot. It does not

affect the statistics. You may also set the markers by entering the coordinates in the Marker Position fields and clicking **Set**.

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

You may also angle the markers by entering the degrees in the Marker Position Angle fields and clicking **Set**.



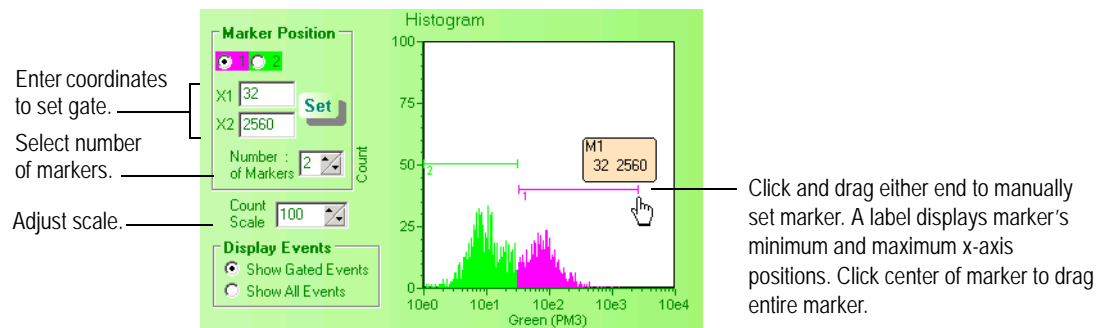
Quadrant markers separating dead stimulated (UL), dead resting (UR), live proliferating (LL), and live resting (LR).

Histogram Marker

You may use the histogram markers instead of the quadrant markers to generate statistics. The histogram markers allow you to get statistics on any population within the Green (PM3) parameter. You can set two or three markers to identify and generate statistics on the resting cell population (M1) and up to two proliferating populations (M2 and M3). If you are interested only in the live populations, set the gate to include live cells only [Red (PM2) negative]. The Analysis Results are automatically updated each time you move the markers.

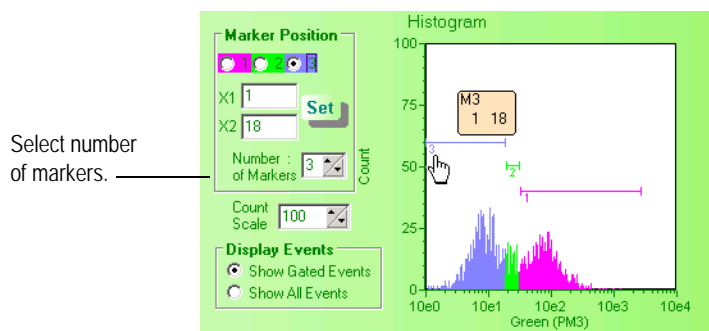
- To set histogram markers 1 and 2, click and drag either side of the marker to size it. Click the horizontal line to drag the entire marker. You cannot overlap markers 1 and 2. Events that fall within marker 1 appear in pink, events that fall within marker 2 appear in light green.

You may also set the markers by entering the coordinates in the Marker Position fields and clicking **Set**.



Green (PM3) histogram with marker 1 set on resting cells and marker 2 set on proliferating cells.

- 8 To set a third marker, click the Number of Markers up arrow. Adjust the marker appropriately. You can extend marker 3 to any position.



Green (PM3) histogram with marker 3 activated.

- 9 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the list, or use the keyboard arrow keys to select samples.
- 10 You can apply markers settings from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Then, click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
- 11 When you have finished analyzing the samples in the current file, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
 - **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting files.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
 - **NOTE:** Exporting to the CSV file may take several minutes, especially with a large number of samples.
- 12 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

CellGrowth Results

The results appear immediately after the acquisition of the sample is complete. The percent of proliferating cells and statistics for each quadrant are displayed. Results include the count, cells/mL, % Total (all data in the dot plot) or % Gated (if a gate was enabled), and the mean FSC intensity and mean fluorescence intensities for PM3 and PM2. The total number

of events acquired, number of events in the gate, and the percentage of events inside and outside the gate are also displayed.

Quadrant Marker Statistics

Analysis Results - Dot Plot						
% of Live Cells Proliferating: 100.00 %						
	Count	Cells/mL	% Gated	PM3 MFI	PM2 MFI	FSC Int
Proliferating Cells (LL)	1888	3.78e05	94.40	3.17	3.72	1167.02
Resting Cells (LR)	0	0.00e00	0.00	0.00	0.00	0.00
Dead Resting Cells (UR)	0	0.00e00	0.00	0.00	0.00	0.00
Dead Stimulated Cells (UL)	112	2.24e04	5.60	4.13	154.11	1066.55
Gated Events	Total Count	% Inside Gate		% Outside Gate		
	2000	2330		85.84		
				14.16		

Statistics include the percentage of live, proliferating cells.

Results for each quadrant are displayed as a count, cells/mL, and percentage of the total or gated data in the dot plot. Additionally, the mean FSC intensity and mean fluorescence (PM3 and PM2) intensities are displayed.

If a gate was set, the number of cells within the gate, as well as the percentage of cells inside and outside the gate are displayed.

CellGrowth quadrant marker statistics.

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

Quadrant	Staining	Population	Color
lower left	Green (PM3) negative, Red (PM2) negative	live, proliferating cells	green
lower right	Green (PM3) positive, Red (PM2) negative	live, resting cells	teal
upper right	Green (PM3) positive, Red (PM2) positive	dead, resting cells	pink
upper left	Green (PM3) negative, Red (PM2) positive	dead, stimulated cells	purple

Histogram Marker Statistics

M1 provides statistics on the resting cell population. M2 and M3 provide statistics on the proliferating populations. Results include the statistics within each marker—count, cells/mL, percentage of gated (or total), PM3 mean fluorescence intensity, and FSC intensity. If a gate was set, the number of cells within the gate and the percentage of cells inside and outside the gate are also included.

Analysis Results - Histogram					
	Count	Cells/mL	% Gated	PM3 MFI	FSC Int
Resting Cells (M1)	811	2.99e05	40.55	106.99	1582.79
Proliferating Cells 1 (M2)	1189	4.39e05	59.45	10.94	1813.28
Proliferating Cells 2 (M3)	-	-	-	-	-
Gated Events	Total Count	% Inside Gate		% Outside Gate	
	2000	2159		92.64	
				7.36	

Results for each marker are displayed as a count, cells/mL, and percentage of the total or gated data in the dot plot. Additionally, the mean FSC intensity and PM3 fluorescence intensity are displayed.

If a gate was set, the number of cells within the gate, as well as the percentage of cells inside and outside the gate are displayed.

Exporting CellGrowth Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

■ **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.

- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting CellGrowth Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.
The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

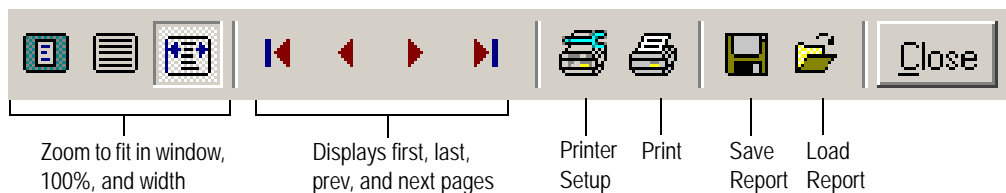
Printing Results

You can print results from the Analysis screen only. If you are currently at the Acquisition screen, click **Pause**, then **Go to Analysis**, then **Print**.

- 1 Select any sample from the Analysis Sample List and click **Print**.
The Print dialog box appears. If you wish to preview the results before you print, click **Print Preview** before clicking **Print**.
- 2 Click **OK** in the Print dialog box, or click the Print icon from the Print Preview window.

Print Preview Icons

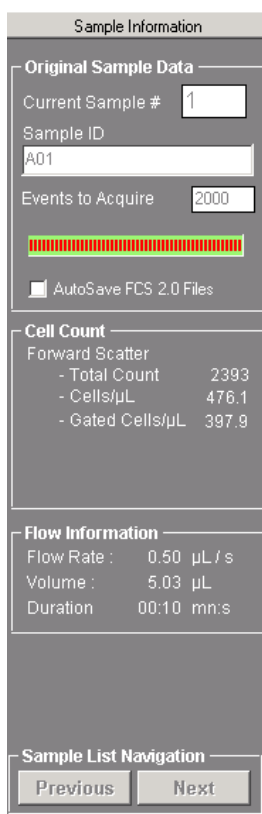
Move the cursor over the icon to display the description.



CellGrowth Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information



The screenshot shows the 'Sample Information' control panel. It has a title bar 'Sample Information' and four main sections: 'Original Sample Data', 'Cell Count', 'Flow Information', and 'Sample List Navigation'. The 'Original Sample Data' section contains input fields for 'Current Sample #' (set to 1), 'Sample ID' (set to A01), and 'Events to Acquire' (set to 2000), along with a progress bar and an 'AutoSave FCS 2.0 Files' checkbox. The 'Cell Count' section displays 'Forward Scatter' data: Total Count (2393), Cells/μL (476.1), and Gated Cells/μL (397.9). The 'Flow Information' section shows 'Flow Rate : 0.50 μL / s', 'Volume : 5.03 μL', and 'Duration 00:10 mn:s'. The 'Sample List Navigation' section has 'Previous' and 'Next' buttons.

Original Sample Data	
Current Sample #	1
Sample ID	A01
Events to Acquire	2000
<input type="checkbox"/> AutoSave FCS 2.0 Files	

Cell Count	
Forward Scatter	
- Total Count	2393
- Cells/μL	476.1
- Gated Cells/μL	397.9

Flow Information	
Flow Rate :	0.50 μL / s
Volume :	5.03 μL
Duration	00:10 mn:s

Sample List Navigation	
Previous	Next

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample.
- Displays the number of events to acquire. The default is 2000.
- The progress bar provides an estimate of the target event count during acquisition.

Cell Count

Displays the number of cells (total count) detected by the FSC detector, as well as the concentration (cells/μL) and concentration of cells within the gate.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Unit Control

The screenshot shows the 'Unit Control' software interface. It has a title bar 'Unit Control' and several sections:

- Detection:** Includes a 'Laser' status indicator (red dot), 'Setting' for PM 3 (500 V) and PM 2 (500 V), and 'FSC Gain: x 8'.
- Pump Status:** Includes indicators for 'End of Stroke' (red), 'Waste' (red), and 'Pump Reset' (red). It also shows 'Position' (10 Steps) and 'Actual Speed' (0 Steps/s).
- Pump Action:** Includes indicators for 'Pump on' (red), 'Waste' (yellow), 'Pump Reset' (yellow), 'Purge' (yellow), and 'Sample' (green). It also shows 'Speed' (0 Half Steps/s).
- Threshold parameters:** Includes 'Offset' (1.04 V) and 'Threshold' (239 units) for FSC.
- Sample List Navigation:** Includes 'Previous' and 'Next' buttons.

Detection

Displays laser status, the PM3 and PM2 voltage settings, and the FSC gain.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the FSC offset and threshold settings.

■ **NOTE:** You can edit the Threshold units during the adjust settings step by typing a value in the field.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava CellGrowth Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save CellGrowth spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save CellGrowth FCS file to another directory or give it a new name.
CellGrowth Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled.	1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	1. Sample tube or plate not properly loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate).	1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in PM3 vs PM2 dot plot or PM3 histogram.	1. Laser not warmed up. 2. Threshold set too low or debris included in gate.	1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM3 dot plot but not in PM3 vs PM2 dot plot.	FSC vs PM3 gate excludes events.	Ensure FSC vs PM3 gate is set to include population of interest.
Events appear in FSC vs PM2 dot plot but not in PM3 histogram.	FSC vs PM2 gate excludes events.	Ensure FSC vs PM2 gate is set to include population of interest.

Problem	Possible Cause	Solutions
Poor resolution between resting and proliferating cell populations.	<ol style="list-style-type: none"> 1. PM3 voltage too low to detect PM3 signal. 2. Incomplete staining with guava painting reagent. 3. Debris included in analysis. 4. guava painting reagent expired or not stored properly. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase PM3 signal. 2. Refer to package insert for proper staining instructions. 3. Adjust FSC threshold or set gate to remove debris. 4. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light. 5. Adjust settings to increase FSC threshold to remove debris.
Poor resolution between dead and live cell populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with PI. 3. Too much guava painting reagent used. 4. PM3 signal too high causing guava painting reagent to bleed into PM2. 5. PM2 voltage too low to optimally detect PI signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check PI expiration date. 3. Repeat experiment, painting cells with a lower reagent concentration. 4. Adjust settings to reduce PM3 voltage. 5. Adjust settings to increase PM2 voltage. 6. Adjust settings to increase FSC threshold to remove debris.
Events appear off scale in dot plots.	PM3 and/or PM2 voltages and/or FSC gain set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM3 and/or PM2 voltages and/or FSC gain so positive populations appear on scale. Repeat Adjust Settings.

CHAPTER 16

guava RapidQuant Assay

Introduction

The guava RapidQuant™ Assay provides quantification of human and mouse IgG antibodies, typically found in hybridoma supernatants, using protein-bound beads.

The Guava RapidQuant™ Human IgG and Guava RapidQuant™ Mouse IgG Kits contain IgG Capture Beads (Protein G-bound beads), which bind human or mouse antibodies (analyte) through the Fc region. The bound analyte is detected and quantified using the FITC-labeled goat anti-human (or goat anti-mouse) IgG antibody provided in the kit. A human (or mouse) antibody standard (whole IgG), also provided with the kit, is used at different concentrations to obtain a standard curve. The human IgG kit quantifies IgG₁, IgG₂, IgG₃, and IgG₄ antibodies in the range of 0.5–20 µg/mL using the single standard provided. The mouse IgG kit quantifies IgG₁, IgG_{2a}, and IgG_{2b} antibodies in the range of 2.5–40 µg/mL using the single standard provided. It also detects IgG₃, but at a lower efficiency than the other subtypes. Both kits work only with intact antibodies that include an Fc region. If the antibody to be tested is an F(ab')₂, Fab, sFv, or other variant without an Fc region, these kits should not be used.

To run the assay, stain samples using the appropriate kit. Define samples as standard, control, unknown, or blank. Acquire the samples on the guava easyCyte HT System using guavaSoft Software. The data are displayed in two plots—a Forward Scatter (FSC-HLog) vs Green Fluorescence (GRN-HLog) dot plot and a Green Fluorescence (GRN-HLog) histogram. The software automatically plots a standard curve and calculates a linear regression equation, then determines the unknown concentrations based on the standard curve. If necessary, you may set a gate to eliminate any undesirable populations, or eliminate samples from the analysis, then recalculate.

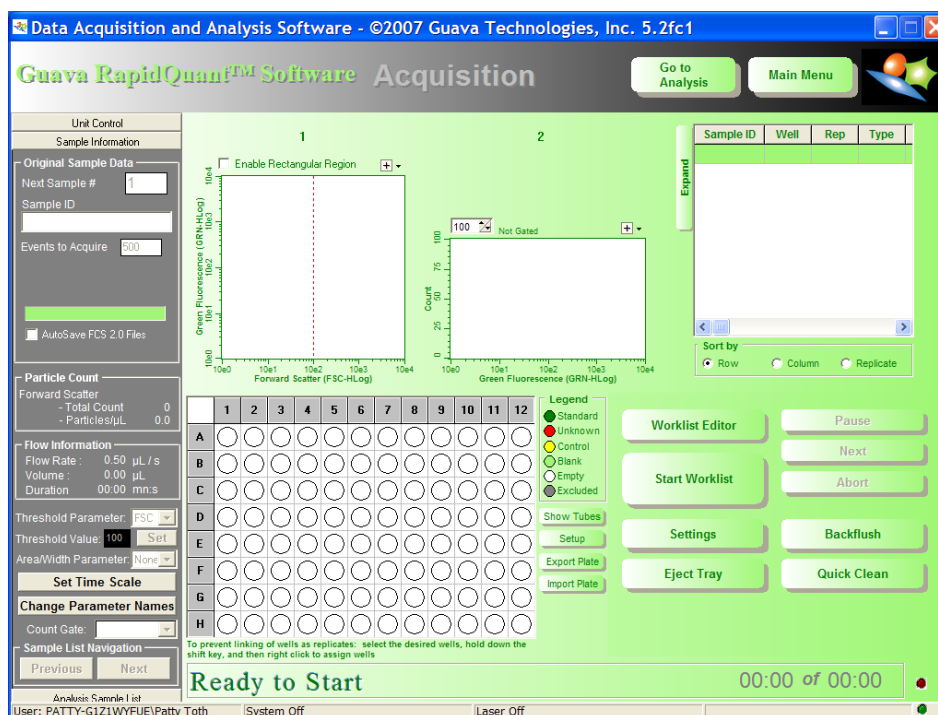
Histogram and dot plot statistics include the bead count, % total, % gated, geometric mean, mean, median, %CV, and particles/mL.

The RapidQuant data for all samples within a data set are saved to an FCS 3.0 file. The data can be analyzed immediately after all samples are acquired using guavaSoft Software, or later using guavaSoft Software or an FCS 2.0-compatible program, if you selected to save FCS 2.0 files. In addition to the saved data file, user-selectable statistics, instrument settings, and the acquisition summary information are exported to a spreadsheet file.

Running the guava RapidQuant Assay

For details on software screen buttons, see “Acquisition Buttons” on page 1-13. For details on the information displayed within the control panels, see “guava RapidQuant Software Module Control Panels” on page 16-20.

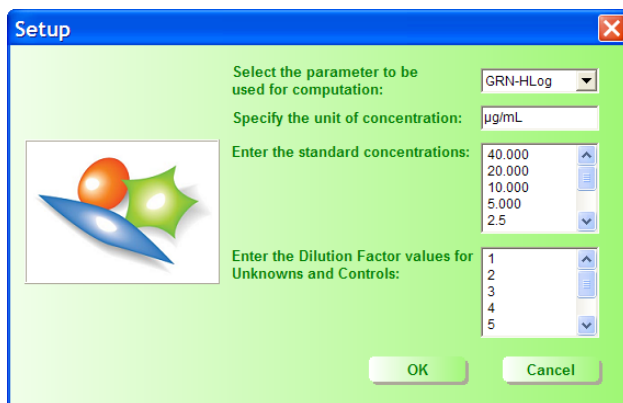
- 1 Open guavaSoft Software by double-clicking the guavaSoft icon on the desktop.
- 2 Click **RapidQuant** from the main menu.
If the tray is ejected, click **OK** in the dialog box to load the tray. Be sure to keep the area clear as the tray loads.
Allow the easyCyte HT System to warm up for 10 minutes before acquiring samples.



- 3 Prepare samples for analysis in a microplate or 0.5-mL tubes. Refer to the *Guava RapidQuant Human IgG Kit* or *Guava RapidQuant Mouse IgG Kit* package insert for information.
- 4 Click **Setup** to the right of the plate map.
Setup allows you to enter details about the samples and how the plate is set up.
 - **NOTE:** If you wish to apply a previously saved plate template to this run, click **Import Plate** to the right of the plate map. Select the file name for the data set, then click **Open**. You can also wait until after acquisition is complete to define the plate. In either case, proceed to step 7 on page 16-4.
 - Select GRN-HLog for the computation parameter.
 - Enter μg/mL for the unit of concentration.
 - Ensure that all the standard concentrations used in your plate are present in the standard concentration list. The default list includes 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0.313. If necessary, you can add a standard concentration (a maximum of 20)

to the list by scrolling to the bottom of the list, placing the cursor in the empty row, and entering a new value.

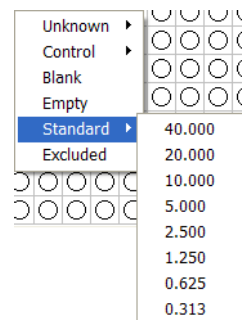
- Ensure that the dilution factors used for your unknowns and controls are included in the dilution factor list. The default list includes 1, 2, 3, 4, 5, 8, and 10. If necessary, you can add a dilution factor to the list by scrolling down to the bottom of the list, placing the cursor in the empty row, and entering a new value.
- **NOTE:** The dilution factor applies only to unknowns and controls. Prepare standards according to the instructions in the package insert. Do not dilute.



- 5 Define each well in the plate map. Each well must be designated as Standard, Unknown, Control, Blank, or Excluded. Any wells not defined are designated Empty by default.

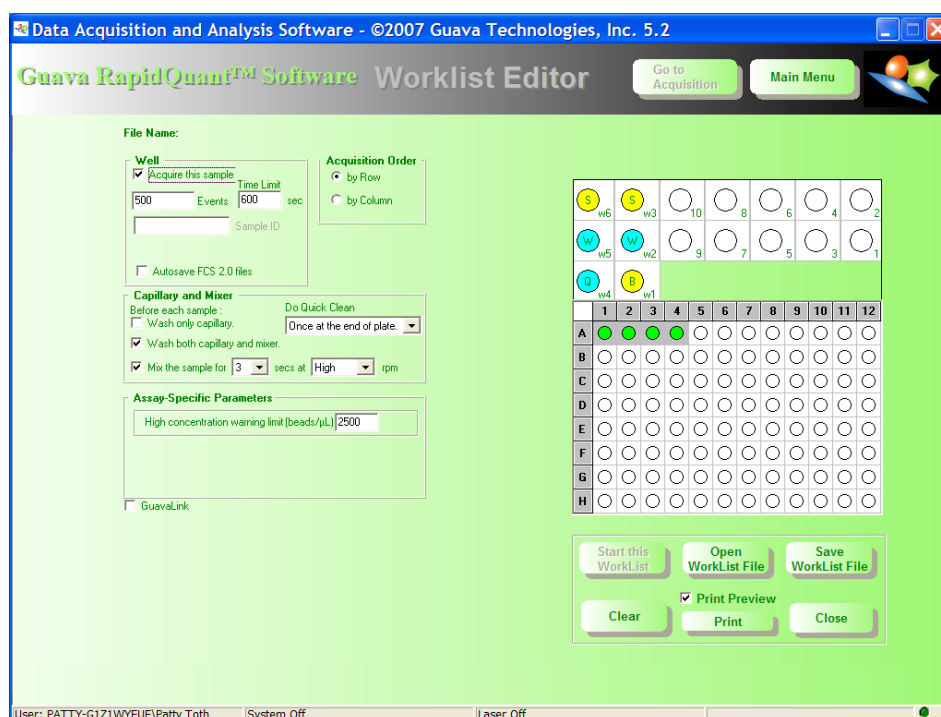
Right-click the well, then select the appropriate sample type. You may also click and drag to select a group of wells.

- **NOTE:** Assigning well types can be done in the acquisition or analysis prior to running the plate, or after the plate has been run.
- For unknowns and controls, select the appropriate dilution factor. Right-click individual wells and choose the sample type, then the dilution factor from the pop-up menu. Select 1 for undiluted. Wells highlighted together will be treated as replicates.
- For standards, select the appropriate concentration. Right-click individual wells and choose the appropriate concentration from the pop-up menu. Wells highlighted together will be treated as replicates



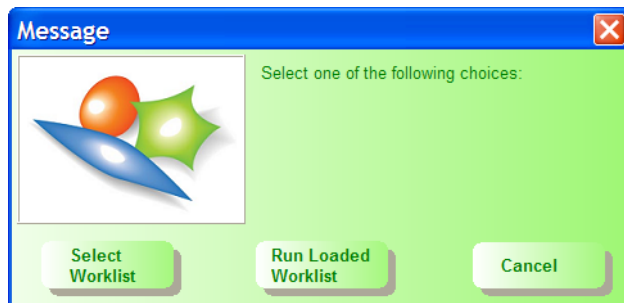
- **NOTE:** To prevent linking of wells as replicates, select the desired wells, hold down the shift key, and then right-click to assign wells. This will assign all of the selected wells as independent samples (ie, not replicates) and thus assigns them different sample numbers.
- **Standard** – A standard will be used to calculate the standard curve. When selecting standards, you will need to select the appropriate concentration from the pop-up menu. A well designated as a standard appears green.

- **Unknown** – An unknown is a sample whose concentration is determined using the standard curve. Unknowns have dilution factors associated with them. A well designated as an unknown appears red.
 - **Controls** – Controls are negative controls consisting of beads with FITC goat anti-human [or mouse] IgG antibody only. A well designated as a control appears yellow.
 - **NOTE:** If you wish to run positive controls, you must assign those wells as unknowns.
 - **Blank** – A blank is a sample containing beads only (with no FITC goat anti-human [or mouse] IgG antibody). A well designated as a blank appears light green.
 - **Empty** – A blank is a well that has no sample, has not yet been defined, or was not acquired. A well designated as a blank appears white.
 - **Excluded** – Samples which were acquired but you do not wish to consider in the analysis. A well designated as excluded appears gray.
- 6 If you wish to use this plate again you may save it as a template. Click **Export Plate** to the right of the plate map. Enter a file name for the plate map template, then click **Save**.
- 7 Click **Worklist Editor**. The Worklist Editor application opens allowing you to define the worklist parameters and select wells for acquisition.



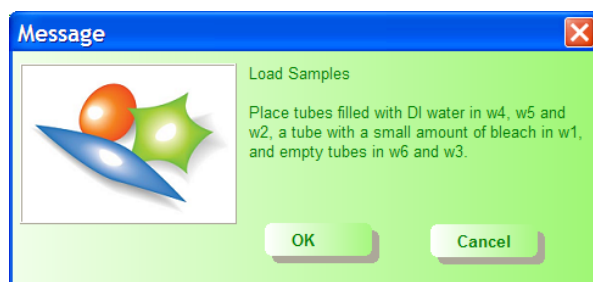
- **NOTE:** If you already created a worklist, you can click **Start Worklist**. A dialog box prompts you to select a worklist or run the loaded worklist. The tray ejects and dialog

boxes appear prompting you to load samples, select the worklist file, save the data set, and select the sample to adjust instrument settings.



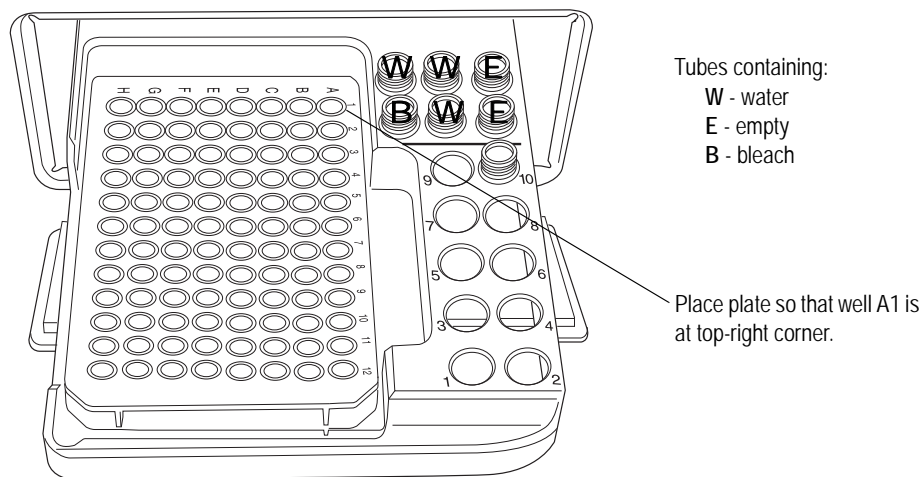
- 8 Define the worklist parameters for the run and click **Start this Worklist**. See “Creating a Worklist” on page 1-29 for more information on setting up a worklist. See “Worklist Editor Software” on page 1-27 for information on the buttons below the plate map. When you start a worklist, the sample tray ejects. A dialog box appears prompting you to load samples.

◆ **WARNING:** Keep the area in front of the tray clear as the tray ejects.



- 9 Place the microplate, any sample tubes (0.5-mL microcentrifuge tubes), and the cleaning tubes in the tray. Make sure well A1 of the plate is in the top-right corner. Load the following 1.5-mL microcentrifuge tubes in these positions:
 - Load tubes containing water in positions w2, w4, and w5 (for Quick Clean and washing the capillary and mixer).
 - **IMPORTANT:** Always load a tube filled with water in position w4 (for Quick Clean).
 - Load empty tubes in positions w3 and w6 (for spinning/drying the mixer).

- Load a tube containing 100 μ L of bleach in position w1 (for performing a backflush).

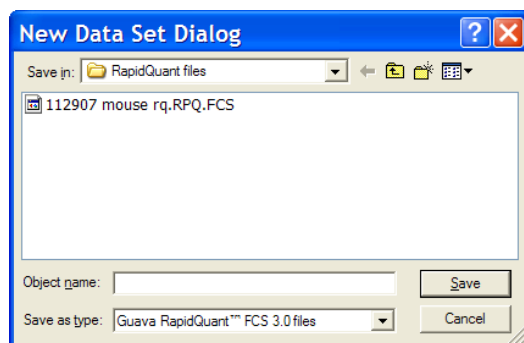


10 Click **OK** in the Load Samples dialog box after you are finished loading samples and cleaning tubes to load the sample tray.

- ◆ **WARNING:** Keep the area clear as the tray loads.
- ◆ **WARNING:** Always use the **Eject Tray** button in guavaSoft Software to open the door. Click **Pause** first, if necessary. Never open the door with your fingers.

11 Select the folder where you want to save the file, and enter a file name. Click **Save**. The file name you enter for the FCS file will also be used for the spreadsheet (.csv) file. If you wish, you may select an existing data file and either overwrite it or append it with the data from this session.

- **NOTE:** Your system administrator may have configured guavaSoft Software to disable overwriting and/or appending files.

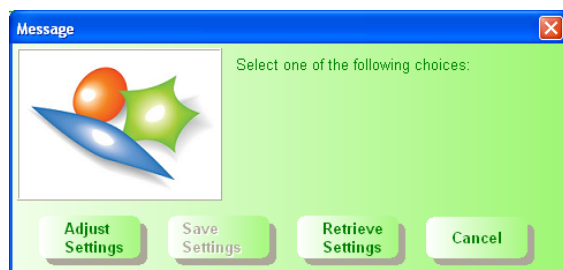


Enter a file name for the data set and click Save.

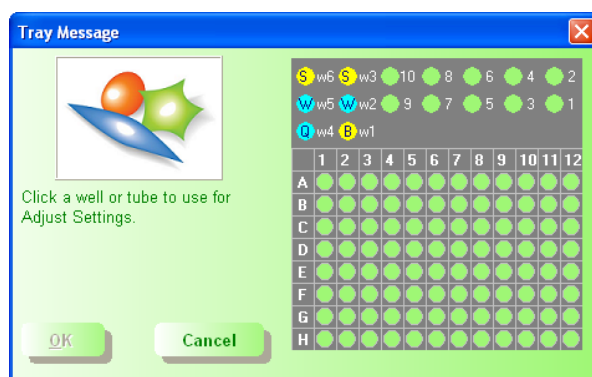
12 Select an instrument settings option.

- To adjust instrument settings, click **Adjust Settings**.

- To retrieve instrument settings, click **Retrieve Settings**. Select a settings file and click **Open**. The settings are automatically downloaded to the guava easyCyte HT System.



- 13 A dialog box appears prompting you to select the sample for adjusting settings. Click to select the well containing beads only (blank sample), then click **OK**.



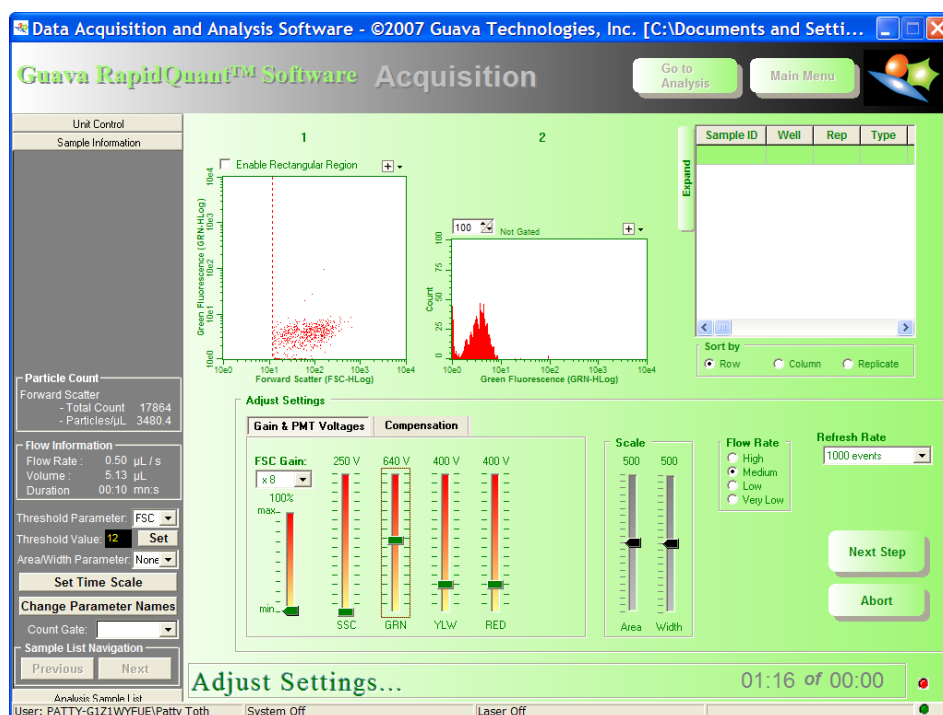
The Adjust Settings screen appears, displaying a Forward Scatter (FSC-HLog) vs Green Fluorescence (GRN-HLog) dot plot and a Green Fluorescence (GRN-HLog) histogram. This screen allows you to adjust the threshold and optimize the display of the data. The system automatically sets the threshold to exclude background signals.

- 14 Check the Particles/ μ L value in the Sample Information control panel and ensure that it is less than or equal to 2500 particles/ μ L.
If the sample has greater than 2500 particles/ μ L, you must prepare the sample again, ensuring you add the correct amount of bead suspension.
- 15 To adjust or fine tune the settings, you can make the following adjustments once events start to appear on the screen:
- Set the **Refresh Rate** to the maximum number of events you want to display.
 - Set the **Flow Rate** to Very Low (0.12), Low (0.24 μ L/s), Medium (0.59 μ L/s) or High (1.2 μ L/s). The recommended flow rate is Medium.
 - NOTE: If you change the flow rate during the adjust settings step, EMD Millipore recommends that you repeat the adjust settings step at the new flow rate to ensure that the markers and threshold are still set correctly.
 - Select the threshold parameter (FSC) from the drop-down menu in the Sample Information control panel.
 - The Capture Bead population has a low FSC signal. To see the entire bead population, click and drag the threshold marker (dotted red line) to



the left. You can also enter a numerical value in the Threshold Value text box in the Sample Information control panel, then click **Set**.

- **NOTE:** When setting the threshold, place it at least 2 to 3 mm to the left of the bead population to ensure that you are not excluding beads. Any debris that gets acquired can be excluded during analysis with an optional dot plot gate.
- If necessary, increase the **FSC Gain** setting so that the entire bead population is visible. Use the drop-down menu to change the FSC signal by a factor of 2. Use the FSC slider to change the FSC signal by steps of 1% between two coarse gain levels. If the slider is set to 100% (min), the gain is equal to the value appearing in the drop-down box. If the slider is set to 200% (max), the gain is 2 times the value appearing in the drop-down box.
- If necessary, change the Green Fluorescence voltage by moving the GRN slider up or down so that the entire bead population is distributed in the first decade of the histogram.
- **NOTE:** It is not necessary to move the SSC, YLW, RED, or NIR sliders. It is also not necessary to make any adjustments to the Compensation.



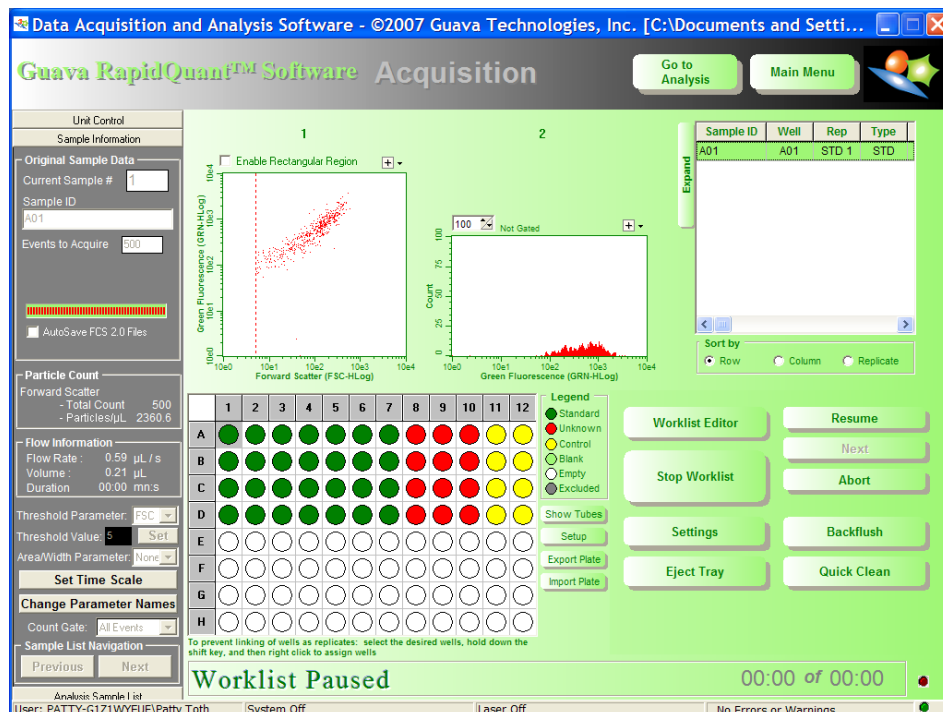
16 Ensure the Enable Rectangular Region box above the dot plot is *not* checked.

17 When you are finished adjusting settings, click **Next Step** to advance to the data acquisition screen.

If you made changes to an existing settings file, when you click **Next Step** you will be prompted to save it as a new file or overwrite the existing file.

18 Click **Resume**.

The system acquires the first sample and automatically displays the results. The tube/well currently being acquired is highlighted in gray.



■ ACQUISITION NOTES

- You may click **Pause** at any time during the run, then click **Backflush**, **Quick Clean**, **Eject Tray**, or **Go to Analysis**. The system will complete the current step before pausing. Click **Resume** to continue.
- If you wish to adjust the instrument settings during the run, click **Pause**, **Settings**, then **Adjust Settings**. When the settings are set, click **Next Step**, then **Resume**.
- **NOTE:** If you adjust the settings during a run, you must restart the acquisition of the plate, as MFI values may be affected.
- The progress bar provides an estimate of the target event count during the acquisition period, which times out after 1.75 minutes (high flow rate), 3.5 minutes (medium flow rate), 7 minutes (low flow rate), or 10 minutes (very low flow rate).

The system automatically performs a Quick Clean at the end of the assay.

guava RapidQuant Analysis

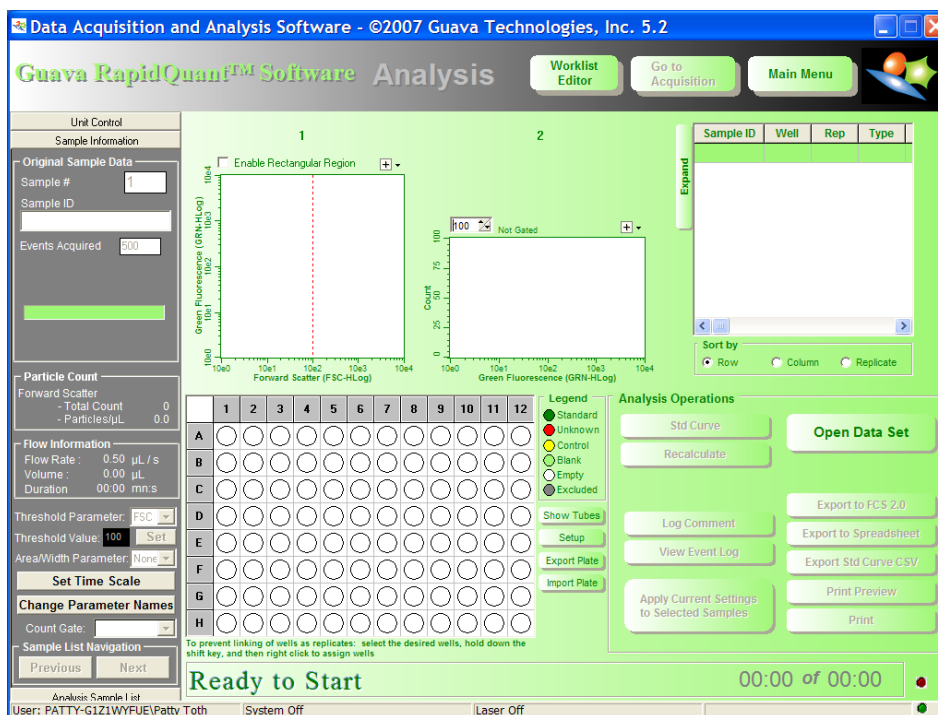
Use the Analysis screen to analyze samples, print results, log comments, or view the event log from a data set that was saved previously. The Worklist Editor can be accessed from the Analysis screen as well. You can also export data to FCS 2.0 files or a spreadsheet file, as well as export the standard curve to a CSV file.

You can save any changes made within Analysis by overwriting the existing file or saving a new file.

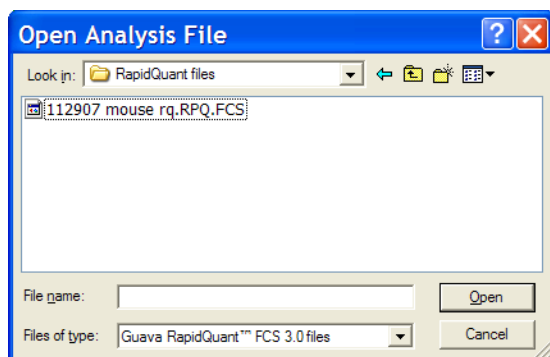
■ **NOTE:** If your system administrator has configured guavaSoft Software to disable overwriting files, you must save any changes to a separate file with a new name.

If you access the Analysis screen during data acquisition you can view the data plots for any samples already acquired. You may also log comments or view the event log. However, you cannot change analysis settings (plots, axes parameters, gates, etc) from the Analysis screen during acquisition. Any analysis settings you wish to change during acquisition should be done from the Acquisition screen.

- 1 Click **RapidQuant** from the main menu.
- 2 Click **Go to Analysis** from the Acquisition screen.



- Click **Open Data Set**. Select an FCS file for analysis and click **Open**.

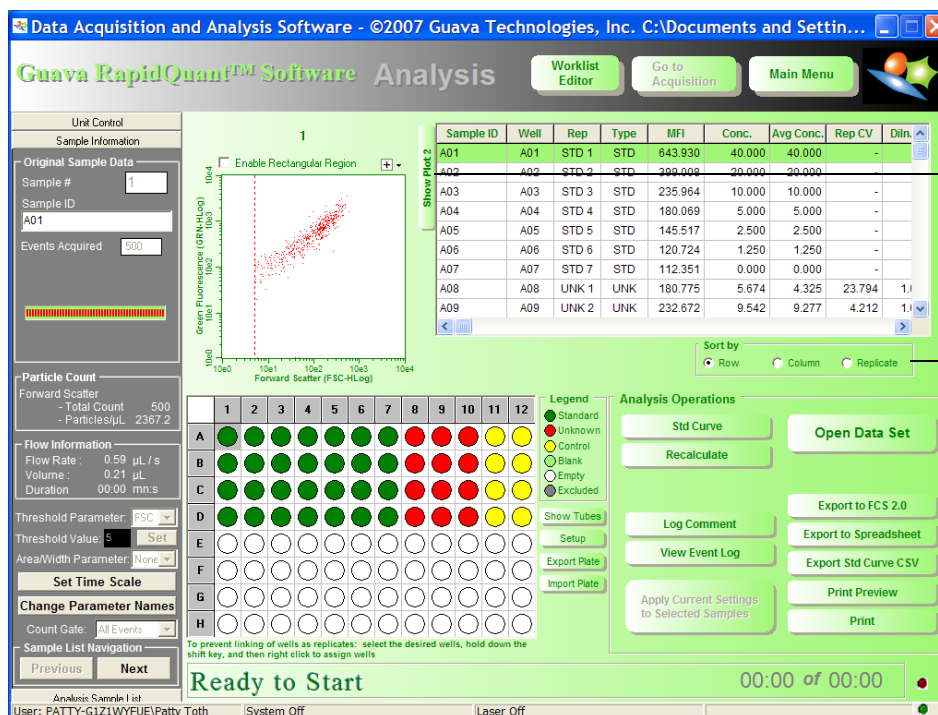


The data and results for the first sample in the data set appear. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel. To select data for a particular sample, you can click on the sample in the Analysis Sample List, or click on a well in the plate map. If you want a picture of the plots, you can copy the screen to the clipboard by pressing Alt + Print Screen on the keyboard. You can then paste the screen into most third-party applications.

The software automatically plots a standard curve and calculates a linear regression equation for the standards, then determines the unknown concentrations from the equation that best fits the average of the standards.

If you did not define the well types prior to acquisition, all wells are defined as empty.

■ **NOTE:** To expand the data table, click **Expand** to the left of the table.



Click to expand the table.

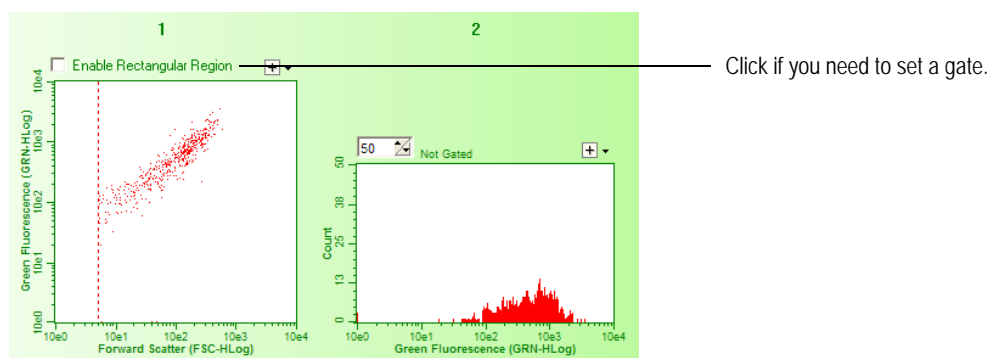
Click to sort the table by plate row, column, or replicate.

Data for RapidQuant run of standards, unknowns, and controls.

- If you did not define well types prior to acquisition, do so now. Refer to step 5 on page 16-3. When well definition is complete, click **Recalculate** to calculate a standard

curve and corresponding equation. If you modify the plate setup from what was originally defined, a message appears below the data table indicating that you must recalculate.

- 5 Observe the dot plot (or histogram) and confirm that the bead population is homogeneous. Repeat this for all samples in the data set. If multiple populations appear for any sample, you will need to set a gate. The green fluorescence will increase in proportion to the amount of IgG bound to the Capture Beads. The MFI for the bead population is displayed in the data table.
If gating is necessary to exclude outliers, refer to “Setting a Gate” below. If you do not need to set a gate, proceed to step 9 on page 16-13.



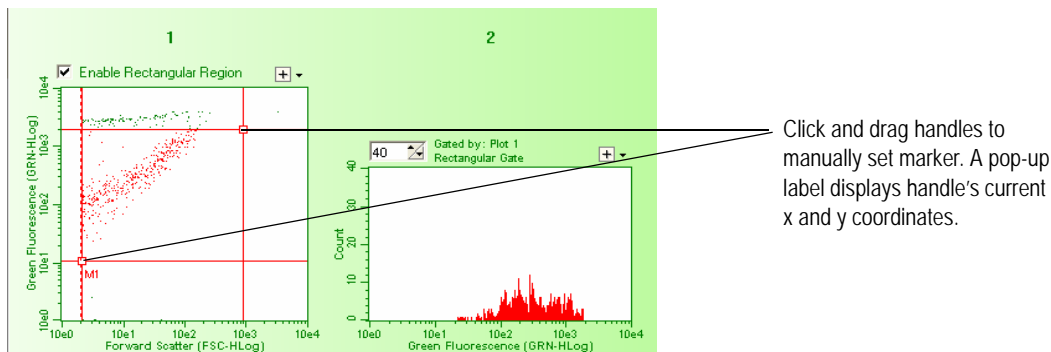
Sample with IgG bound Capture Beads.

Setting a Gate

In rare instances, one or more samples may contain a heterogeneous population of beads. If this occurs, you may set a gate to avoid erroneous results. Ideally, at least 95% of the Capture Bead population should be included in the gate; however, this may not be possible. If necessary, you can exclude the sample from analysis and recalculate, using only the sample's replicate(s), or proceed with setting a gate, keeping in mind that the assay error may be higher in that sample.

- 6 Select the sample by clicking on the sample ID in the Analysis Sample List or the well in the plate map.
- 7 Click Enable Rectangular Region above plot 1 to place a check mark in the box.
- 8 Set the gate to exclude the beads in question.
To set a rectangular gate, position the cursor over the upper-left handle. Click and drag the handle to a new location. Repeat with the lower-left handle. Events that fall

within the center rectangle appear in red and are included in the gate. The gate is immediately applied to the data appearing in plot 2 and the data table.

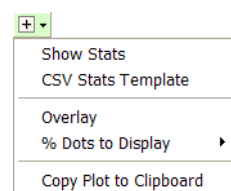


Rectangular marker set on CD3+ lymphocytes.

- 9 Click **Next** under Sample List Navigation in the Sample Information control panel or Unit Control panel. You can also click on the next sample in the Analysis Sample List, use the keyboard arrow keys to select samples, or click directly on a well in the plate map.
- 10 You can apply gates, as well as changes to parameter names from one sample to another sample(s), whether you have made changes or the samples were acquired with different settings. Select the sample with the changes first, then select the samples to which you want to apply the settings from the Analysis Sample List. Click **Apply Current Settings to Selected Samples**.
Hold down the Shift key while clicking and dragging to select groups of samples. Or, hold down the Ctrl key while clicking to select multiple samples.
 - **NOTE:** Apply Current Settings to Selected Samples applies to parameter names as well, so make name changes last and apply only to the samples with the same custom name.
- 11 When you have finished analyzing the samples for this plate, you can save any analysis changes you made by exiting Analysis or clicking **Open Data Set**. A dialog box appears prompting you to save the changes. Click **Yes** and either overwrite the existing file or save the file with a new name.
Results are automatically exported to a CSV file that is given the same name as the FCS file.
- 12 If you wish to view the event log, click **View Event Log**. Refer to “Event Log” on page 1-19 for information. You can also enter comments related to the assay and save these comments to the event log. Click **Log Comment** and type in the information. Then, click **Save Comments to Log**.

Plot Menu

Some analysis features are available from the plot menu. To open the plot menu, click the plus sign (+) in the upper-right corner above the plot or right-click the plot. See “Statistics” on page 16-16 and “Selecting Statistics for the Spreadsheet File” on page 16-17 for information on those options.

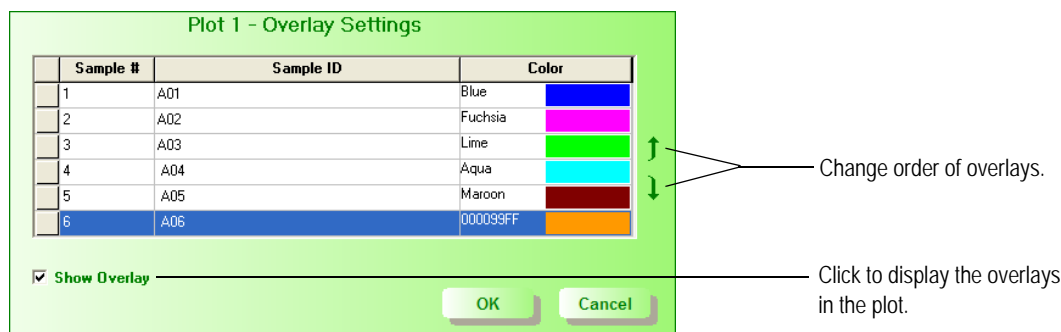


Overlaying Plots

You can overlay the data of up to six samples in a dot plot and seven samples in a histogram plot. The data for each sample will be displayed in a different color, allowing you to see the different sample data overlaid within the same plot.

- 1 Select **Overlay** from the plot menu.

The Overlay Settings dialog box appears (histogram overlay dialog box shown).



- 2 Click the Sample # column and select the sample from the drop-down list. You may select up to six samples for dot plots and seven samples for histograms.
- 3 To change the line or fill color, click the color in the Line and/or Fill Color column for a sample, then click the palette icon and select the desired color from the color palette. Click **OK**.
- 4 Click **Show Overlay** and then click **OK**.
The selected sample data in the selected colors appears. The first sample's dot plot/histogram is drawn. The other plots are layered over it, with the last plot appearing on top.
To change the overlay order, click a sample in the list then click the up or down arrow to the right of the list to reorder the sample. To remove an overlaid plot, right-click on the overlay and select **Remove**. The overlay plots are gated from the same gate as the initial plot.

Copying a Plot to the Clipboard

You can copy a plot to the clipboard.

- 1 Select **Copy Plot to Clipboard** from the plot menu.
You can also copy the screen to the clipboard by pressing **Alt + Print Screen** on the keyboard. You can then paste the screen into most third-party applications.

Changing the Number of Dots to Display

You can change the number of dots displayed in the plot.

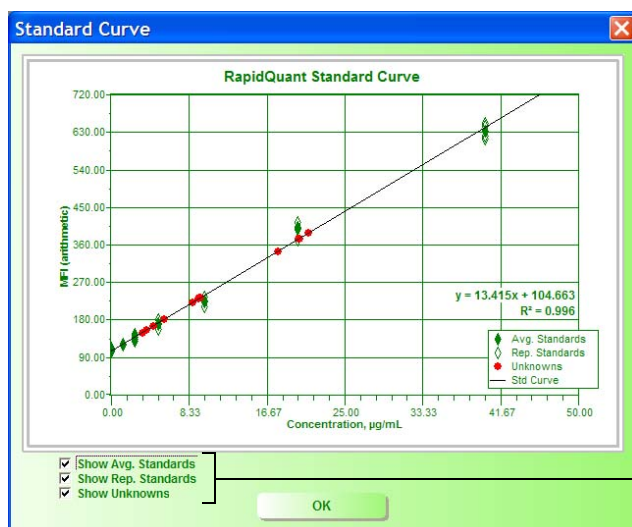
- 1 Select **% Dots to Display** from the plot menu, then select a percentage from the menu. If you choose **Custom**, you can enter the number of events you wish to display.

guava RapidQuant Results

Results for individual samples appear in the data table. You can click a particular sample in the Analysis Sample List or on an individual well to display the results for that sample in the data table. You can also place the cursor over a well to see a pop-up menu with the well, type, replicate, concentration (for standards and unknowns only), and dilution (for unknowns and controls only).

Standard Curve

- 1 Click **Std Curve** to view the standard curve and linear regression equation. You can uncheck the boxes for Show Avg. Standards, Show Rep. Standards, or Show Unknowns if you do not wish to view them on the graph. Any unknowns with MFI values that fall significantly above the range of the standard curve may not appear on the plot. Antibody concentration data for any unknowns with MFI values that fall outside the range of the standard curve will not be accurate. For accurate data, you must repeat the assay, diluting the unknowns with high MFI values and concentrating the unknowns with low MFI values, so that their MFI values now fall within those obtained with the standards.
 - **NOTE:** You can copy and paste the standard curve graph to another application by right-clicking on it and selecting **Copy**.



Hide or view the Averages of the Standards, the Replicates of the Standards, and/or the Unknowns.

- 2 Click **OK** to close the Standard Curve window.

Data Table

The data table provides the sample ID, well number, Rep (replicate), sample type, MFI, concentration value, average concentration, Rep CV, dilution factor, and original concentration. The values for Avg. Conc., Rep CV, dilution factor, and original concentration are identical for each replicate of a sample. You can expand the size of the table by clicking **Expand**.

You can sort the data by plate rows, columns, or replicates by clicking the appropriate option under Sort by at the bottom of the table. The printed results and the exported Std Curve CSV summary file will both reflect the view feature selected here.

Sample ID	Well	Rep	Type	MFI	Conc.	Avg Conc.	Rep CV	Diln.
A01	A01	STD 1	STD	643.930	40.000	40.000	-	
A02	A02	STD 2	STD	399.008	20.000	20.000	-	
A03	A03	STD 3	STD	235.964	10.000	10.000	-	
A04	A04	STD 4	STD	180.069	5.000	5.000	-	
A05	A05	STD 5	STD	145.517	2.500	2.500	-	
A06	A06	STD 6	STD	120.724	1.250	1.250	-	
A07	A07	STD 7	STD	112.351	0.000	0.000	-	
A08	A08	UNK 1	UNK	180.775	5.674	4.325	23.794	1.0
A09	A09	UNK 2	UNK	232.672	9.542	9.277	4.212	1.0

Sort by: ☒ Row ☐ Column ☐ Replicate

- To view all the data in the table as well as the standard curve and the color-coded plate map, click **Print Preview**.
- To print the report, click the Print.

Statistics

You can view the statistics for the dot plot and the histogram.

- To view the stats, click the plus sign (+) at the upper-right corner of a plot or right-click the plot to open the plot menu. Then click **Show Stats**.

The statistics provide you with the count, % total, % gated, x and y geometric mean, mean, median, and %CV, and particles/mL for all data in the plot (if ungated) or for data within the gate.

You can resize the stats windows by clicking and dragging the bottom or right edge of the window. You can resize the columns by clicking and dragging the right edge of the column header. If the column becomes too small to adjust, close, then reopen the window to restore the column width.

Plot 1 - DotPlot: FSC-HLog vs GRN-HLog										
Not Gated										
	Count	%Total	%Gated	x-Geo. Mean	y-Geo. Mean	x-Mean	y-Mean	x-Median	y-Median	x-%CV
M1	467	93.40%	...	98.03	510.26	141.15	676.39	123.06	551.06	77.00
All Events	500	100.00%	...	84.11	442.08	133.63	643.93	111.99	505.67	83.40

Dot plot statistics

Plot 2 - Histogram: GRN-HLog
Gated by: Plot 1 Rectangular Gate

	Count	%Total	%Gated	x-Geo. Mean	x-Mean	x-Median	x-%CV	Particles/mL
All Events	488	97.60%	100.00%	383.71	585.90	443.58	87.41	2.08e06

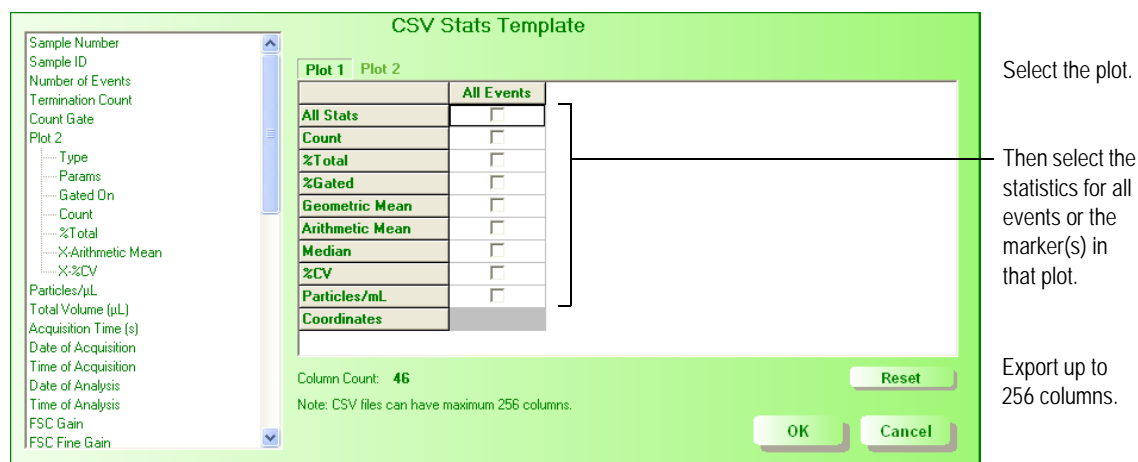
Histogram statistics

You must manually select any statistics that you wish to export. See “Selecting Statistics for the Spreadsheet File” on page 16-17.

Selecting Statistics for the Spreadsheet File

Although you can view any statistics on screen, you must manually select the statistics that you wish to export to the spreadsheet file.

- 1 Open the plot menu from either plot and select **CSV Stats Template**. The CSV Stats Template dialog box appears.



The left side of the screen displays a list of instrument settings and sample information that is always exported to the spreadsheet file. The right side of the screen allows you to select the specific statistics that will be exported for each plot. If a plot has more than one marker, you can select the stats for each marker. When you click **OK**, the statistic you selected will be remembered the next time you open the CSV Stats Template.

- Click to select the statistic for export. Stats are shown for each marker in the plot or total events in the plot if no markers are set.
- Click **Reset** to ignore any changes made and revert to the stats that were selected when you opened the CSV Stats Template.

- **NOTE:** If you exceed 256 columns, a warning message appears requesting that you review and remove stats until there are no more than 256 columns of stats for export.

Exporting guava RapidQuant Results to an FCS 2.0 File

You can export the results from the current sample to an FCS 2.0 file. One FCS file is saved for each sample acquired. You can analyze FCS 2.0 files using a third-party flow cytometry analysis application.

- 1 To export analysis results to an FCS 2.0 file, click **Export to FCS 2.0**.
You can also select specific samples from the Analysis Sample List and click **Export to FCS 2.0**. The selected files are saved to individual FCS 2.0 files.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**. The sample number is automatically appended to the file name you enter. For example, if the sample number is 1, the file will be named *filename_0001.FCS*.

Exporting guava RapidQuant Results to a Spreadsheet File

You can export the results to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel. The CSV file will contain only the sample information and instrument settings unless you select the stats you wish to export.

- **NOTE:** If you saved an FCS file after making analysis changes, a spreadsheet file will be saved automatically.
- 1 To export analysis results to a spreadsheet file, click **Export to Spreadsheet**.
 - 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Exporting guava RapidQuant Standard Curve to a Spreadsheet File

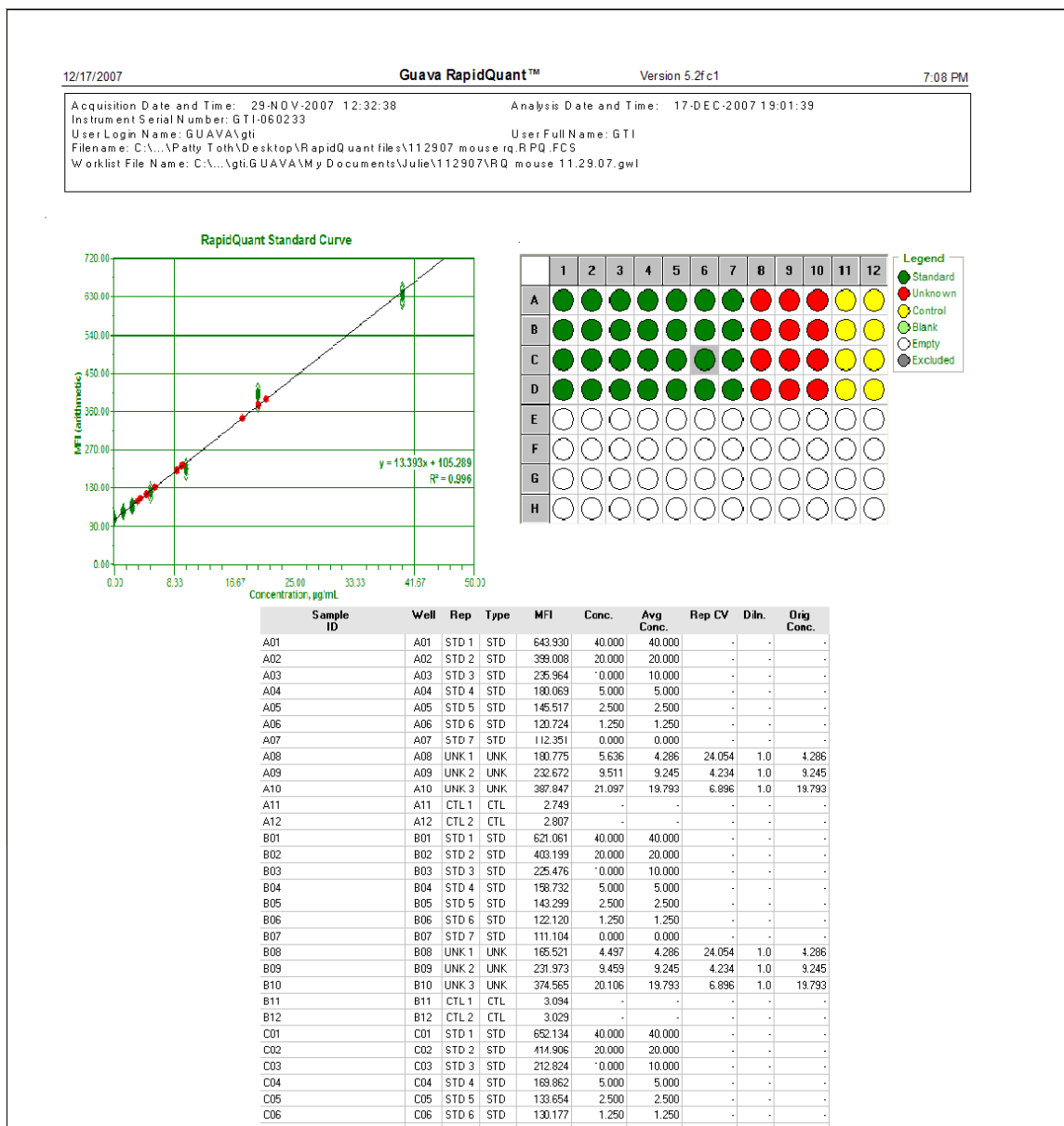
You can export the standard curve, as well as all values within the data table, to a comma-separated values (CSV) file for analysis using a spreadsheet program such as Microsoft Excel.

- 1 To export the standard curve results to a spreadsheet file, click **Export Std Curve CSV**.
- 2 Select the folder where you want to save the file, and enter a file name. Click **Save**.

Printing Results

You can print results from the Analysis screen only. The printed results include the a standard curve, color-coded plate map, and the data table.

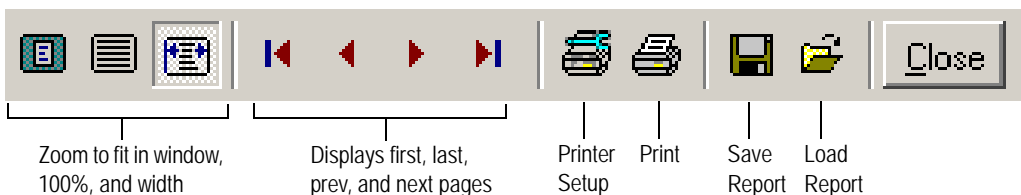
1 To view the results, click **Print Preview**.



2 To print the results, click **Print**, then click **OK** in the print dialog box. Or, click the Print icon from the Print Preview window.

Print Preview Icons

Move the cursor over the icon to display the description.



guava RapidQuant Software Module Control Panels

To display the Unit Control or Sample Information control panel, click the title bar.

Sample Information

Sample Information

Original Sample Data

Sample #

Sample ID

Events Acquired

Progress Bar

Particle Count

Forward Scatter

- Total Count 500
- Particles/μL 2367.2

Flow Information

Flow Rate : 0.59 μL / s

Volume : 0.21 μL

Duration 00:00 mn.s

Threshold Parameter:

Threshold Value:

Area/Width Parameter:

Count Gate:

Sample List Navigation

Original Sample Data

- Displays the sample #. This number defaults to 1 and advances at the completion of sample acquisition.
- Displays the Sample ID for the individual sample. The default is the well number.
- Displays the number of events acquired. The default is 500.
- The progress bar provides an estimate of the target event count during acquisition.

Particle Count

Displays the total number of beads and beads/μL that have exceeded the threshold.

Flow Information

Displays the sample flow rate, volume of sample acquired, and acquisition duration.

Threshold Parameter and *Threshold Value* allow you to select the threshold parameter and enter its value. Click **Set** after entering the Threshold Value.

Area/Width Parameter allows you to save area and width measurements for a selected parameter.

Set Time Scale allows you to adjust the axis scale that displays the time parameter.

Click **Change Parameter Names** to enter your own parameter names. See “Changing Parameter Names” on page 16-21.

Count Gate allows you to select a gate used as a counting gate. All events above the threshold are saved to the file whether they are in the gate or not. However, the number of Events to Acquire is applied to events that fall within the gate.


Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

Changing Parameter Names

You can change the default long (or stain) name of any parameter. The short name, for example, GRN-HLog, will still appear in parentheses after the new long name.

- 1 Click **Change Parameter Names** in the Sample Information control panel. The Customize Parameter Names dialog box appears. Only the parameters installed on your instrument appear in the list of parameters. Area and width appear only if they were selected during the adjust settings step.
- 2 Highlight the existing name in the *Long or Stain Name* text field and type in the new name.
- 3 Click **Update**.



Param No.	Short Name	Long or Stain Name
1	FSC-HLin	Forward Scatter
2	SSC-HLin	Side Scatter
3	GRN-HLog	Green Fluorescence
4	YLW-HLog	Yellow Fluorescence
5	RED-HLog	Red Fluorescence
6	FSC-HLog	Forward Scatter
7	SSC-HLog	Side Scatter
8	GRN-HLin	Green Fluorescence
9	YLW-HLin	Yellow Fluorescence
10	RED-HLin	Red Fluorescence
11	TIME	Time

- You can change the long or stain names for individual samples.
- If you change the names before starting acquisition, the new names will apply to all samples in the run.
- If you use the *Apply Current Settings to Selected Samples* button during analysis, any changes made to long names will be applied to the selected samples.

Unit Control

The Unit Control panel is divided into several sections:

- Detection:** Includes a Laser status indicator (red dot), FSC Gain: x 2 (100%), and voltage settings for SSC (250 V), GRN (400 V), YLW (400 V), RED (400 V), and NIR (250 V).
- Pump Status:** Includes indicators for End of Stroke (red dot), Waste (red dot), and Pump Reset (red dot). It also shows Position (0 Steps) and Actual Speed (0 Steps/s).
- Pump Action:** Includes indicators for Pump on (red dot), Waste (yellow dot), Pump Reset (yellow dot), Purge (yellow dot), and Sample (green dot). It also shows Speed (0 Steps/s).
- Threshold parameters:** Includes Offset (0.24 V) and Threshold (100 units) settings. Below this is a section for Baseline Values with sliders for NIR, GRN, RED, YLW, SSC, and FSC, all set to 0.
- Sample List Navigation:** Includes buttons for Previous and Next.

Detection

Displays the laser status, the SSC (if installed), GRN, YLW, RED, and NIR (if installed) voltage settings, and the FSC gain.

- **NOTE:** Do not change the voltages from this panel. Use the sliders in Adjust Settings to adjust the GRN voltage. For this assay, it is not necessary to adjust the SSC, YLW, RED, and NIR voltages.

Pump Status

Displays the current status of the pump.

Pump Action

Indicates the current pump position.

Threshold Parameters

Displays the offset and threshold settings for the threshold parameter.

Sample List Navigation

Allows you to select the previous or next sample from the Analysis Sample List during a data set analysis.

guava RapidQuant Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava RapidQuant spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava RapidQuant FCS file to another directory or give it a new name.

Problem	Possible Cause	Solutions
RapidQuant Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter registration code. The code is case sensitive. NOTE: If registration code is correct, contact EMD Millipore Technical Support. You may need a firmware upgrade to run RapidQuant Software Module.
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Beads in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 3. Undesirable population in sample. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold. 3. Set gate to exclude undesirable population, or exclude sample from analysis.

Problem	Possible Cause	Solutions
FSC Count under Particle Count shows events, but events appear in the wrong place in FSC vs GRN dot plot.	<ol style="list-style-type: none"> 1. Supernatant or FITC goat anti-mouse (or human) IgG antibody not added. 2. Instrument settings not optimal. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, prepare sample again from original supernatant. 2. Repeat Adjust Settings step.
Events appear in dot plot but not in histogram.	Gate set incorrectly.	Ensure gate includes Capture Bead population.
Events appear off scale in dot plot or histogram.	FSC gain or GRN voltage set incorrectly.	Adjust gain setting or voltage settings so bead-only control population appears on scale in FSC and between 0 and 10 in GRN fluorescence parameter.
Standard curve is not linear.	<ol style="list-style-type: none"> 1. Pipettes not calibrated. 2. Dilution steps performed improperly. 3. Standards or other kit components not stored properly. 	<ol style="list-style-type: none"> 1. Ensure pipettes are calibrated. Pipetting accuracy is necessary for optimal results. 2. Check accuracy of dilution steps. 3. Order new kit.

Problem	Possible Cause	Solutions
Unknown concentration predictions are not accurate.	<ol style="list-style-type: none"> 1. Unknowns are under- or over-diluted, resulting in MFI values for unknowns that are higher or lower, respectively, than those obtained with standards. 2. Unknowns not diluted correctly. 3. Standards were not diluted correctly. 4. Samples were not properly shaken during incubation. 5. Plate map was set up incorrectly in RapidQuant Software Module. 	<ol style="list-style-type: none"> 1. Repeat assay diluting unknowns so that their MFI values fall within range of MFIs obtained with standards. NOTE: It may be necessary to make different dilutions of same unknown to ensure that some diluted samples fall within linear range. 2. Be sure unknown dilutions were performed correctly and that proper dilution factors were entered into software. 3. Check that protocol was followed. 4. A rocker or shaker should be used during incubation for optimal results. 5. Be sure that replicates and individual samples are defined correctly. Ensure all unknown and control dilutions were properly selected.

CHAPTER 17

Troubleshooting

The following troubleshooting sections can also be found in their corresponding chapters.

General System Troubleshooting

Problem	Possible Causes	Solutions
Laptop prompts for user ID or password.	Laptop is set up for authorization.	Do not enter password. Click OK or Cancel to continue. Contact your IT department for assistance with any modifications. The original laptop setup does not require a password.
During start-up, laptop freezes on particular screen.	System may be searching for directory during startup.	Press Enter to continue. Reboot computer, if necessary.
Message: <i>The instrument appears to be either off or not connected. You can run in Analysis mode only.</i>	<ol style="list-style-type: none"> 1. guava easyCyte HT System is not turned on or is not getting power. 2. Cable connection between easyCyte HT System and laptop is loose. 3. easyCyte HT System and laptop were not powered on in correct sequence or have lost communication. 	<ol style="list-style-type: none"> 1. Ensure easyCyte HT System power cord is properly plugged in and system is turned on. 2. Ensure USB cable is securely connected to laptop. Reboot computer, if necessary. 3. Turn off easyCyte HT System, exit guavaSoft Software, restart laptop, turn on easyCyte HT System, start guavaSoft Software.
guavaSoft Software launches, but only Analysis mode is available when an assay is launched.	Registration code not entered or not entered correctly.	Enter registration code and ensure all characters are correct.

Problem	Possible Causes	Solutions
Laptop keeps shutting down.	<ol style="list-style-type: none"> 1. Power supply to laptop is faulty. 2. Screen saver is interfering. 	<ol style="list-style-type: none"> 1. Ensure laptop is plugged in correctly. Use surge protector and ensure it is plugged in and turned on. 2. Adjust power scheme screen saver options. Click Start> Settings>Control Panel. Double-click Display, select Screen Saver tab, click Settings under <i>Energy saving features of monitor</i>. Make sure "Setting for Always On power scheme" are all set to Never. Laptop should not be allowed to "sleep." guavaSoft Software will stop acquiring data until the laptop is woken up.
For InCyte only Message: <i>Sorry - this unlock is invalid.</i>	Incorrect or no unlock key.	Ensure correct unlock key is entered. If necessary, contact customer support to obtain unlock key.
Noise occurring during sampling.	<ol style="list-style-type: none"> 1. Mixer paddle is making contact with the plate. 2. Mixer paddle may be misaligned or bent. 	<ol style="list-style-type: none"> 1. Ensure you are using a compatible plate. See "Compatible Microplates" on page D-6. 2. Contact EMD Millipore Technical Support.
Message: <i>The tray door is open.</i> appears when the door is shut.	The door sensor switch is damaged.	Contact EMD Millipore Technical Support.
Message: <i>TRAY HOLD OFF STATE.</i>	<ol style="list-style-type: none"> 1. The program is waiting for the automation to reset. 2. The tray door was opened. 3. The program lost detection of automation position or status. 4. There is a mechanical or electrical problem with the automation or mixer function. 	<ol style="list-style-type: none"> 1. Wait approximately 30 seconds for re-initialization. 2. Keep tray door closed during acquisition. 3. Exit guavaSoft, turn the instrument off, then on again. Restart guavaSoft, then open the worklist. 4. Contact EMD Millipore Technical Support.

easyCheck Procedure Troubleshooting

Problem	Possible Cause	Solutions
No event counts appear for RED2 and NIR2.	<ol style="list-style-type: none"> 1. Wrong beads used. 2. Red laser not operating or problem with the signal. 	<ol style="list-style-type: none"> 1. Use easyCheck beads. Do not use Guava Check beads. 2. Contact EMD Millipore Technical Support.
One or more Particles/mL results falls outside the acceptance range (appear in red).	<ol style="list-style-type: none"> 1. System is not clean. 2. Incorrect information entered in easyCheck fields. 3. Bead suspension incorrectly prepared. 	<ol style="list-style-type: none"> 1. Run Quick Clean, then rerun easyCheck Procedure. If results still fall outside range, run Guava Clean. 2. Ensure correct Bead Lot # and Expected Particles/mL are entered. Refer to easyCheck Beads vial label and information card for values. 3. Prepare fresh bead sample and rerun easyCheck Procedure. Refer to <i>guava easyCheck Kit</i> package insert for preparation instructions.
FSC, SSC, GRN, YLW, RED, NIR, RED2, and/or NIR2 intensity is >10% outside the acceptable range.	<ol style="list-style-type: none"> 1. System is not clean. 2. Problem with detector or laser. 	<ol style="list-style-type: none"> 1. Run Quick Clean. If results are still outside range, run Guava Clean. 2. If problem persists, contact EMD Millipore Technical Support.
Particle counts for FSC, SSC, GRN, YLW, RED, NIR, RED2, and/or NIR2 intensity is not within 100 events of each other.	<ol style="list-style-type: none"> 1. If FSC count is low, capillary may not be seated correctly. 2. If any of the counts is low, possible problem with detector. 	<ol style="list-style-type: none"> 1. Remove metal plate. Unscrew tubing from top of flow cell and firmly push down on top of flow cell assembly. If problem persists, contact EMD Millipore Technical Support. 2. Rerun easyCheck Procedure. If counts are still low, contact EMD Millipore Technical Support.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells and 150 µL for 0.5-mL tubes. <i>Use 0.5-mL tubes in the tube position, as a higher volume (1 mL) is required for 1.5-mL tubes.</i>
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. No beads in sample. 4. Clogged flow cell. 5. Broken flow cell. 6. Sample pump not working. 7. Laser not operational. 8. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells and 150 µL for 0.5-mL tubes. <i>Use 0.5-mL tubes in the tube position, as a higher volume (1 mL) is required for 1.5-mL tubes.</i> 3. Ensure correct sample is loaded. 4. Perform a Backflush. Follow with Quick Clean. 5. Remove flow cell and inspect for damage, including chipped capillary. Replace if necessary. 6. Run Quick Clean and watch for fluid in waste vial. 7. Contact EMD Millipore Technical Support. 8. Ensure tubing connector is secure.
<p>Message: <i>The login user does NOT have read/write permission to the file GuavaCheckLog.csv in the Log folder. Contact the system administrator for assistance.</i></p>	The user was not assigned access control to the system.	Contact your system administrator for user access to the software.

guava ViaCount Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save ViaCount spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save ViaCount FCS file to another directory or give it a new name.
ViaCount Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Message: <i>Less than 10 particles/μL. Sample is too dilute. Accuracy may be compromised.</i>	<ol style="list-style-type: none"> 1. Sample concentration is too low. 2. Clogged flow cell. 3. Settings were not adjusted correctly. 	<ol style="list-style-type: none"> 1. Recommended concentration range for accurate counting is 1×10^4 to 5×10^5 cells/mL. Refer to <i>Guava PCA-96 ViaCount Flex Reagent</i> package insert for proper dilution instructions. 2. Perform a Backflush. Follow with Quick Clean. 3. Adjust Settings and make necessary changes.
Message: <i>Sample is too concentrated. Please dilute or accuracy may be compromised.</i>	Sample is too concentrated.	Recommended concentration range for accurate counting is 1×10^4 to 5×10^5 cells/mL. Refer to <i>Guava PCA-96 ViaCount Flex Reagent</i> package insert for proper dilution instructions.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 4. Cells were lysed during staining. 5. Sample was not properly stained. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software. 4. Check sample. If necessary, restrain sample from original suspension. 5. Check buffers used to process cells. Check expiration date of reagent.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Events appear off scale in dot plots.	FSC gain or PM1 and /or PM2 voltages set incorrectly, or samples staining brightly.	Adjust gain setting or voltage settings so positive populations appear on scale.

Problem	Possible Cause	Solutions
Viability is significantly lower than expected.	<ol style="list-style-type: none"> 1. Sample was not mixed properly before acquisition. 2. Residual bleach in flow cell. 3. Buffer used is non-isotonic or pH is out of physiological range. 4. Cells are fragile and stressed by preparation procedure. 	<ol style="list-style-type: none"> 1. Ensure sample mixing option was selected in WorkEdit Software. 2. Run Quick Clean. Restain fresh cell sample. 3. Check osmolarity and pH. pH should be 7.2–7.4. 4. Resuspend cells in medium containing 10–20% FBS for 30 min with frequent, gentle agitation. Restain.
Cell count is significantly different than expected.	<ol style="list-style-type: none"> 1. Dilution factor or original volume entered incorrectly. 2. Sample was not mixed properly before acquisition. 3. Adherent cells were not adequately removed from culture surface. 4. Sample was not stained completely. 5. Sample dilution factor is too low. 6. Clogged flow cell. 7. Cells are aggregated. 8. Instrument settings not optimal. 9. Incorrect sample tubes or plates used. 	<ol style="list-style-type: none"> 1. Ensure dilution factor and original volume are correct. 2. Ensure sample mixing option was selected in WorkEdit Software. 3. Check culture vessel to ensure cells were removed. Repeat procedure to release cells if necessary. 4. Ensure reagent is not expired or was not exposed to excessive light. Ensure sufficient amount of ViaCount Flex Reagent was used. 5. Use at least a 1:10 of sample to Viacount reagent, or use Viacount Flex CS Reagent (Catalog No. 4500-0110). 6. Perform a Backflush. Follow with Quick Clean. 7. Prepare uniform suspension. Use ViaCount CDR to disaggregate cells. 8. Adjust settings so positive populations appear on scale and debris is below threshold. 9. See Order Information for list of supported tubes and plates.

Problem	Possible Cause	Solutions
guava Excel Utility will not start Microsoft Excel when you try to open a CSV file	<ol style="list-style-type: none"> 1. File is not a ViaCount file or it was created by a version of CytoSoft Software prior to version 2.0. 2. Templates folder is not located in same directory as utility program. 	<ol style="list-style-type: none"> 1. Excel Utility can be used only for ViaCount files created in CytoSoft Software, version 2.0 or later. 2. Be sure Templates folder and executable are in same folder.

guava ExpressPlus Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava ExpressPlus spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava ExpressPlus FCS file to another directory or give it a new name.
guava ExpressPlus Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Cell Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.

Problem	Possible Cause	Solutions
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying GRN, YLW, or RED.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but the events appear in the wrong places in plots displaying GRN, YLW, and/or RED.	<ol style="list-style-type: none"> 1. Sample was not stained. 2. Cell lysis. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, restrain sample from original suspension. 2. Check buffers used to process cells.
Events appear in Plot 1 but not in Plot 2 or Plot 3.	Plot 1 gate excludes events, and gate is applied to Plot 2 and/or Plot 3.	Uncheck Plot 1 (under Gated On) for Plots 2 and 3 to see if events appear. Or, set Plot 1 gate to include population of interest.
Events appear in Plot 2 but not in Plot 3.	Plot 2 gate excludes events, and gate is applied to Plot 3.	Uncheck Plot 2 (under Gated On) for Plot 3 to see if events appear. Or, set Plot 2 gate to include population of interest.
Events appear off scale in dot plots or histograms.	FSC gain or SSC, GRN, YLW, and/or RED voltages set incorrectly, or samples staining brightly.	Adjust gain setting or voltage settings so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.

Problem	Possible Cause	Solutions
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Voltages too low to detect fluorescent signals. 2. Incomplete staining with fluorescent probe, or fluorescent probe inappropriate for cell type. 3. Fluorescent probes over-exposed to light, stored improperly, or expired. 4. Non-specific binding of fluorescent probes. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Ensure positive control is staining adequately and with correct reagent. 3. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 4. If using antibody-based probes, try Fc blocking reagent during staining to minimize non-specific binding. Otherwise, titer the fluorescent probes down to reduce the nonspecific staining. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.
Poor resolution between positive populations in plots displaying GRN, YLW, and/or RED.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s). 2. Too much reagent in staining tube. 3. Fluorescence background too high. 4. Voltage too high causing signal to bleed into other parameters. 5. Voltage too low to optimally detect positive signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. 2. Washing cells may remove residual reagent. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce voltage. Adjust compensation settings. 5. Adjust settings to increase voltage. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris. Or, select one of the fluorescence parameters as the threshold.

guava ExpressPro Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava ExpressPro spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava ExpressPro FCS file to another directory or give it a new name.
ExpressPro Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Cell Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.

Problem	Possible Cause	Solutions
Unexpected events appearing in plots displaying GRN, YLW, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but the events appear in the wrong places in plots displaying GRN, YLW, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Sample was not stained. 2. Cell lysis. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, restain sample from original suspension. 2. Check buffers used to process cells.
Events appear in some plots but not in others.	Ensure correct gate is selected for plot in question.	<ol style="list-style-type: none"> 1. Open plot menu, point to Apply Gates and select gate. 2. Check gate definition to ensure it includes the correct regions and operators.
Events appear off scale in dot plots or histograms.	FSC, SSC, GRN, YLW, RED, NIR, RED2, and/or NIR2 gains set incorrectly, or samples staining brightly.	Adjust gain setting so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.
Cannot resolve dim positive staining from background signal.	Dirty capillary.	<p>Perform at least one cycle of Guava Clean.</p> <p>While acquiring samples, select Clean & Rinse instead of Quick Clean, and if necessary, run it frequently.</p>

Problem	Possible Cause	Solutions
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Gains too low to detect fluorescent signals. 2. Incomplete staining with fluorescent probe, or fluorescent probe inappropriate for cell type. 3. Fluorescent probes over-exposed to light, stored improperly, or expired. 4. Non-specific binding of fluorescent probes. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Ensure positive control is staining adequately and with correct reagent. 3. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 4. If using antibody-based probes, try Fc blocking reagent during staining to minimize non-specific binding. Otherwise, titer the fluorescent probes down to reduce the nonspecific staining. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.
Poor resolution between positive populations in plots displaying GRN, YLW, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s). 2. Too much reagent in staining tube. 3. Fluorescence background too high. 4. Gains too high causing signal to bleed into other parameters. 5. Gain too low to optimally detect positive signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. 2. Washing cells may remove residual reagent. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce gain. Adjust compensation settings. 5. Adjust settings to increase gain. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris. Or, select one of the fluorescence parameters as the threshold.

guava InCyte Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava InCyte spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava InCyte FCS file to another directory or give it a new name.
Incyte Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Gates and/or events in plots disappear.	<ol style="list-style-type: none"> 1. Data file or method is selected. 2. Data was acquired in tubes. 3. More than 96 samples were acquired. 	Click on the Analyzed Group in the Analysed Data pane to display data and gates.
Few events, as indicated in Cell Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.

Problem	Possible Cause	Solutions
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but the events appear in the wrong places in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Sample was not stained. 2. Cell lysis. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, restrain sample from original suspension. 2. Check buffers used to process cells.
Events appear in some plots but not in others.	Ensure correct gate is selected for plot in question.	<ol style="list-style-type: none"> 1. Open plot menu, point to Apply Gates and select gate. 2. Check gate definition to ensure it includes the correct regions and operators.
Events appear off scale in dot plots or histograms.	FSC SSC, GRN, YLW, RED, NIR, RED2, and/or NIR2 gains set incorrectly, or samples staining brightly.	Adjust gain setting so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.

Problem	Possible Cause	Solutions
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Gains too low to detect fluorescent signals. 2. Incomplete staining with fluorescent probe, or fluorescent probe inappropriate for cell type. 3. Fluorescent probes over-exposed to light, stored improperly, or expired. 4. Non-specific binding of fluorescent probes. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Ensure positive control is staining adequately and with correct reagent. 3. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 4. If using antibody-based probes, try Fc blocking reagent during staining to minimize non-specific binding. Otherwise, titer the fluorescent probes down to reduce the nonspecific staining. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.
Cannot resolve dim positive staining from background signal.	Dirty capillary.	<p>Perform at least one cycle of Guava Clean.</p> <p>While acquiring samples, select Clean & Rinse instead of Quick Clean, and if necessary, run it frequently.</p>

Problem	Possible Cause	Solutions
Poor resolution between positive populations in plots displaying GRN, YEL, RED, NIR, RED2, and/or NIR2.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s). 2. Too much reagent in staining tube. 3. Fluorescence background too high. 4. Gain too high causing signal to bleed into other parameters. 5. Gain too low to optimally detect positive signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. 2. Washing cells may remove residual reagent. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce gain. Adjust compensation settings. 5. Adjust settings to increase gain. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris. Or, select one of the fluorescence parameters as the threshold.

guava Nexin Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava Nexin spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava Nexin FCS file to another directory or give it a new name.
Nexin Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in PM1 vs PM2 dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM1 dot plot but not in PM1 vs PM2 dot plot.	FSC vs PM1 gate excludes events.	Ensure FSC vs PM1 gate is set to include population of interest.
Events appear off scale in dot plots.	PM1 and /or PM2 voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM1 and/or PM2 voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample.

Problem	Possible Cause	Solutions
Poor resolution between PE ⁺ and PE ⁻ populations.	<ol style="list-style-type: none"> 1. PM1 voltage too low to detect PE signal. 2. Incomplete staining with Annexin V-PE. 3. Incomplete induction of apoptosis in test suspension. 4. Uninduced population reacting with Annexin V because cells have been stressed. 5. Apoptotic bodies included in analysis. 6. Annexin V expired, or not stored properly. 7. Background noise too high. 8. Fluorescence background too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase PM1 signal. 2. Refer to package insert for proper staining instructions. 3. Check induction control. 4. Stabilize unstained cells by resuspending in 10–20% FBS for 30 min and restain. 5. Adjust FSC threshold or set gate to remove debris. 6. Refer to package insert for proper storage instructions. Do not use expired reagent. Do not expose reagent to light. 7. Adjust settings to increase FSC threshold to remove debris. 8. Washing cells may remove residual reagent.
Poor resolution between 7-AAD ⁺ and PE ⁺ populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with 7-AAD. 3. Too much Annexin V-PE in staining tube. 4. Fluorescence background too high. 5. PM1 signal too high causing PE to bleed into PM2. 6. PM2 voltage too low to optimally detect 7-AAD signal. 7. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check 7-AAD expiration date. 3. Washing cells may remove residual reagent. 4. Washing cells may remove residual reagent. 5. Adjust settings to reduce PM1 voltage. 6. Adjust settings to increase PM2 voltage. 7. Adjust settings to increase FSC threshold to remove debris.

guava Caspase Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save Caspase spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save Caspase FCS file to another directory or give it a new name.
Caspase Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.

Problem	Possible Cause	Solutions
Unexpected events appearing in a two-color dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in gating dot plot but not in analysis dot plot.	Gate excludes events.	Ensure gate is set to include population of interest.
Poor resolution between Caspase Reagent–positive and Caspase Reagent–negative populations.	<ol style="list-style-type: none"> 1. Voltage too low to detect fluorescence signal. 2. Incomplete staining with Caspase Reagent. 3. Incomplete induction of apoptosis in test suspension. 4. Uninduced population reacting with Caspase Reagent because cells have been stressed. 5. Apoptotic bodies included in analysis. 6. Caspase Reagent expired or not stored properly. 7. Background noise too high. 8. Fluorescence background too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescence signal. 2. Refer to package insert for proper staining instructions. 3. Check induction control. 4. Stabilize unstained cells by resuspending in 10–20% FBS for 30 min and restain. 5. Adjust FSC threshold or set gate to remove debris. 6. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light. 7. Adjust settings to increase FSC threshold to remove debris. 8. Washing cells may remove residual reagent.

Problem	Possible Cause	Solutions
Poor resolution between 7-AAD ⁺ and Caspase Reagent–positive populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with 7-AAD. 3. Too much Caspase Reagent in staining tube. 4. Fluorescence background too high. 5. Fluorescence signal too high causing Caspase Reagent to overlap into other fluorescence parameter. 6. RED voltage too low to optimally detect 7-AAD signal. 7. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check 7-AAD expiration date. 3. Washing cells may remove residual reagent. 4. Washing cells may remove residual reagent. 5. Adjust settings to reduce voltage corresponding to parameter used to detect Caspase Reagent. 6. Adjust settings to increase RED voltage. 7. Adjust settings to increase FSC threshold to remove debris.
Events appear off scale in dot plots.	GRN, YLW, and /or RED voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease GRN, YLW, and/or RED voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample.

guava TUNEL Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save TUNEL spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save TUNEL FCS file to another directory or give it a new name.
TUNEL Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Poor resolution between TRITC ⁺ and TRITC ⁻ populations.	<ol style="list-style-type: none"> 1. Fluorescence background too high. 2. PM1 voltage too low to detect PM1 signal. 3. Incomplete induction of apoptosis in test suspension. 4. Reagent expired or not stored properly. 	<ol style="list-style-type: none"> 1. Washing cells may remove residual reagent. Wash with Rinsing Buffer or PBS. 2. Adjust settings to increase PM1 signal. 3. Check induction control. 4. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light.

Problem	Possible Cause	Solutions
Apoptotic population shows dim TRITC signal.	Sample was not stained completely.	<ol style="list-style-type: none"> 1. Increase the incubation time during the DNA labeling reaction. 2. Increase the amount of TdT enzyme. Refer to the <i>Guava TUNEL Kit</i> package insert. 3. Ensure reagent is not expired or was not exposed to excessive light.
Staining artifacts appear in dot plot or histogram.	Cells were not properly resuspended in the DNA Labeling Mix or Anti-BrdU Staining Mix.	<ol style="list-style-type: none"> 1. Aspirate carefully to avoid disturbing pellet. 2. Wash sides of tube when resuspending cells.

guava MitoPotential Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save MitoPotential spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save MitoPotential FCS file to another directory or give it a new name.
MitoPotential Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Cell Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.

Problem	Possible Cause	Solutions
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in plots displaying PM1, PM2, or PM3.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold.
FSC Count under Cell Count shows events, but there are no events in plots displaying PM1, PM2, and/or PM3.	<ol style="list-style-type: none"> 1. Sample was not stained. 2. Cell lysis. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, restrain sample from original suspension. 2. Check buffers used to process cells.
Events appear in Plot 1 but not in Plot 2 or Plot 3.	Plot 1 gate excludes events, and gate is applied to Plot 2 and Plot 3.	Set Plot 1 gate to include population of interest.
Events appear off scale in dot plots or histograms.	FSC gain or PM1, PM2, and / or PM3 voltages set incorrectly, or samples staining brightly.	Adjust gain setting or voltage settings so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.

Problem	Possible Cause	Solutions
Poor resolution between positive and negative populations.	<ol style="list-style-type: none"> 1. Voltages too low to detect fluorescent signals. 2. Voltages too high to detect fluorescent signals. 3. Incomplete staining with JC1 and/or 7-AAD. 4. Reagents over-exposed to light, stored improperly, or expired. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase fluorescent signal. Adjust compensation settings. 2. Adjust settings to decrease fluorescent signal. Adjust compensation settings. 3. Ensure positive control is staining adequately. Otherwise, titer reagents to determine optimal concentration. 4. Refer to reagent package insert for proper storage instructions. Do not expose reagent to excessive light. Do not use expired reagents. 5. Adjust settings to increase FSC threshold to remove debris. Or, wash stained sample and reacquire.
Poor resolution between positive populations in plots displaying PM1, PM2, and/or PM3.	<ol style="list-style-type: none"> 1. Incomplete staining with reagent(s), cell concentration too high, or staining time too short. 2. Too much reagent in staining tube. 3. Voltage too high causing signal to bleed into other parameters. 4. Voltage too low to optimally detect positive signal. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date and amount of reagent(s) used in staining. Ensure sample is not too concentrated. Ensure staining time was appropriate (see package insert). 2. Titer both dyes to determine their optimum concentration. Repeat experiment with optimized reagents. 3. Adjust settings to reduce voltage. Adjust compensation settings. 4. Adjust settings to increase voltage. Adjust compensation settings. 5. Adjust settings to increase FSC threshold to remove debris.

Problem	Possible Cause	Solutions
Cell concentration decreases during acquisition.	<ol style="list-style-type: none"> 1. Cells are settling. 2. Adherent cells are reattaching. 	<ol style="list-style-type: none"> 1. Be sure that samples are mixed prior to acquisition. 2. Use low-binding, 96-well plates. If that is not sufficient to prevent reattachment, lower the FBS in the medium to 5%.

guava Cell Cycle Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save Cell Cycle spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save Cell Cycle FCS file to another directory or give it a new name.
Cell Cycle Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.

Problem	Possible Cause	Solutions
No events, as indicated in Particle Count section of Sample Information control panel.	1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate).	1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μ L for round-bottom wells, 150 μ L for 0.5-mL tubes, and 900 μ L for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Debris appearing in the PM2 histogram.	FSC threshold set too low.	Increase the FSC threshold to remove as much debris as possible.
Poor resolution among G0/G1, S, and G2/M phases.	Poor sample quality or protocol not properly followed.	Ensure cells were healthy and growing prior to fixing and staining. Ensure proper protocol was followed and correct amount of PI was used.

guava CellPaint Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save CellPaint spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save CellPaint FCS file to another directory or give it a new name.
CellPaint Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube not properly loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in PM3 vs PM1 dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM3 dot plot but not in PM3 vs PM1 dot plot.	FSC vs PM3 gate excludes events.	Ensure FSC vs PM3 gate is set to include population of interest.

Problem	Possible Cause	Solutions
Poor resolution between Green (PM3)-positive and double-negative populations.	<ol style="list-style-type: none"> 1. PM3 voltage too low to detect signal. 2. Incomplete staining with Guava CFSE reagent. 3. Debris included in analysis. 4. Guava CFSE expired, or not stored properly. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase Green (PM3) signal. Repeat compensation adjustment. 2. Refer to package insert for proper staining instructions. 3. Adjust FSC threshold or set gate to remove debris. 4. Refer to package insert for proper storage instructions. Do not use expired reagent. Do not expose reagent to light. 5. Adjust settings to increase FSC threshold to remove debris.
Poor resolution between Green (PM3)-positive and double-positive populations.	<ol style="list-style-type: none"> 1. Incomplete staining with orange fluorescent antibody probes. 2. Too much Guava CFSE reagent used. 3. Fluorescence background too high. 4. Green (PM3) signal too high, causing Guava CFSE reagent to bleed into Orange (PM1). 5. PM1 voltage too low to optimally detect orange signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Check expiration date of probes and/or titer reagent to optimize staining. 2. Repeat experiment, painting cells with a lower reagent concentration. 3. Washing cells may remove residual reagent. 4. Adjust settings to reduce PM3 voltage. Repeat compensation adjustment. 5. Adjust settings to increase PM1 voltage. Repeat compensation adjustment. 6. Adjust settings to increase FSC threshold to remove debris.
Events appear off scale in dot plots.	PM3 and /or PM1 voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM3 and/or PM1 voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample. Repeat compensation adjustment.

guava CellToxicity Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save CellToxicity spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save CellToxicity FCS file to another directory or give it a new name.
CellToxicity Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not properly loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.

Problem	Possible Cause	Solutions
Unexpected events appearing in PM3 vs PM2 dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM3 dot plot but not in PM3 vs PM2 dot plot.	FSC vs PM3 gate excludes events.	Ensure FSC vs PM3 gate is set to include population of interest.
Poor resolution between target and effector cell populations.	<ol style="list-style-type: none"> 1. PM3 voltage too low to detect PM3 signal. 2. Incomplete staining with Guava CFSE reagent. 3. Debris included in analysis. 4. Guava CFSE reagent expired or not stored properly. 5. Background noise too high. 6. Some effector cells appear to be picking up Guava CFSE dye and can't be separated from target cells using a gate. 	<ol style="list-style-type: none"> 1. Adjust settings to increase PM3 signal. Adjust compensation settings. 2. Refer to package insert for proper staining instructions. 3. Adjust FSC threshold or set gate to remove debris. 4. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light. 5. Adjust settings to increase FSC threshold to remove debris. 6. Use quadrant markers to separate CFSE-positive effector cells from dead target cells.
Poor resolution between dead and live target cell populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with 7-AAD. 3. Too much Guava CFSE reagent used. 4. PM3 signal too high causing Guava CFSE reagent to bleed into PM2. 5. PM2 voltage too low to optimally detect 7-AAD signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check 7-AAD expiration date. 3. Repeat experiment, painting target cells with a lower reagent concentration. 4. Adjust settings to reduce PM3 voltage. Adjust compensation settings. 5. Adjust settings to increase PM2 voltage. Adjust compensation settings. 6. Adjust settings to increase FSC threshold to remove debris.

Problem	Possible Cause	Solutions
Events appear off scale in dot plots.	PM3 and /or PM2 voltages set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM3 and/or PM2 voltages so positive populations appear on scale. Repeat Adjust Settings with negative sample. Adjust compensation settings.
All samples contain target cells that are 100% killed.	FSC vs PM2 dot plot axis selected with gate enabled.	Select FSC vs PM3 dot plot and adjust gate if necessary to include all target cells.

guava CellGrowth Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save CellGrowth spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save CellGrowth FCS file to another directory or give it a new name.
CellGrowth Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter the registration code. The code is case sensitive.
Few events, as indicated in Particle Count section of Sample Information control panel.	1. Clogged flow cell. 2. Insufficient sample volume. 3. Cells in suspension have settled.	1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μ L for round-bottom wells, 150 μ L for 0.5-mL tubes, and 900 μ L for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.

Problem	Possible Cause	Solutions
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not properly loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in PM3 vs PM2 dot plot or PM3 histogram.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Threshold set too low or debris included in gate. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. If threshold did not exclude debris when sample was acquired, set gate to remove excess debris.
Events appear in FSC vs PM3 dot plot but not in PM3 vs PM2 dot plot.	FSC vs PM3 gate excludes events.	Ensure FSC vs PM3 gate is set to include population of interest.
Events appear in FSC vs PM2 dot plot but not in PM3 histogram.	FSC vs PM2 gate excludes events.	Ensure FSC vs PM2 gate is set to include population of interest.
Poor resolution between resting and proliferating cell populations.	<ol style="list-style-type: none"> 1. PM3 voltage too low to detect PM3 signal. 2. Incomplete staining with guava painting reagent. 3. Debris included in analysis. 4. guava painting reagent expired or not stored properly. 5. Background noise too high. 	<ol style="list-style-type: none"> 1. Adjust settings to increase PM3 signal. 2. Refer to package insert for proper staining instructions. 3. Adjust FSC threshold or set gate to remove debris. 4. Refer to package insert for proper storage instructions. Do not use expired reagent or reagent exposed to light. 5. Adjust settings to increase FSC threshold to remove debris.

Problem	Possible Cause	Solutions
Poor resolution between dead and live cell populations.	<ol style="list-style-type: none"> 1. High viability sample. 2. Incomplete staining with PI. 3. Too much guava painting reagent used. 4. PM3 signal too high causing guava painting reagent to bleed into PM2. 5. PM2 voltage too low to optimally detect PI signal. 6. Background noise too high. 	<ol style="list-style-type: none"> 1. Resolution may be difficult if few dead cells in sample. 2. Check PI expiration date. 3. Repeat experiment, painting cells with a lower reagent concentration. 4. Adjust settings to reduce PM3 voltage. 5. Adjust settings to increase PM2 voltage. 6. Adjust settings to increase FSC threshold to remove debris.
Events appear off scale in dot plots.	PM3 and/or PM2 voltages and/or FSC gain set incorrectly, or samples staining brightly.	Adjust settings to increase or decrease PM3 and/or PM2 voltages and/or FSC gain so positive populations appear on scale. Repeat Adjust Settings.

guava RapidQuant Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: <i>This file already exists. You must pick a new name.</i>	Spreadsheet file with same file name already exists in selected directory.	Save guava RapidQuant spreadsheet file to another directory or give it a new name.
Message: <i>This file exists with read-only attributes. Please use a different file name.</i>	FCS file with same file name already exists in selected directory.	Save guava RapidQuant FCS file to another directory or give it a new name.
RapidQuant Software Module starts in Analysis mode. Acquisition mode is not available.	A registration code was not entered or was entered incorrectly.	Enter registration code. The code is case sensitive. NOTE: If registration code is correct, contact EMD Millipore Technical Support. You may need a firmware upgrade to run RapidQuant Software Module.

Problem	Possible Cause	Solutions
Few events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Clogged flow cell. 2. Insufficient sample volume. 3. Beads in suspension have settled. 	<ol style="list-style-type: none"> 1. Perform a Backflush. Follow with Quick Clean. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Ensure sample mixing option was selected in WorkEdit Software.
No events, as indicated in Particle Count section of Sample Information control panel.	<ol style="list-style-type: none"> 1. Sample tube or plate not loaded. 2. Insufficient sample volume. 3. Clogged flow cell. 4. Broken flow cell. 5. Sample pump not working. 6. Laser not operational. 7. Loose fitting on minstac tubing (under metal plate). 	<ol style="list-style-type: none"> 1. Ensure tube or plate is in place and tray is loaded. 2. Minimum sample volume is 100 μL for round-bottom wells, 150 μL for 0.5-mL tubes, and 900 μL for 1.5-mL tubes. 3. Perform a Backflush. Follow with Quick Clean. 4. Remove flow cell and inspect for damage. Replace if necessary. 5. Run Quick Clean and watch for fluid in waste vial. 6. Contact EMD Millipore Technical Support. 7. Ensure tubing connector is secure.
Unexpected events appearing in dot plot.	<ol style="list-style-type: none"> 1. Laser not warmed up. 2. Instrument settings not optimal. Acquiring debris. 3. Undesirable population in sample. 	<ol style="list-style-type: none"> 1. Allow laser to warm up 10 min before acquisition. 2. Adjust settings so debris is below threshold. 3. Set gate to exclude undesirable population, or exclude sample from analysis.
FSC Count under Particle Count shows events, but events appear in the wrong place in FSC vs GRN dot plot.	<ol style="list-style-type: none"> 1. Supernatant or FITC goat anti-mouse (or human) IgG antibody not added. 2. Instrument settings not optimal. 	<ol style="list-style-type: none"> 1. Check sample. If necessary, prepare sample again from original supernatant. 2. Repeat Adjust Settings step.
Events appear in dot plot but not in histogram.	Gate set incorrectly.	Ensure gate includes Capture Bead population.

Problem	Possible Cause	Solutions
Events appear off scale in dot plot or histogram.	FSC gain or GRN voltage set incorrectly.	Adjust gain setting or voltage settings so bead-only control population appears on scale in FSC and between 0 and 10 in GRN fluorescence parameter.
Standard curve is not linear.	<ol style="list-style-type: none"> 1. Pipettes not calibrated. 2. Dilution steps performed improperly. 3. Standards or other kit components not stored properly. 	<ol style="list-style-type: none"> 1. Ensure pipettes are calibrated. Pipetting accuracy is necessary for optimal results. 2. Check accuracy of dilution steps. 3. Order new kit.
Unknown concentration predictions are not accurate.	<ol style="list-style-type: none"> 1. Unknowns are under- or over-diluted, resulting in MFI values for unknowns that are higher or lower, respectively, than those obtained with standards. 2. Unknowns not diluted correctly. 3. Standards were not diluted correctly. 4. Samples were not properly shaken during incubation. 5. Plate map was set up incorrectly in RapidQuant Software Module. 	<ol style="list-style-type: none"> 1. Repeat assay diluting unknowns so that their MFI values fall within range of MFIs obtained with standards. NOTE: It may be necessary to make different dilutions of same unknown to ensure that some diluted samples fall within linear range. 2. Be sure unknown dilutions were performed correctly and that proper dilution factors were entered into software. 3. Check that protocol was followed. 4. A rocker or shaker should be used during incubation for optimal results. 5. Be sure that replicates and individual samples are defined correctly. Ensure all unknown and control dilutions were properly selected.

Appendix A

EasyFit Case Study Examples

guava ViaCount EasyFit uses computer-assisted population analysis to determine cell count and differentiate viable from non-viable (dead and apoptotic) cells and whole cells from debris. The EasyFit analysis algorithm was designed to improve upon the ViaCount analysis ease-of-use and provide better discrimination of live cells and dead cells using 3-dimensional data analysis, rather than the 2-dimensional analysis available when you manually adjust in the ViaCount Software Module. EasyFit is not constrained by the linear FSC and PM1 markers found in manual ViaCount analysis, meaning that your results are less sensitive to the presence of large amounts of contaminating debris. Hence more accurate results can be obtained.

There are biological circumstances in which the EasyFit analysis method is not recommended for the most accurate results. For example, when cell cultures contain a significant amount of apoptotic cells, especially if those apoptotic cells are in close proximity to the viable cells based on FSC and PM1 values, or if all of the cells in the culture are apoptotic. This happens most often when cultures are induced to undergo apoptosis, but it can also happen to cells under normal culture conditions.

Another instance in which the EasyFit analysis method yields inaccurate results is with cultures of very low viability. If your cultures have less than 10% viable cells, EMD Millipore recommends that you perform manual ViaCount analysis.

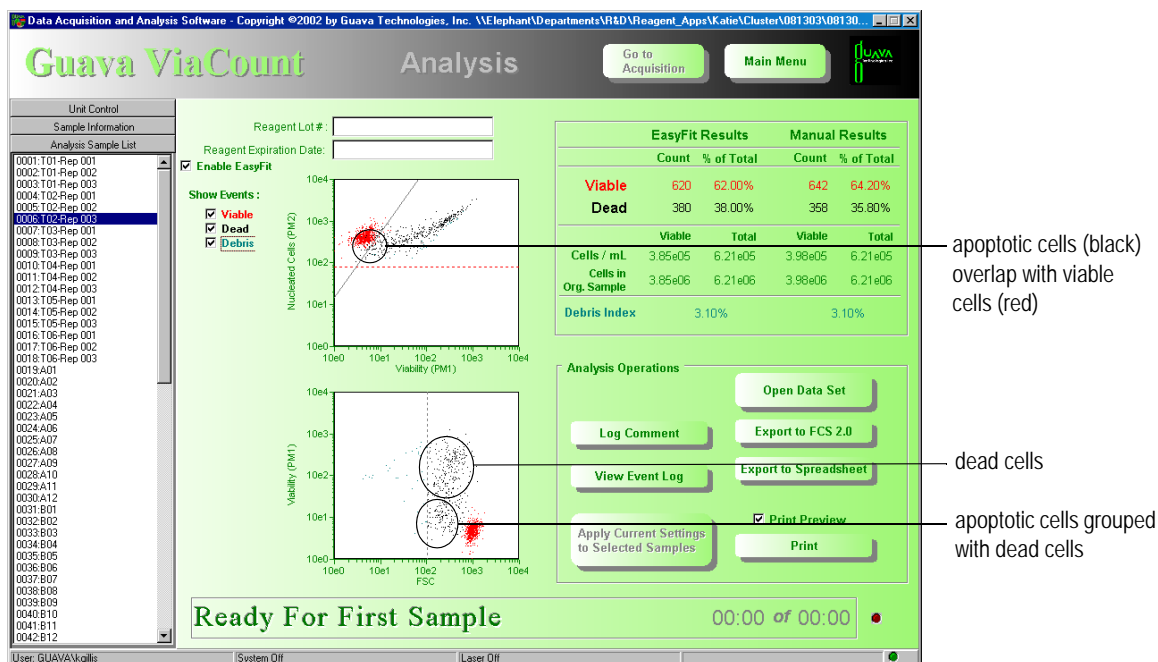
This appendix provides examples of where EasyFit analysis works as well as or better than manual analysis, and where manual analysis works better than EasyFit analysis. EMD Millipore recommends that you validate EasyFit for your particular cell lines before routinely using it, and after validation you should continue to monitor the dot plots and results, by comparing manual results to EasyFit results, for each sample to ensure that the analysis is correct.

Use EasyFit Under These Circumstances

Following are examples of when EasyFit analysis is recommended, and would work as well as or better than manual analysis.

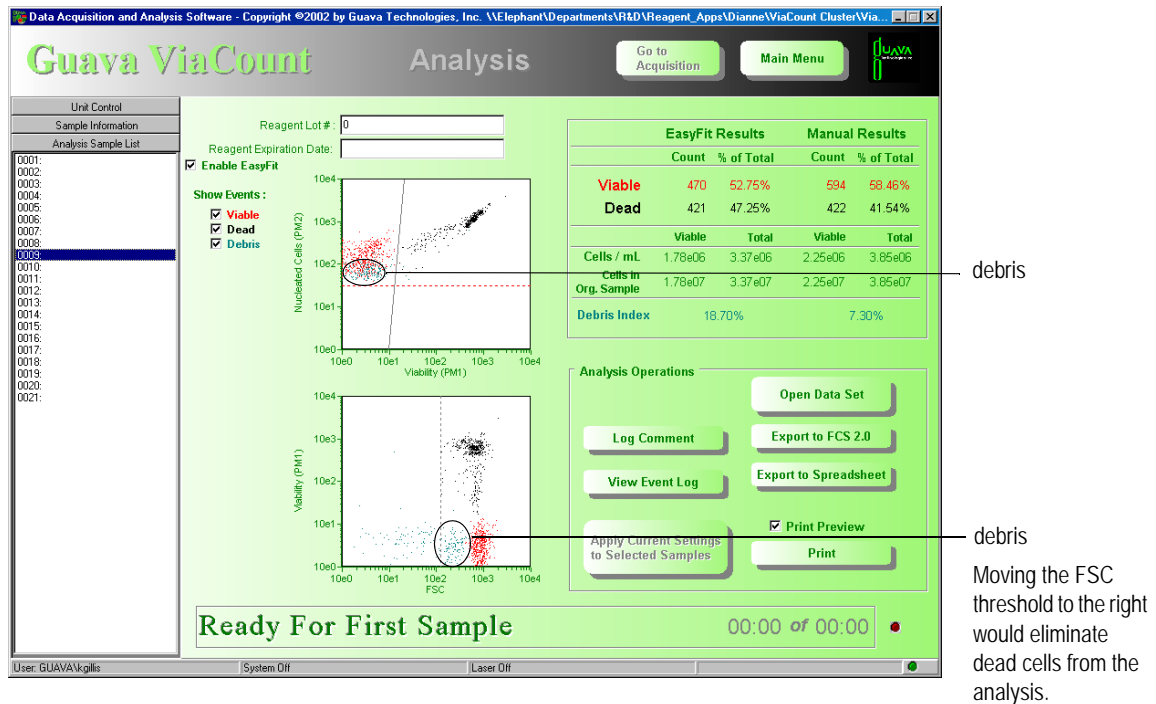
1. Use EasyFit When Viable and Apoptotic Cells Overlap in PM1 and PM2

Use EasyFit when it is difficult to determine where to place the viability marker because the viable cells and apoptotic/dead cells do not show a clean separation. With manual analysis there is no way to adjust the PM1 viability marker to completely separate the viable from the apoptotic cells. Because EasyFit analyzes the data using three parameters at once, it can separate the apoptotic cells from viable cells based on their differential FSC signals, as seen in the FSC vs Viability (PM1) dot plot.



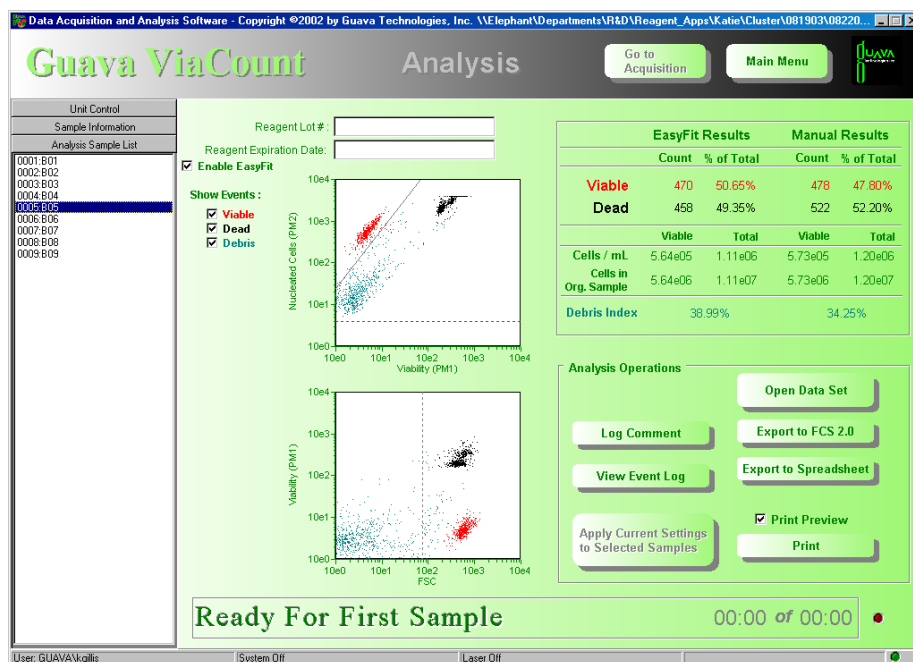
2. Use EasyFit When Debris is Adjacent to Viable Cell Population

Use EasyFit when debris appears directly to the left of the viable population in the FSC vs Viability (PM1) dot plot, and when you cannot move the FSC threshold to separate the viable cells from the debris without excluding some of the dead cells and hence overestimating the viability of the culture. The 3-dimensional algorithm can easily identify and separate out the debris.



3. Use EasyFit With Low PM2 Marker Settings

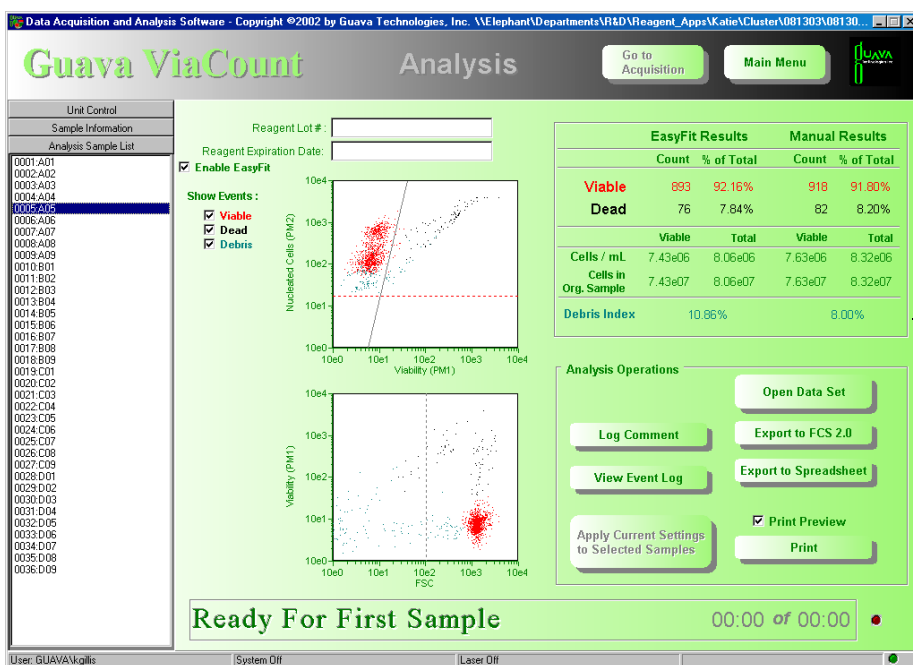
EasyFit works as well as manual analysis when the PM2 threshold is set too low, allowing a large amount of debris to be acquired. The populations are still distinct and well defined.



EasyFit and manual results are comparable.

4. Use EasyFit When Incomplete Staining Occurs

EasyFit works as well as manual analysis when two viable cell populations appear based on PM2. This staining pattern may appear if an insufficient amount of ViaCount Reagent is used. Some cultures require a higher concentration of PM2 dye to adequately stain all viable cells. While there are two viable populations based on PM2, there is only one based on PM1 and FSC.



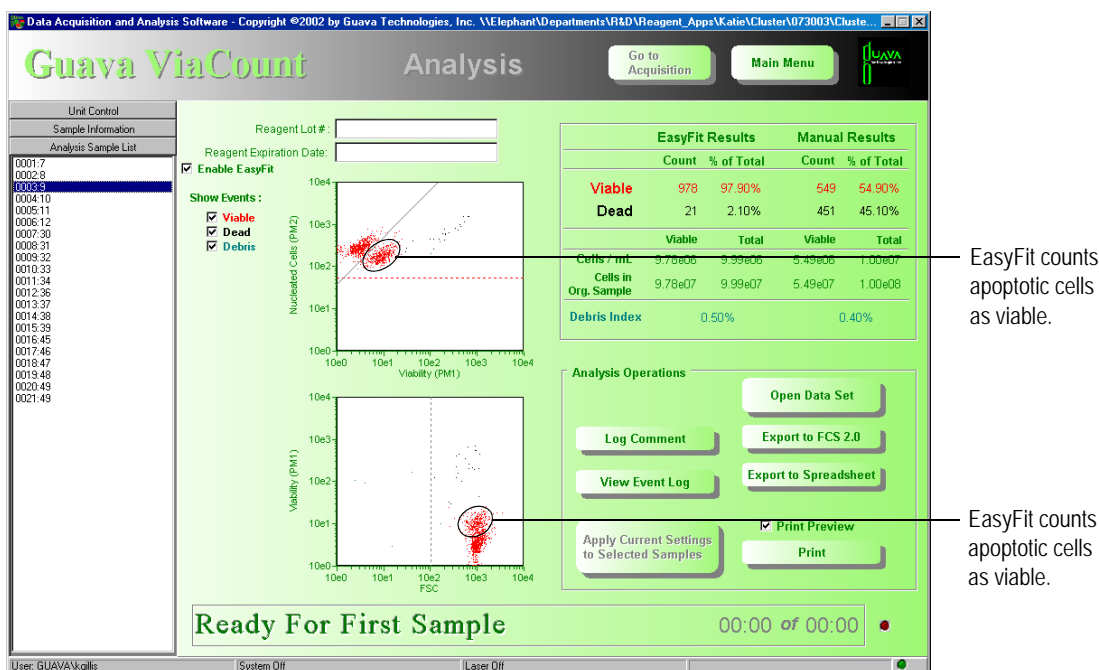
EasyFit and manual results are comparable.

Use Manual ViaCount Analysis Under These Circumstances

EasyFit is not recommended under general conditions where there are a predominant number of apoptotic cells located close to the viable cells based on FSC, or there is a very large amount of debris. EasyFit does not accurately determine the percent viability and the viable cells/mL if cultures contain <10% viable cells. EasyFit also does not work optimally if the instrument is not set up properly prior to sample acquisition. Following are specific examples of where manual analysis is preferred over EasyFit analysis.

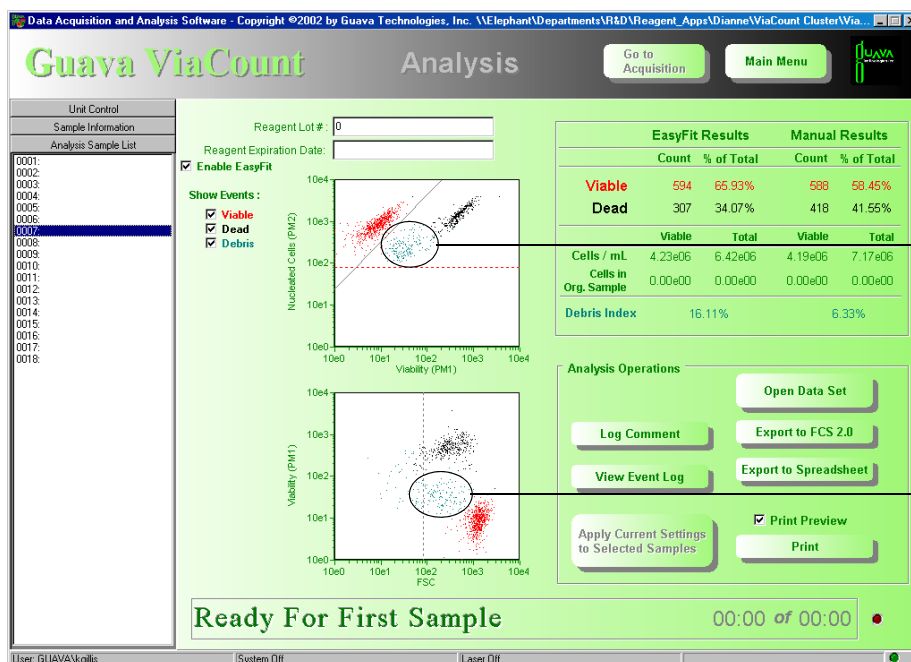
1. Use Manual ViaCount Analysis With Only a Slight Shift in Apoptotic Cells

Use manual analysis with the apoptotic marker enabled, instead of EasyFit, if the apoptotic population is close to the viable population and EasyFit is counting the apoptotic cells as viable cells rather than dead.



2. Use Manual ViaCount Analysis to Correctly Identify Apoptotic Cells

EasyFit may incorrectly identify apoptotic cells as debris. Apoptotic cells have slightly lower FSC intensity and slightly higher PM1 intensity than viable cells.

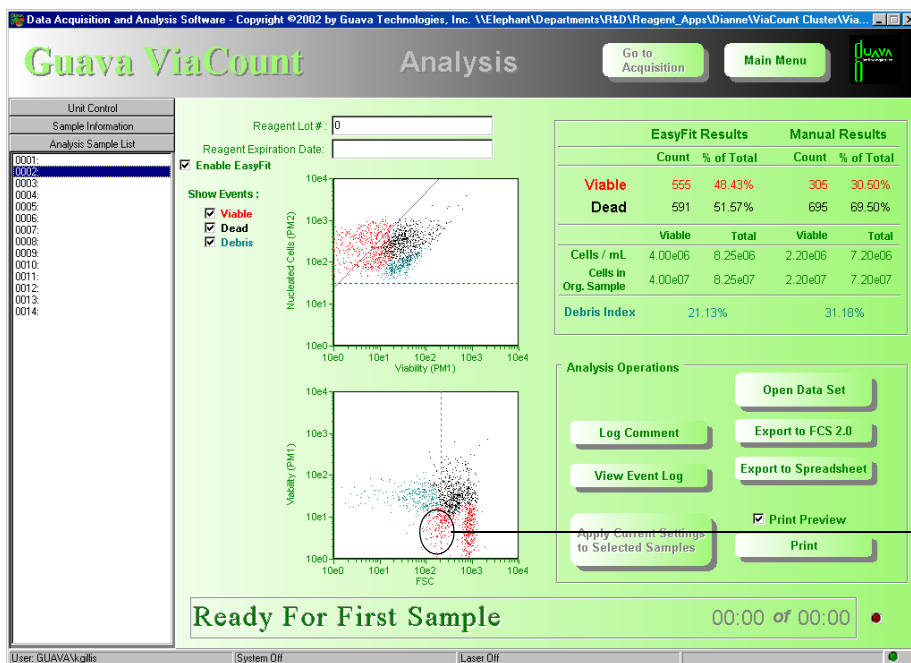


EasyFit counts apoptotic cells as debris.

EasyFit counts apoptotic cells as debris.

3. Use Manual ViaCount Analysis With a Large Amount of Apoptotic and Dead Cells

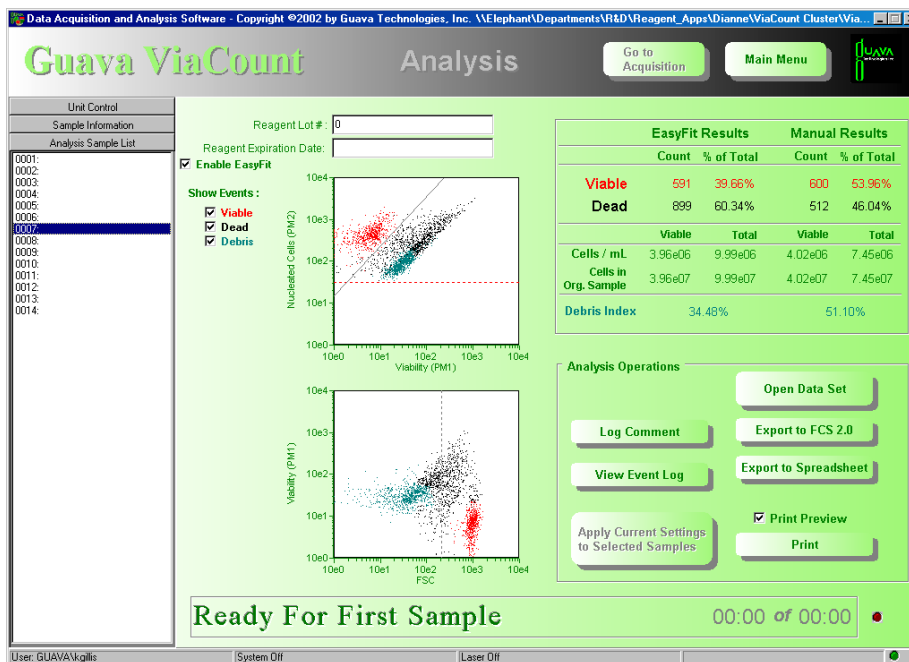
EasyFit does not work well when there is large amount of apoptotic and dead cells.



EasyFit detects an erroneous viable population.

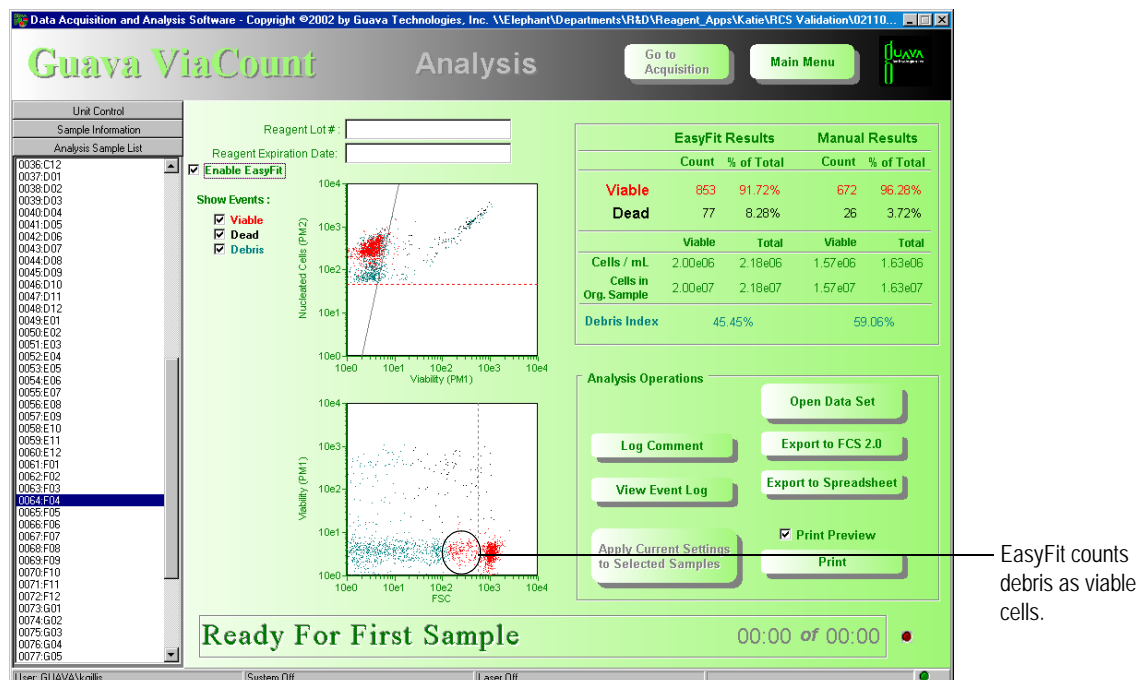
4. Use Manual ViaCount Analysis With Poor Resolution Between Dead Cells and Debris

EasyFit cannot distinguish between dead cells and debris in cultures with a large amount of debris. For samples with a large amount of debris where it may also be difficult to manually discriminate populations, you may want to use an alternate viability test method.



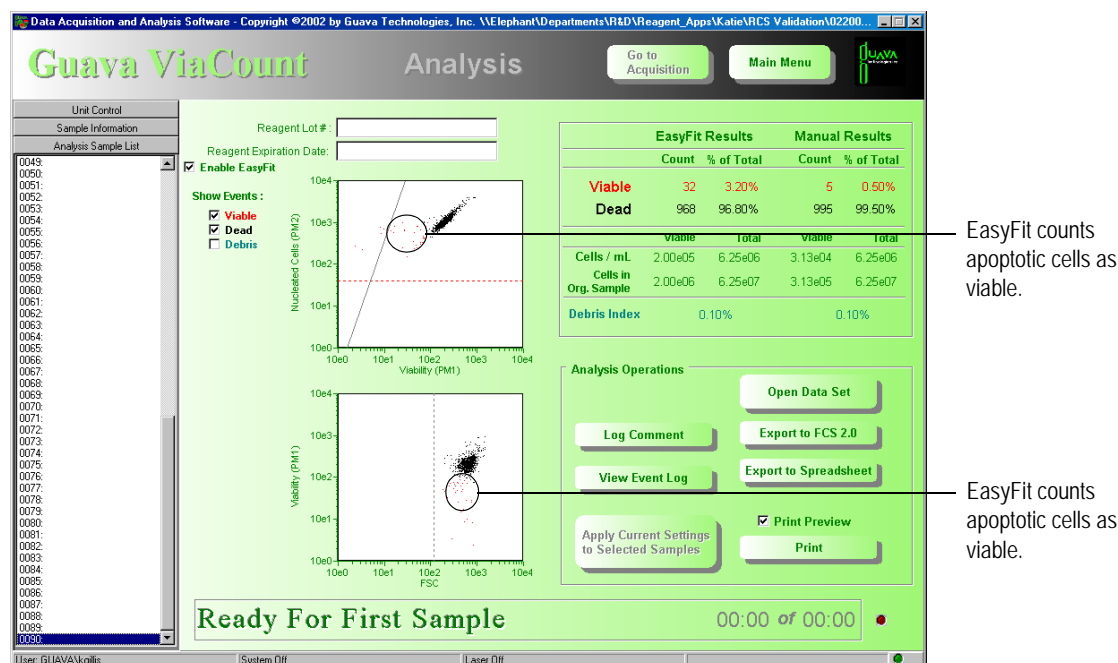
5. Use Manual ViaCount Analysis When Large Amounts of Debris are Not Well Separated from Viable Cells

EasyFit may count some of the debris as viable cells if there is not a clear separation between debris and viable cells.



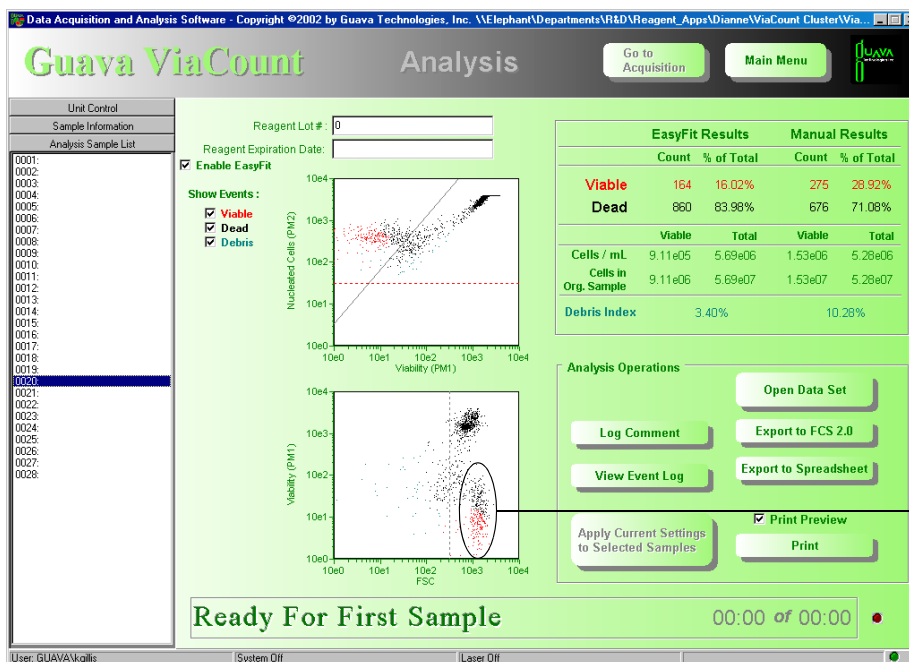
6. Use Manual ViaCount Analysis With Low Viability Cultures

If the percentage of viable cells is low (<10%), EasyFit overestimates the number of viable cells. The few apoptotic cells in this sample are counted as live instead of dead.



7. Use Manual ViaCount Analysis With a High PM1 Voltage Setting

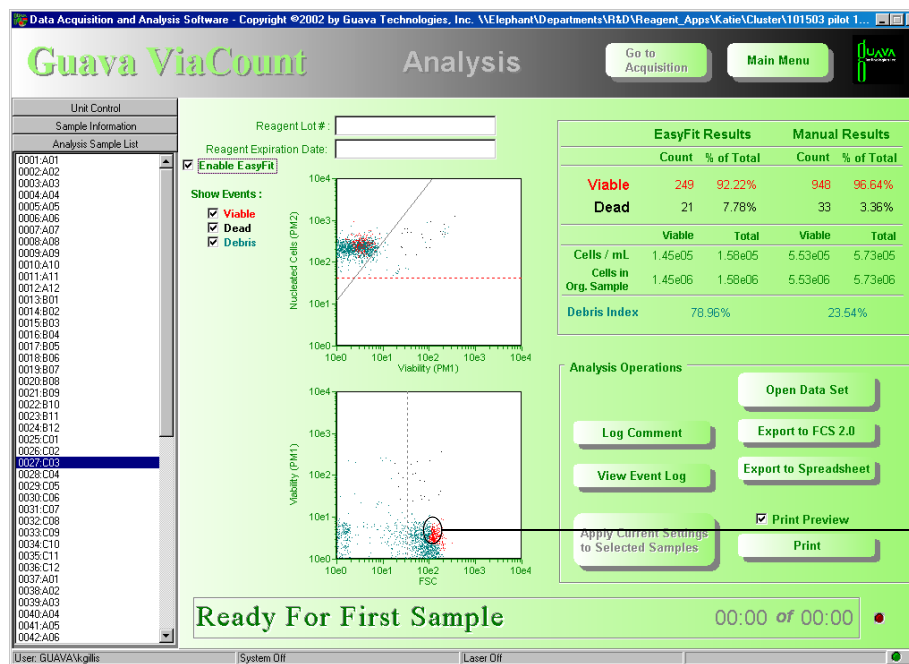
PM1 voltage is set too high. EasyFit is counting viable cells as dead cells. To prevent this, use a PM1 voltage setting that places the viable cells between $10e0$ and $10e1$.



PM1 voltage set too high. Viable cell population approaching PM1 positive.

8. Use Manual ViaCount Analysis With a Low FSC Gain Setting

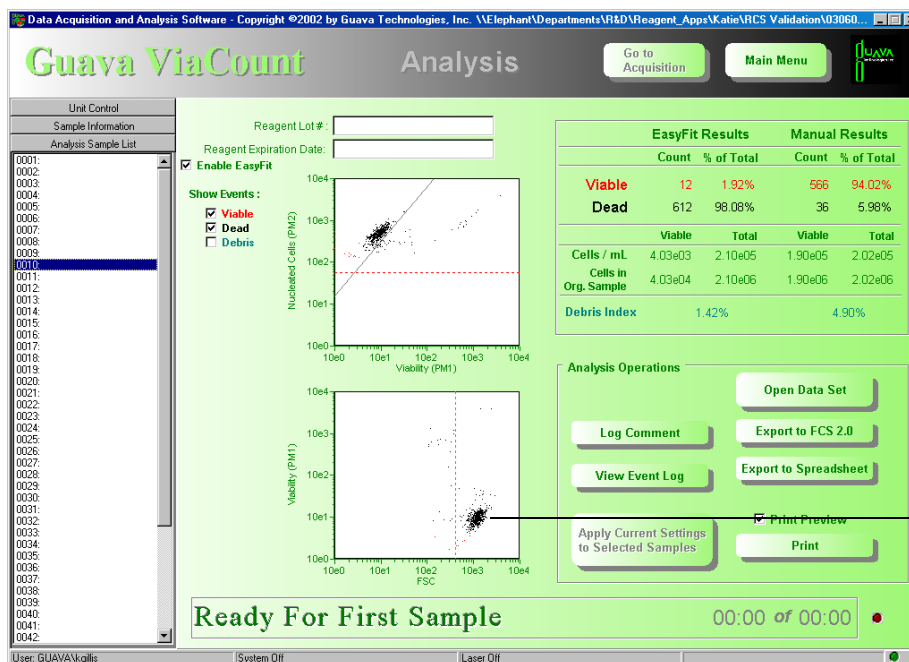
EasyFit incorrectly identifies viable cells as debris when the FSC gain is too low. To prevent this, adjust the FSC gain to place the viable cells at approximately $10e3$ on the FSC scale.



Small portion of viable cells counted as viable by EasyFit.

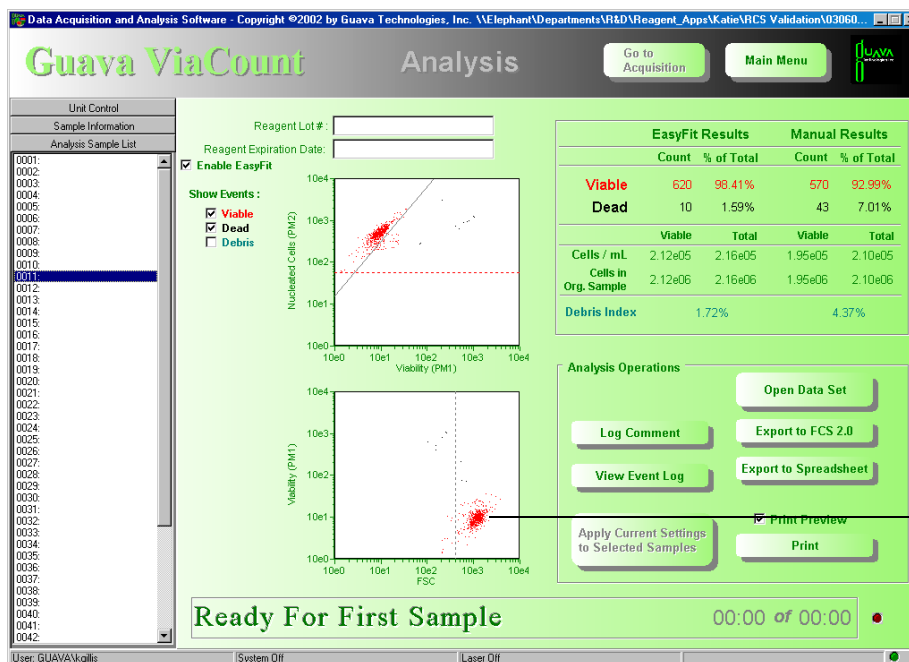
9. Manual ViaCount Analysis May be More Reproducible

The following two screens show the replicate samples collected at different times. In the first sample (below) EasyFit incorrectly identifies the viable cells as dead. This was most likely because the PM1 voltage was not adjusted so that the viable cell population was placed between $10e0$ and $10e1$. However, even with the correct instrument settings, EasyFit can on occasion fail to yield the same results for replicate samples.



EasyFit incorrectly counts viable cells as dead cells.

The same sample as above, acquired a few minutes later. This time even though the PM1 mean fluorescence was still too high, EasyFit correctly identifies the viable cells.



EasyFit correctly identifies viable cells.

Appendix B

Administrator Features

Setting Up Access Control

guavaSoft Software, version 2.7 supports features which allow a laboratory administrator in controlled environments to restrict users from access to certain features of the guava easyCyte HT System in order to help ensure that standard procedures are followed correctly. Users whose access has been restricted cannot change instrument or analysis settings, and may only operate the guava easyCyte HT System according to previously saved Settings files or the instrument default settings.

Who Should Read This Section on Administrator Features

By default, after installation there are no access control restrictions, and the default “GTI” user account has access to all features of the software. If such unrestricted access is sufficient for your needs, then no further steps are necessary and you may begin using the software.

You should read this section if:

- you want to use access control in your laboratory or environment, or
- you want to create new user accounts, or
- you are a network administrator and need information about the guava environment in order to create guava user accounts in a manner consistent with your local security policy

EMD Millipore recommends that you contact your local network administrator for help in setting up user accounts and access control.

Access Control Levels

guavaSoft Software, version 2.7 supports three levels of access control—*Administrator*, *Supervisor*, and *Operator*. Each user account under Windows has one of these levels, which is assigned by the lab administrator on a case-by-case basis. A higher access control level always has privileges of all access control levels below.

Administrator - level user accounts have unrestricted access to all features of the software including the “Administration Configuration” on page B-9, and are assumed to be guavaSoft Software Administrators who know how to properly configure all features of the software for supervisor-level and operator-level users.

Supervisor - level user accounts have unrestricted access to all features of the software except the “Administration Configuration” on page B-9, and are assumed to be expert users who know how to properly use all features of the software at the supervisor and operator levels.

All user accounts can:

- perform the Adjust Settings step
- retrieve instrument settings files
- start new data sets
- run the guava easyCyte HT System and acquire data (entering all relevant information such as sample IDs, number of events to acquire, and dilution factor and original volume, when applicable)
- perform Quick Clean, Backflush, and Cleaning operations
- run the easyCheck Procedure
- abort an acquisition, when necessary
- export data to FCS 2.0 or CSV format
- add comments to event log
- view event log file

Operator - level user accounts cannot:

- change instrument settings (FSC gain, PMT voltages, pump speed)
- adjust markers or gates
- make any adjustments during the Adjust Settings step
- use Next Step, except to (a) finish Adjust Settings, or (b) terminate a normal acquisition
- save instrument settings

Before Setting Up Access Control

The guavaSoft Software, version 2.7 installer automatically creates the three different guava User Groups under Windows, namely, GuavaAdmin, GuavaSupervisor, and GuavaOperator. The correct access to the various guava files needed to function properly are also automatically set up by the installer.

- **IMPORTANT NOTE:** If you plan to connect the laptop to a network, be sure to contact your local network administrator for help, since the procedures described here may need to be modified in order to comply with security policies in effect for your local network.

“First-Time Setup After Installation” describes steps which need to be taken only once, after installation of the software. It explains how to set up the guava Settings files folder access control.

“How to Create A New User Account” describes how to create a new user account.

“Assigning a guava Access Control Level to a User Account” describes how to assign the access control level (Administrator, Supervisor, or Operator) to a user account.

First-Time Setup After Installation

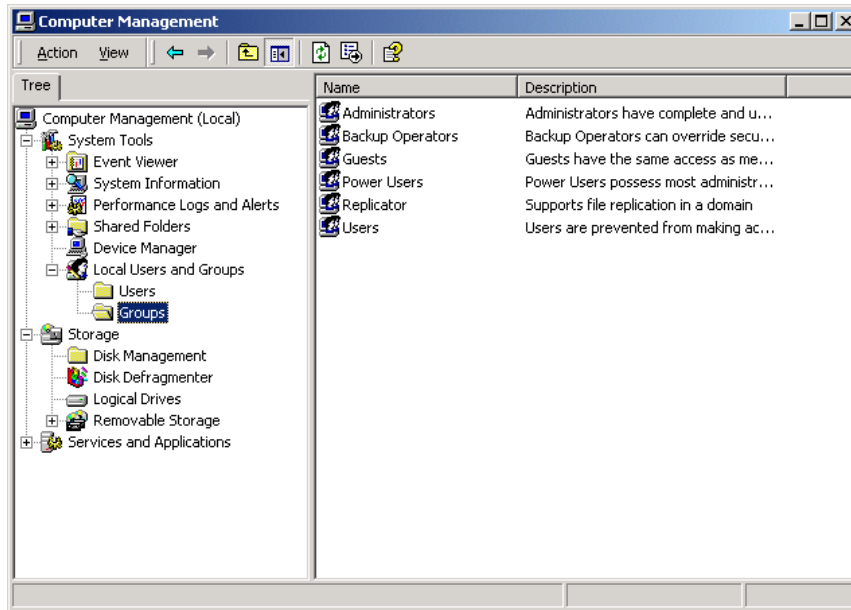
This section shows you after installation, the three User Groups, GuavaAdmin, GuavaSupervisor, and GuavaOperator are automatically created. The steps to set up the folder for the guava Setting Files are also described in this section.

The GuavaAdmin group is used by guavaSoft Software to allow the guava Administrator to configure the “Administration Configuration” features. This option is available from the guavaSoft Software main menu to a guava Administrator only. The GuavaAdmin group consists of only those users who are to share responsibility for administering Access Control. All members of the GuavaAdmin group should also be a Windows Administrator. Only these users (or a Windows administrator) will be able to assign or change the access control levels for other users.

Three User Groups Automatically Created Upon Installation

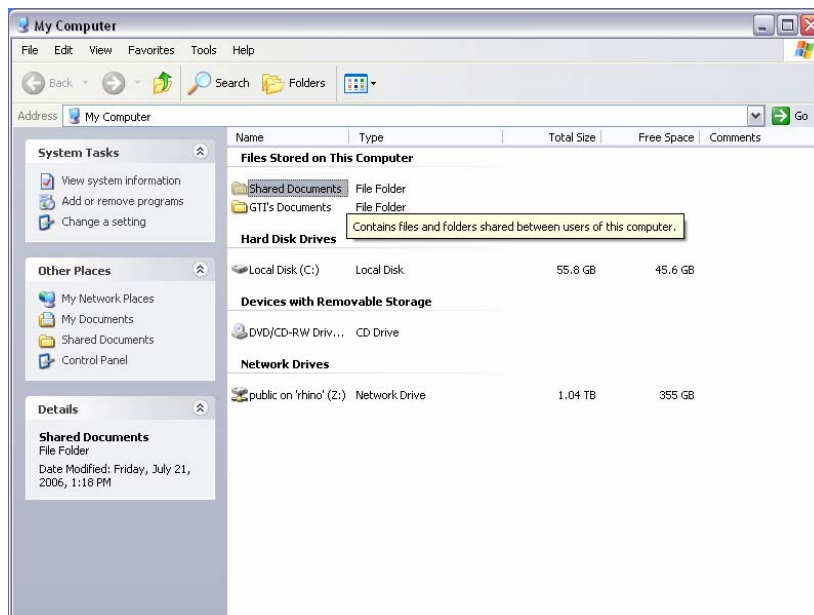
- 1 Log onto the laptop as administrator. (No password is required by default.)

- 2 Right-click My Computer and select **Manage** from the menu. The Computer Management window appears. Expand the content of the “Local Users and Groups” folder and click on the “Groups” folder.



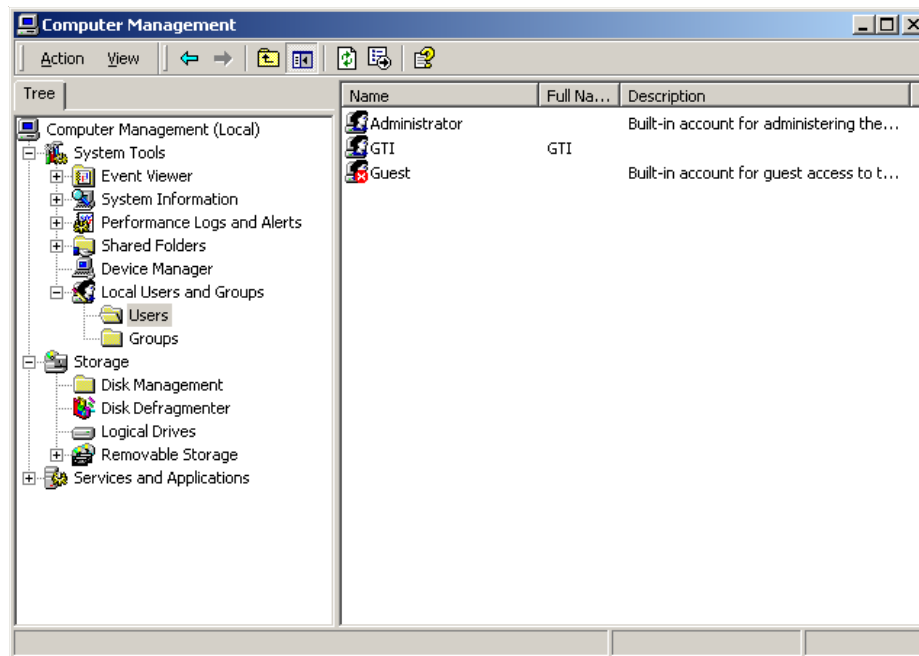
Create the guavaSoft “guavaSettings” Folder

- 1 Double-click My Computer. Double-click Shared Documents. Create a new folder in the Shared Documents folder called guavaSettings.

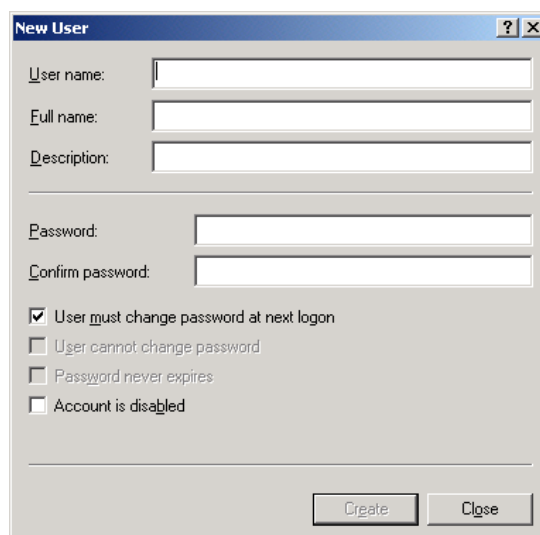


How to Create A New User Account

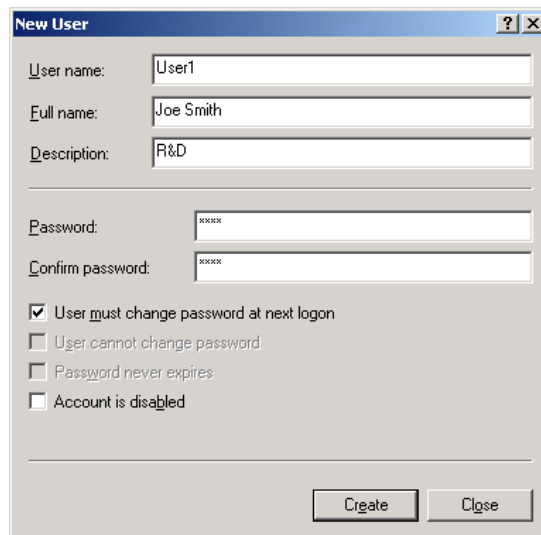
- 1 Log onto the laptop as administrator.
- 2 Right-click My Computer and select **Manage** from the menu.
- 3 Find Local Users and Groups and click to expand its contents. Click the Users folder.



- 4 Select **New User** from the Action menu in the menu bar at the top of the window. The New User dialog box appears.



- 5 Enter a user name to be added to the system. Type in a full name (first and last), a password and confirmation, and a description. In the example below, a user account “User1” for “Joe Smith” in “R&D” is created.



The screenshot shows a 'New User' dialog box. The 'User name' field contains 'User1', 'Full name' contains 'Joe Smith', and 'Description' contains 'R&D'. The 'Password' and 'Confirm password' fields are masked with 'x' characters. The 'User must change password at next logon' checkbox is checked, while the others are unchecked. The 'Create' and 'Close' buttons are at the bottom right.

- 6 Click **Create**. You may continue adding more users by repeating the steps starting with step 4. Click **Close** when you are finished adding users.
- 7 You will need to assign each user to the proper access control group. Proceed to “Assigning a guava Access Control Level to a User Account” for this information.

Assigning a guava Access Control Level to a User Account

This section shows you how to assign the Access Control level to a user account for guavaSoft Software. You do this by assigning the user to one of the groups created in “First-Time Setup After Installation” on page B-3.

If you want the user to be able to administer Access Control for other users or to be able to configure the “Administration Configuration” feature (normally, very few users should have this right), add the user to the GuavaAdmin group as shown below. Also make sure that the user you have added to the GuavaAdmin group is also a Windows Administrator.

In a similar manner, a user account can be assigned to have either Supervisor- or Operator-level access control simply by adding the user to either the GuavaSupervisor or GuavaOperator groups, respectively, as shown below.

Be careful not to assign a user to more than one guava group. The higher Group privilege takes precedence, which may not be what you want.

- 1 Log onto the laptop as a Windows Administrator.
- 2 Right-click My Computer and select **Manage** from the menu.
- 3 Navigate to the Groups folder under “Local Users and Groups.”

How to Give a User Account GuavaAdmin-Level Access

- 1 Right-click on the GuavaAdmin group and select **Properties**.
- 2 Click **Add** in the GuavaAdmin Properties window.
The Select Users window appears.



- 3 Enter the user login name for the administrator and click **OK**.
 - 4 Repeat these steps to add additional administrators.
- **NOTE:** If a user in the GuavaAdmin group is NOT a Windows Administrator, you can add the user to the Administrators group:
- Right-click My Computer and select **Manage** from the menu.
 - Click the Groups folder under the “Local Users and Groups” folder.
 - Right-click on the “Administrators” group in the right side of the window and select **Add to Group** from the menu.
 - Click **Add** in the Administrators Properties window.
 - Select the user you want to add to the Administrators group and click **Add**.
 - Click **OK** when you are finished.

How to Give a User Account GuavaSupervisor-Level Access

- 1 Right-click on the GuavaSupervisor group and select **Properties**.
- 2 Click **Add** in the GuavaSupervisor Properties window.
The Select Users window appears.



- 3 Enter the user login name for the supervisor and click **OK**.
- 4 Repeat these steps to add additional supervisors.

How to Give a User Account GuavaOperator-Level Access

- 1 Right-click on the GuavaOperator group and select **Properties**.
- 2 Click **Add** in the GuavaOperator Properties window.
The Select Users window appears.



- 3 Enter the user login name for the operator and click **OK**.
- 4 Repeat these steps to add additional operators.
- 5 Reboot the laptop when finished.

Information For Network/IT Administrators

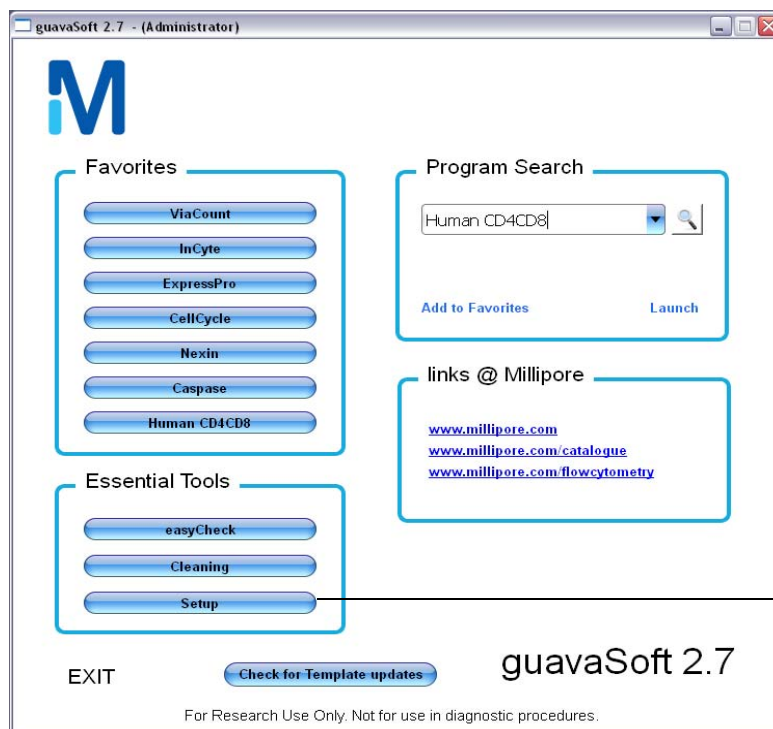
- User accounts which function as administrators for guava Access Control do not need to have Windows Administrator privileges, they only need full control of the AccessRights folder within the guavaSoft folder hierarchy.
- The GuavaSettings folder described in “First-Time Setup After Installation” on page B-3 does not need to be in C: drive. It can be located anywhere as long as all users can access it. Although the setup procedures above do not detail how to do this, the Operator-level accounts do not need access to the GuavaSettings folder, since they cannot create Settings files. Therefore, you could obtain greater security by arranging that the GuavaOperator Group has only Read access to the GuavaSettings folder.
- All guavaSoft Software users need Read/Write/Modify access to the Log folder when running the easyCheck Procedure, since this assay updates the GuavaCheckLog.

Administration Configuration

Administrators can configure, or customize, certain software features for supervisors and/or operators.

You must be logged in as an administrator to gain access to the Setup button on the guavaSoft Software main menu.

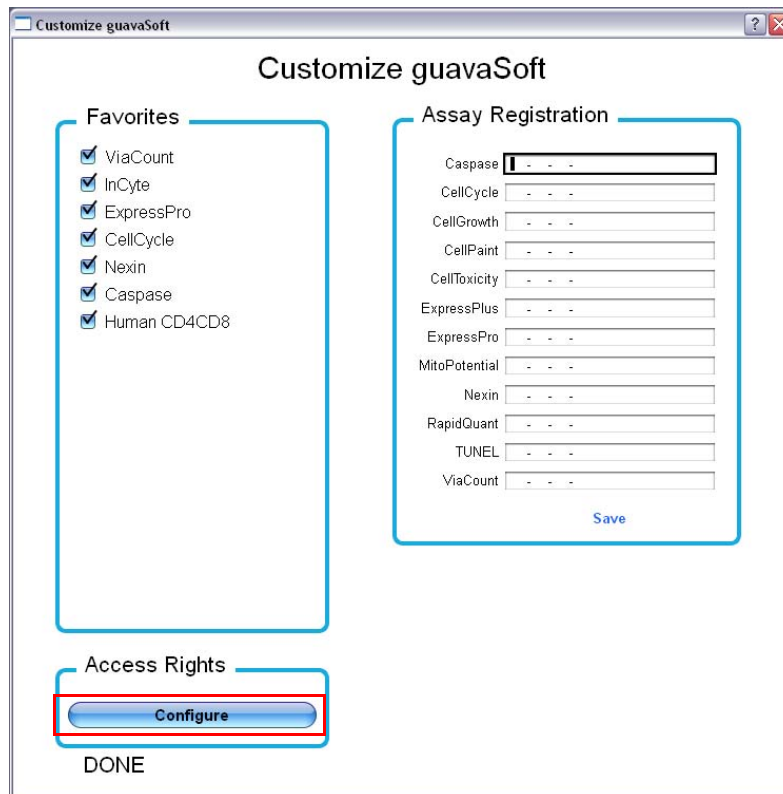
- 1 Click **Setup** from the main menu.



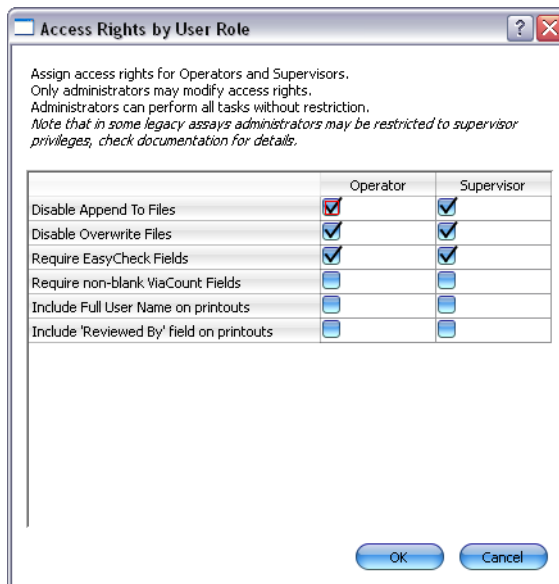
Available to administrators who log onto the system.

The Customize guavaSoft screen appears.

2 Click **Configure** under Access Rights.



3 Click the check boxes for the features you wish to allow for supervisors and/or operators.



The following table describes each feature.

Feature	Description
Disable Append to Files	<p>If selected, will not allow user to append to an existing FCS 3.0 file. Users may overwrite the existing file or create a new file. This applies to a data file when you click New Data Set at the start of a session.</p> <p>If overwriting is disabled (see below), you will be appending to a <i>copy</i> of the existing file since you cannot overwrite it. If overwriting is allowed, you will be appending to the end of the existing file.</p>
Disable Overwrite Files	<p>If selected, will not allow users to overwrite an existing FCS 3.0 file. Users may append to the existing file or create a new file. This applies to a data file when you click New Data Set at the start of a session, as well as to a data file when you make changes during analysis.</p>
Require easyCheck Fields	<p>If selected, the user must enter values for the Bead Lot #, Bead Expiration Date, and Expected Particles/mL before starting every easyCheck run. If this option is not selected, the information will default to the last information entered.</p>
Require non-blank ViaCount Fields	<p>If selected, the user must enter values for the Reagent Lot # and Reagent Expiration Date before a ViaCount Assay can be started. If this option is not selected, the reagent information is not required.</p>
Include Full User Name on Printouts	<p>If selected, the user's full name is included on all printouts generated by that user. This information is taken from the "Full Name" field in the user's Windows account record.</p>
Include "Review By" Field on Printouts	<p>If selected, places a <i>Reviewed by:</i> _____ field on all printouts generated by the user.</p>

- 4 Click **OK** to save the settings.

Appendix C

Glossary

acquisition	The electronic and software function of collecting various types of information from a cell sample.
Analysed Group	An FCS file created in InCyte software. It contains the sample data and the Analysis Method. When data is acquired in InCyte, the software will automatically pair the data set and Method for analysis. If data were acquired in any other guavaSoft module, you will need to pair the data with a new or existing Method during analysis to form the Analysed Group.
analysis	The software function of numerically and graphically manipulating data to identify and separate cell populations for the purpose of calculating relevant statistical information.
antibodies	A class of proteins secreted by sensitized B lymphocytes following contact with an antigen. Also referred to as immunoglobulins. Monoclonal antibodies, derived from a unique secreting clone of the parent B cell, are characterized by their highly specific antigen binding capabilities.
area	The area under the electronic pulse. The area measurement provides a more accurate assessment of total signal fluorescence.
caspases	CysteinyI-directed aspartate-specific proteases. A family of enzymes that initiate the apoptotic cascade, carry out cellular breakdown, and process cytokines.

coefficient of variation (%CV)	<p>The ratio of the standard deviation to the mean, expressed as a percent. It is calculated using the formula:</p> $\%CV = \frac{SD}{\bar{x}} \times 100$
compensation	A process of reducing the unwanted fluorescence signal of one fluorochrome overlapping into the range of wavelengths of another fluorochrome.
data set	A series of samples included within one file for a selected assay. A data file and an Excel file are saved for each data set.
depolarized cells	Cells in which the mitochondrial membrane potential has collapsed. Because of this loss of negative potential, the JC-1 dye no longer accumulates in the mitochondria, causing cells to fluoresce mostly green.
detector	A device used to measure light intensity. The side scatter detector (PM4) and the fluorescence detectors (PM1, PM2, PM3, and PM5) are photomultiplier tubes, and the FSC detector is a photodiode. Both output a current that is proportional to the intensity of incident light.
dot plot	A graphical representation of two-parameter data. Each axis of the plot displays values for one parameter. A dot represents the values for a cell or particle.
FBS	Fetal bovine serum. A cell-free extraction of blood obtained from fetal calves used to supplement growth media in tissue culture.
FCS file	Flow Cytometry Standard file. A data file containing the results for an individual sample as well as all acquisition information at the time of data collection. FCS files are defined by the Data Files Standards Committee of the Society for Analytical Cytology. <i>Cytometry</i> . 1990;11:323–332.
flow cell	A optical assembly within the guava easyCyte HT System where the laser beam illuminates the sample stream.
fluorescence	The phenomenon of light emission that occurs when a fluorochrome's excited electrons drop to a lower energy level.

fluorochrome	A fluorescent dye used as a detection reagent in cell analysis applications. A molecule capable of absorbing light at a certain wavelength, then emitting light at a longer wavelength (fluorescence) as it releases energy. For a list of all fluorochromes compatible with the easyCyte HT System, see “Fluorochromes” on page 3 of the Specifications section.
FSC	Forward scatter. Light scattered as a particle passes in front of the laser beam. Forward scattered light is used as an indicator of relative particle size and incorporates both the particle’s cross-sectional dimension and refractive index.
gate	A graphical boundary that defines a subset of data. Gates may be set on a single-parameter histogram or a two-parameter dot plot.
helper/inducer T cells	T cells that promote the immune response by releasing soluble helper factors such as interleukin 2 and interleukin 4.
histogram	A graphical representation of single-parameter data. The horizontal axis of the graph represents the increasing signal intensity of the parameter and the vertical axis represents the number of events (cells).
isotype control	An antibody of the same immunoglobulin class and fluorescent capacity as the test reagent, but having no specificity for the target antigen or other antigens present on the test cells. The isotype control is used to estimate nonspecific binding of the test reagent.
laser	Light amplification by stimulated emission of radiation. A light source that is highly directional, monochromatic, coherent, and bright. The emitted light is in one or more narrow spectral bands, and is concentrated in an intense, narrow beam.
marker	A boundary or set of boundaries used to segregate data into subsets for statistical analysis. Set a marker on a histogram to obtain statistics on a certain region. Set quadrant markers on a dot plot to obtain statistics on data within four quadrants.
mean fluorescence	The average of the fluorescence intensities of each event acquired within a given set of events.

median	The axis value for the event that falls in the middle of the distribution.
Method	The part of an InCyte FCS file that contains all the analysis components (gates, regions, markers, plots, parameters, and statistical setup). Methods can be part of the FCS file and can also be saved to a separate file (.gsy). Data files acquired using a program other than InCyte will not have Methods associated with them, therefore you must use a new or existing Method before you can analyze the file using InCyte.
monocytes	Immature macrophages found in the blood.
NK cells	Natural killer cells. Non-T, non-B lymphocytes found in normal individuals and capable of killing some tumor cells and some virus-infected cells.
offset	A baseline detector setting for an individual assay to ensure that the cells are being detected.
parameter	A specific cell property that is measured as the cell passes in front of the laser beam. Each parameter is the output from a photomultiplier (which measures fluorescence or side scatter) or a photodiode (which measures forward scatter).
PM1 (YLR)	Photomultiplier 1. A device used for measuring light intensity. The PM1 in the guava easyCyte HT System can measure fluorescent light from PE, PI, Cy3, TRITC, sulforhodamine, DS Red, and AlexaFluor 532.
PM2 (RED)	Photomultiplier 2. A device used for measuring light intensity. The PM2 in the guava easyCyte HT System can measure fluorescent light from PE-Cy5, PerCP, 7-AAD, PI, Cychrome, and PE-Cy5.5.
PM3 (GRN)	Photomultiplier 3. A device used for measuring light intensity. The PM3 in the guava easyCyte HT System can measure fluorescent light from FITC, GFP, and Alexa 488.
PM4 (SSC)	Photomultiplier 4. A device used for measuring light intensity. The PM4 in the guava easyCyte HT System can measure laser light scattered at a 90 degree angle to the laser beam.

PM5 (NIR)	Photomultiplier 5. A device used for measuring light intensity. The PM5 in the guava easyCyte HT System can measure fluorescent light from PE-Cy7 or PE-AlexaFluor 750 from the blue laser, or can measure fluorescent light from APC-Cy7 from the red laser.
PM6 (RED2)	Photomultiplier 6. A device used for measuring light intensity. The PM6 in the guava easyCyte HT System can measure fluorescent light from APC or Cy5.
PM7 (NIR2)	Photomultiplier 7. A device used for measuring light intensity. The PM7 in the guava easyCyte HT System can measure fluorescent light from APC-Cy7.
PMNs	Polymorphonuclear cells. Myeloid cells that are derived from the bone marrow. These cells possess a lobulated, irregular nucleus and cytoplasm that is filled with granules. PMNs consist of neutrophils, eosinophils, and basophils.
PMT	Photomultiplier tube. A device used for measuring light intensity.
polarized cells	Cells which exhibit a normal mitochondrial membrane potential, and hence when exposed to the JC-1 dye, fluoresce mostly orange.
population	A group of cells that express similar values within one or more parameters. For example, cells that are positive for a particular antibody appear in the same location within a histogram or dot plot.
SR-VAD-FMK	Sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone. A cell-permeable, noncytotoxic caspase inhibitor that covalently binds to multiple caspases that have been activated during apoptosis.
SSC	Side scatter. Light scattered as a particle passes in front of the laser beam. Side scattered light is measured at approximately 90° from the incident laser beam. Side scattered light is used as an indicator of a particle's relative internal granularity.
suppressor/cytotoxic T cells	T cells that suppress the response of other cells to antigen, or kill other cells in an antigen-specific manner.

T cells	Lymphocytes that have undergone a period of processing in the thymus and are responsible for mediating the cell-mediated immune response.
threshold	The minimum level of discrimination to electronically eliminate unwanted signal. A threshold setting allows you to specify events you wish to acquire based on signal intensity of the event. Anything below the threshold is not acquired.
width	The width of the electronic pulse. The width measurement helps you discriminate doublets from singlets.

Appendix D

Order Information

For ordering information contact the nearest EMD Millipore office by calling 1 800 645-5476, or visiting us on our website at www.millipore.com/offices.

In addition, EMD Millipore and its distribution network will provide guava[®] Products to all sectors of life science research in certain countries outside North America and Europe.

Instrument Components

Parts	Catalog Number
guava easyCyte 8HT System (software not included)	0500-4008
laptop computer	0110-3670
cleaning solution vial assembly (40 mL)	0110-5780
waste vial assembly (40 mL)	0110-5790
flow cell assembly	0500-2260
flow cell removal/tightening tool (screw-in model)	6000-2410
flow cell removal tool (tweezer model)	6000-3020
syringe assembly cleaning tool	0110-0210
<i>guava easyCyte HT System User's Guide text</i>	0110-5820
instrument shipping box	0110-5690

Software

guava easyCyte HT System Software Modules	Catalog Number
guava ExpressPro Software (required for 6-color operation)	0500-4125
guava InCyte Software Module	0500-4120
guavaSuite Software Modules for guava easyCyte HT Systems	0500-4130

Essential Tools

Kit	Catalog Number
guava easyCheck Kit (50 tests)	4500-0025
Guava ICF (Instrument Cleaning Fluid) [100 mL]	4200-0140

Reagent Kits

Cell Health	Catalog Number
<u>ViaCount Assay Kits</u>	
Guava ViaCount Reagent (100 tests)	4000-0040
Guava ViaCount Reagent (600 tests)	4000-0041
Guava ViaCount Flex Reagent (100 tests)	4500-0110
Guava ViaCount Flex Reagent (500 tests)	4700-0060
CDR (Cell Dispersal Reagent) [100 tests]	4700-0050
<u>Cell Cycle Kits</u>	
FlowCollect® Bivariate Cell Cycle Kit for DNA Replication Analysis (25 tests)	FCCH025102
FlowCollect® Bivariate Cell Cycle Kit for G2/M Analysis (25 tests)	FCCH025103
Guava Cell Cycle Reagent (100 tests)	4500-0220
<u>DNA Damage Kits</u>	
FlowCollect® Multi-Color DNA Damage Response Kit (25 tests)	FCCH025104
FlowCollect® DNA Damage Histone H2A.X Dual Detection Kit (25 tests)	FCCS025153
FlowCollect® Cell Cycle Checkpoint H2A.X DNA Damage Kit (25 tests)	FCCH025142
FlowCollect® Cell Cycle Checkpoint ATM DNA Damage Kit (25 tests)	FCCH025143
FlowCollect® Histone H2A.X Phosphorylation Assay Kit	FCCS100182
<u>MitoHealth Kits</u>	
FlowCollect® MitoPotential Red Kit (100 tests)	FCCH100105
FlowCollect® MitoDamage Kit (100 tests)	FCCH100106
FlowCollect® MitoLive Kit (100 tests)	FCCH100107
FlowCollect® MitoStress Kit (100 tests)	FCCH100109
FlowCollect® Cytochrome <i>c</i> Kit (100 tests)	FCCH100110
FlowCollect® Oxidative Stress Characterization Kit (25 tests)	FCCH025111
guava MitoPotential Kit (100 tests)	4500-0250
<u>Apoptosis Kits</u>	
<i>Early Apoptosis Kits</i>	
FlowCollect® Annexin Red Kit (100 tests)	FCCH100108
guava Nexin Reagent (100 tests)	4500-0450
guava Nexin Reagent (500 tests)	4500-0455
<i>Mid Apoptosis Kits</i>	
guava MultiCaspase SR Kit (100 tests)	4500-0500
guava Caspase 9 SR Kit (100 tests)	4500-0520
guava MultiCaspase FAM Kit (100 tests)	4500-0530
guava Caspase 3/7 FAM Kit (100 tests)	4500-0540
guava Caspase 8 FAM Kit (100 tests)	4500-0550
guava Caspase 9 FAM Kit (100 tests)	4500-0560
guava MultiCaspase SR and Caspase 3/7 FAM Kit (100 tests)	4500-0570
guava MultiCaspase SR and Caspase 8 FAM Kit (100 tests)	4500-0580
guava MultiCaspase SR and Caspase 9 FAM Kit (100 tests)	4500-0590
guava Caspase 9 SR and Caspase 3/7 FAM Kit (100 tests)	4500-0630
guava Caspase 9 SR and Caspase 8 FAM Kit (100 tests)	4500-0640
guava Caspase 9 SR and MultiCaspase FAM Kit (100 tests)	4500-0650

Cell Health	Catalog Number
<i>Late Apoptosis Kits</i>	
guava TUNEL Kit (100 tests)	4500-0121
<i>Apoptosis Signaling Kits</i>	
FlowCelect [®] Bcl-2 Activation Dual Detection Kit (25 tests)	FCCS025108
<i>Autophagy</i>	
FlowCelect [®] GFP-LC3 Reporter Autophagy Assay Kit (CHO) [25 tests]	FCCH100170
FlowCelect [®] Autophagy Reagent Pack (25 tests)	FCCF200097
FlowCelect [®] GFP-LC3 Reporter Autophagy Assay Kit (U2OS) [25 tests]	FCCH100181
FlowCelect [®] RFP-LC3 Reporter Autophagy Assay Kit (25 tests)	FCCH100183
FlowCelect [®] Autophagy LC3 Antibody-based Assay Kit (100 tests)	FCCH100171
Immunology	Catalog Number
<i>Intracellular Cytokine Kits</i>	
<i>Helper T Cell Kits – Mouse</i>	
FlowCelect [®] Mouse Th1 Intracellular Cytokine Kit (25 tests)	FCIM025123
FlowCelect [®] Mouse Th2 Intracellular Cytokine Kit (25 tests)	FCIM025124
FlowCelect [®] Mouse Th17 Intracellular Cytokine Kit (25 tests)	FCIM025125
FlowCelect [®] Mouse Th1/Th2 Intracellular Cytokine Kit (25 tests)	FCIM025137
FlowCelect [®] Mouse Th1/Th17 Intracellular Cytokine Kit (25 tests)	FCIM025138
FlowCelect [®] Mouse Th1 Differentiation Tool Kit (25 tests)	FCIM025161
FlowCelect [®] Mouse Th2 Differentiation Tool Kit (25 tests)	FCIM025162
FlowCelect [®] Mouse Th17 Differentiation Tool Kit (25 tests)	FCIM025163
FlowCelect [®] Mouse Treg Differentiation Tool Kit (25 tests)	FCIM025166
<i>T-Cell Kits – Human</i>	
FlowCelect [®] Human FOXP3 Treg Characterization Kit (25 tests)	FCIM025118
FlowCelect [®] Human CD4/CD8 T Cell Kit (100 tests)	FCIM100158
<i>T-Cell Kits – Mouse</i>	
FlowCelect [®] Mouse FOXP3 Treg Identification Kit (25 tests)	FCIM025126
FlowCelect [®] Mouse Viable Treg Characterization Kit (25 tests)	FCIM025168
<i>B-Cell Identification Kits – Human</i>	
FlowCelect [®] Human Memory B Cell Identification Kit (25 tests)	FCIM025159
FlowCelect [®] Human B Cell FAS Kit (100 tests)	FCCH100137
<i>B-Cell Identification Kits – Mouse</i>	
FlowCelect [®] Mouse Breg Identification Kit (25 tests)	FCIM025154
<i>T-Cell Signaling Kit – Human</i>	
FlowCelect [®] Human Lymphocyte ZAP-70 Characterization Kit (25 tests)	FCIM025122
<i>Immune Cell Health - Human</i>	
FlowCelect [®] Human T Cell Apoptosis Kit (100 tests)	FCCH100138
FlowCelect [®] T Cell MitoDamage Kit (100 tests)	FCCH100139
FlowCelect [®] T Cell Activation Kit (100 tests)	FCCH100141
FlowCelect [®] Human CD8 T Cell FAS Kit (100 tests)	FCCH100140
FlowCelect [®] Human CD4 T Cell FAS Kit (100 tests)	FCCH100154
FlowCelect [®] Human T Cell Caspase 3/7 Kit (100 tests)	FCCH100157
FlowCelect [®] Human T Cell Caspase 8 Kit (100 tests)	FCCH100155

Immunology	Catalog Number
FlowCollect [®] Human T Cell Caspase 9 Kit (100 tests)	FCCH100156
guava CellToxicity Kit (100 tests)	4500-0230
guava CellGrowth Kit (200 tests)	4500-0270
Cell Signalling	Catalog Number
<u>MAPK Pathway</u>	
FlowCollect [®] PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection Kit (25 tests)	FCCS025100
FlowCollect [®] EGFR/MAPK Pathway Activation Detection Kit (25 tests)	FCCS025101
FlowCollect [®] MAPK Activation Dual Detection Kit (25 tests)	FCCS025106
FlowCollect [®] p38 Stress Pathway Activation Detection Kit (25 tests)	FCCS025132
<u>EGFR Pathway</u>	
FlowCollect [®] EGFR/MAPK Pathway Activation Detection Kit (25 tests)	FCCS025101
FlowCollect [®] EGFR/RTK Activation Dual Detection Kit (25 tests)	FCCS025107
FlowCollect [®] EGFR/STAT3 Pathway Activation Detection Kit (25 tests)	FCCS025111
<u>PI3/Akt/m-TOR Pathway</u>	
FlowCollect [®] PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection Kit (25 tests)	FCCS025100
FlowCollect [®] PI3K Activation Dual Detection Kit (25 tests)	FCCS025105
FlowCollect [®] PI3K-mTOR Signaling Cascade Kit (25 tests)	FCCS025210
<u>Jak/STAT Pathway</u>	
FlowCollect [®] EGFR/STAT3 Pathway Activation Detection Kit (25 tests)	FCCS025111
FlowCollect [®] Multi-STAT Activation Profiling Kit (25 tests)	FCCS025550
FlowCollect [®] STAT1 Activation Dual Detection Kit (25 tests)	FCCS025142
FlowCollect [®] STAT3 Activation Dual Detection Kit (25 tests)	FCCS025143
<u>Multiple Pathway</u>	
FlowCollect [®] PLC-gamma 1 Activation Dual Detection Kit (25 tests)	FCCS025145
FlowCollect [®] Src Activation Dual Detection Kit (25 tests)	FCCS025154
<u>Apoptosis Signaling Pathway</u>	
FlowCollect [®] Bcl-2 Activation Dual Detection Kit (25 tests)	FCCS025108
<u>Chemokine Receptor Kits</u>	
FlowCollect [®] Chemokine Receptor CCR1 Surface Expression Identification and Quantification Kit (25 tests)	FCCR100410
FlowCollect [®] Chemokine Receptor CCR2B Surface Expression Identification and Quantification Kit (25 tests)	FCCR200411
FlowCollect [®] Chemokine Receptor CCR3 Surface Expression Identification and Quantification Kit (25 tests)	FCCR300412
FlowCollect [®] Chemokine Receptor CCR4 Surface Expression Identification and Quantification Kit (25 tests)	FCCR400413
FlowCollect [®] Chemokine Receptor CCR6 Surface Expression Identification and Quantification Kit (25 tests)	FCCR600414
FlowCollect [®] Chemokine Receptor CCR7 Surface Expression Identification and Quantification Kit (25 tests)	FCCR700415
FlowCollect [®] Chemokine Receptor CXCR1 Surface Expression Identification and Quantification Kit (25 tests)	FCXR100420
FlowCollect [®] Chemokine Receptor CXCR2 Surface Expression Identification and Quantification Kit (25 tests)	FCXR200421

Cell Signalling	Catalog Number
FlowCelect [®] Chemokine Receptor CXCR3 Surface Expression Identification and Quantification Kit (25 tests)	FCXR300422
FlowCelect [®] Chemokine Receptor CXCR4 Surface Expression Identification and Quantification Kit (25 tests)	FCXR400423
FlowCelect [®] Chemokine Receptor CXCR6 Surface Expression Identification and Quantification Kit (25 tests)	FCXR600424

Stem Cell Kits

Human

FlowCelect [®] Human ESC Nuclear Marker Characterization Kit (25 tests)	FCHEC25102
FlowCelect [®] Human ESC (HESCA-1) Surface Marker Characterization Kit (25 tests)	FCHEC25104
FlowCelect [®] Human ESC (TRA-1-60) Surface Marker Characterization Kit (25 tests)	FCHEC25106
FlowCelect [®] Human NK Cell Characterization Kit (25 tests)	FCIM025164

Mouse

FlowCelect [®] Mouse ESC Nuclear Marker Characterization Kit (25 tests)	FCMEC25110
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Rodent

FlowCelect [®] Rodent NSC Characterization Kit (Neural) [25 tests]	FCRNC25112
FlowCelect [®] Rodent NSC Characterization Kit (Astrocyte) [25 tests]	FCRNC25114

Antibody Quantification	Catalog Number
Guava RapidQuant Human IgG Kit (100 tests)	4500-0340
Guava RapidQuant Mouse IgG Kit (100 tests)	4500-0350

Service Plans

Service and Training	Catalog Number
Guava easyCyte 8HT Base System 1-yr Service Plan (at time of purchase)	0500-2870
Guava easyCyte 8HT Base System 1-yr Service Plan (after purchase)	0500-2875
Guava easyCyte 8HT Base System 2-yr Service Plan	0500-2880
Guava easyCyte Installation and Training (included at no charge with purchase of system)	0500-0450
Guava Introductory On-Site Training, half day (up to three trainees)	0500-1690
Guava Advanced On-Site Training, full day (up to three trainees)	0500-1680
Guava Instrument Relocation and Installation Service	0500-1710

Compatible Microplates

Following is a list of microplates compatible with the guava easyCyte HT System. Plates are listed by type and material—polystyrene (PS), polypropylene (PP), or flexible vinyl (PVC). While PP and PVC plates are mechanically compatible with the guava easyCyte HT System, EMD Millipore strongly recommends using PS plates whenever possible. PP and PVC plates are flexible and the distortions can affect the assay results.

96-Well Plate Type	Manufacturer	Catalog Number	Treatment		
			Tissue Culture	None	Other
Round-bottom-PS	BD Falcon	353076	✓		
		353077	✓		
		353910 (guava 1000-3020)		✓	
		353918		✓	
		353227		✓	
		351177		✓	
		351190		✓	
	Corning-Costar	3360	✓		
		3799	✓		
		3879	✓		
		3358	✓		
		3360	✓		
		3799	✓		
		3367		✓	
		3788		✓	
		3795		✓	
		3798			✓
		7007			✓-hydrogel
	Nunc	262162		✓	
		268152		✓	
		260210		✓	
		268200		✓	
	Greiner	650101		✓	
Round-bottom-PP	BD Falcon	351190		✓	
	Corning-Costar	3356		✓	
		3359		✓	
		3365		✓	
		3371		✓	
	Nalge-Nunc	267245		✓	
		267334		✓	
		267342		✓	
		267350		✓	
		267369		✓	
		267385		✓	
		267407		✓	
C-bottom-PS (well strips)	Nalge-Nunc	473709			✓-Maxisorp

96-Well Plate Type	Manufacturer	Catalog Number	Treatment		
			Tissue Culture	None	Other
Flat-bottom-PS (cont.)	Corning-Costar	3300	✓		
		3585	✓		
		3594	✓		
		3595	✓		
		3596	✓		
		3598	✓		
		3599	✓		
		3628	✓		
		3665	✓		
		3997	✓		
		3591		✓	
		9017		✓	
		3361			✓
		9018			✓
		3590			✓
		3474			✓-hydrogel
(well strips)	Nalge-Nunc	269620		✓	
		269787		✓	
		260836		✓	
		260844		✓	
		260887		✓	
		260895		✓	
		256510		✓	
		439454			✓
		442404			✓
		475094			✓
	Corning-Costar	2388			✓
		2504			✓
		2508			✓
		2510			✓
		2592			✓
		2593			✓
	Nalge-Nunc	467466			✓-Maxisorp
Flat bottom-PVC	Corning-Costar	2595		✓	

EMD Millipore plates, in any filter thickness and with the following prefixes are also compatible:

- MABV
- MADV
- MAGV
- MAHV
- MSDV
- MSDV
- MSGV
- MSHV

■ NOTE: The EMD Millipore plate with the MAMI prefix, in any filter thickness, is compatible *only* if the middle 96-well plastic insert is removed before use on the guava easyCyte HT System.

■ NOTE: Other EMD Millipore plates may be compatible. Contact EMD Millipore Technical Support for information. The manufacturer's website may have helpful information when comparing plates against known compatible plates.

Incompatible Microplates

Following is a list of microplates not compatible with the guava easyCyte HT System.

96-Well Plate Type	Manufacturer	Catalog Number	Material
Round-bottom (well strip)	Thermo Electron	2205 0151	polystyrene polypropylene
	Nalge-Nunc	262146 262180	polystyrene polystyrene
	Nalge-Nunc	464394	polystyrene
V-bottom	Thermo Electron	2605	polystyrene
	Nalge-Nunc	249662 249570 442587	polystyrene polystyrene polypropylene
Flat-bottom (half area)	Corning-Costar	3686 3690 3694 3695 3696 3697	polystyrene polystyrene polystyrene polystyrene polystyrene polystyrene
PCR tube	Nalge-Nunc	251357	polypropylene (strip)

Microcentrifuge Tubes

Following is a list of microcentrifuge tubes compatible with the guava easyCyte HT System.

Microcentrifuge Tubes	Catalog Number
0.5-mL microcentrifuge tubes (free-standing with screw cap) - used for samples - dead volume of ~75 μ L	VWR 16466-036 (guava 1000-2990)
1.5-mL microcentrifuge tubes (conical bottom, no screw cap) 1.5-mL microcentrifuge tubes (conical bottom, screw cap) 1.5-mL microcentrifuge tubes (round bottom, screw cap) - used for cleaning and when washing the capillary and mixer	VWR 16466-030 (guava 1000-0780) VWR 89004-288 VWR 60872-324
screw caps	VWR 89004-346

- **NOTE:** Snap-cap tubes can be used in place of the 1.5-mL screw-cap tubes (for washing and cleaning) if the caps are cut off.

Microplate Minimum Assay Volumes

Dead Volumes:

round-bottom plate	~50 μ L
flat-bottom plate	~75 μ L
V-bottom plate	~25 μ L

Assay Volumes:

dead volume + 50 μ L (at 200 cells/ μ L)

Specifications

guava easyCyte HT System

Operating Environment

temperature:	16°–35°C (60°–95°F)
humidity:	10–90% relative humidity (non-condensing)
power:	100–240 VAC, 50/60 Hz, 120W
fuse rating:	USA and Japan: 2 A, 110 VAC Slo-blo (Time Lag) type Europe: 1.6 A, 220 VAC Time Lag type

easyCyte HT System size

height:	10 in (25 cm)
width:	20 in (51 cm)
depth:	24 in (61 cm)

easyCyte HT System weight:

73 lb (32.4 kg)

laptop size

height:	11.5 in (29.2 cm) when open
width:	14.2 in (36.1 cm)
depth:	10.4 in (26.3 cm)

laptop weight:

8 lbs (3.6 kg)

Optics

laser:	see “easyCyte HT Lasers and Parameters” below
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forward scatter detector:	photodiode
side scatter detector:	photodiode or photomultiplier
fluorescence detectors:	see “easyCyte HT Lasers and Parameters” below

Signal Processing

parameter dynamic range:	3.5 decades (for all modules except as noted below): 4.0 decades (guava ExpressPro, RapidQuant, and Caspase software modules) 5.0 decades (guava InCyte software module)
pulse processing:	digital signal processing
time:	every particle time stamped

Performance

counting accuracy:	±10%
counting precision:	≤10% CV

Fluidics

flow cell dimension:	standard square capillary with ID of 100 μm
pump:	positive displacement
sample flow rate:	7 $\mu\text{L}/\text{min}$ to 36 $\mu\text{L}/\text{min}$ (7 $\mu\text{L}/\text{min}$ to 72 $\mu\text{L}/\text{min}$ for guava InCyte , ExpressPro, RapidQuant, and Caspase Software Modules)
waste and cleaning vials:	30-mL glass vial with screw top
waste generation:	typically <40 mL in 8 hours of continuous use
dead volume:	50 μL (for 96-well microplate); 75 μL (for 0.5-mL microcentrifuge tubes)
sample concentration:	final particle concentration of 10^4 to 5×10^5 particles/mL for accurate results (up to 2.5×10^6 beads/mL for the RapidQuant Assay)
sample requirement:	as few as 2,000 cells/test; typically 25,000-100,000 cells/test, depending on the assay

Data Management

computer:	Dell™ laptop running Windows XP Professional or Windows 7 Ultimate, and including Microsoft Excel. Minimum specifications: Intel Core Duo processor T2400 (1.86 GHz), 4 GB RAM, 60 GB hard drive, 2 USB ports
data file structure:	Output data file formats: <ul style="list-style-type: none">• binary data storage in Flow Cytometry Standard (FCS) 3.0 format• spreadsheet results file in comma-separated value (CSV) format• optional export of binary data in FCS 2.0 format

easyCyte HT Lasers and Parameters

Laser/Parameter	easyCyte 5HT	easyCyte 6HT	easyCyte 6HT-2L	easyCyte 8HT
488 mW (blue) laser power	20 mW	20 mW	40 mW	75 mW
Blue modulated	No	No	Yes	Yes
640 mW (red) laser power	N/A	N/A	40 mW	40 mW
FSC	Yes	Yes	Yes	Yes
SSC	Yes	Yes	Yes	Yes
Green	525/30	525/30	525/30	525/30
Yellow	583/26	583/26	583/26	583/26
Red	680/30	680/30	690/50	690/50
NIR	N/A	785/70	N/A	785/70
Red 2	N/A	N/A	661/19	661/19
NIR 2	N/A	N/A	N/A	785/70

Fluorochromes

The following tables, list all fluorochromes that can be used with the easyCyte HT system, the laser that excites the dye, and the parameter/detector that detects the emitted signal.

			Blue Laser (488-nm excitation)				Red Laser (640-nm excitation)	
		Max Emission (nm)	Green (525/30 nm)	Yellow (583/26 nm)	Red (680/30 nm)* (690/50 nm)**	NIR (785/70 nm)	Red2 (661/19 nm)	NIR2 (785/70 nm)
Fluorescent Labels	Alexa® 555			x				
	Alexa® 568			x				
	Alexa Fluor® 488	519	x					
	Alexa Fluor® 647	668					x	
	APC	660					x	
	APC-Alexa Fluor® 750	774						x
	APC-Cy7	774						x
	CF™ 488		x					
	CF™ 555			x				
	CF™ 568			x				
	CF™ 647						x	
	Cy™ 2	506	x					
	Cy5	666					x	
	FAM	519	x					
	FITC	519	x					
	PE-Alexa Fluor® 750	775				x		
	PE-Alexa Fluor® 647	668			x			
	PE-Alexa Fluor® 700	719			x			
	PE-B, PE-R	578		x				
	PE-Cy5	670			x			
	PE-Cy5.5	692			x			
	PE-Cy7	774				x		
	PE-Texas Red	613		x	x			
	PerCP	675			x			
	PerCP-Cy5.5	690			x			
	Qdot 525	525	x					
	Qdot 565	565		x				
	Qdot 655	655					x	
Qdot 705	705				x			
Qdot 800	800					x	x	
Rhodamine 110	520	x						
Sulforhodamine	595			x				
TRITC	572			x				
DNA or RNA Selective Dyes	7-AAD (DNA)	647			x		x	
	Acridine Orange (DNA)	526 / 650	x	x				
	Acridine Orange (RNA)	526 / 650			x		x	
	DRAQ 5 (DNA)	670			x		x	
Reporter Proteins	dsRED	583		x				
	EGFP	516	x					
	EYFP	532	x					
pH Probes	BCECF	535	x					
	Carboxy SNARF®-1	589	x					
Ca++ Probes	Fluo-3, Fluo-4	527	x					
	Fura Red™	669	x					

			Blue Laser (488-nm excitation)				Red Laser (640-nm excitation)	
		Max Emission (nm)	Green (525/30 nm)	Yellow (583/26 nm)	Red (680/30 nm)* (690/50 nm)**	NIR (785/70 nm)	Red2 (661/19 nm)	NIR2 (785/70 nm)
Nonselective Nucleic Acid Dyes	Ethidium bromide (EB)	605		x	x		x	
	Ethidium homodimer	617		x	x		x	
	LDS-751	712			x		x	
	Propidium iodide (PI)	617		x	x		x	
	SYBR® Gold	537	x	x				
	SYBR Green	520	x	x				
	SYTO® BC	500	x					
	SYTOX® Green	523	x					
	SYTOX Orange	570		x				
	SYTOX Red	660					x	
	TO-PRO®-1	530		x				
	TO-PRO-3	660					x	
	Thiazole Orange	530	x					
	TOTO-3	660					x	
	YO-PRO®-1	515	x					
YOYO®-1	509	x						
Membrane Potential	DiIC1 (5)	658					x	x
	DiOC6 (3)	501	x					
	JC-1	529 / 590	x	x				
	MitoSense Red	658					x	x
	Rhodamine 123	529	x					
	TMRE	574		x				
	TRMR	573		x				
Miscellaneous	BODIPY®-FL	511	x					
	BODIPY 650/665	665					x	
	Calcien	515	x					
	CFSE	517	x	x				
	Nile Red	638		x	x			
	Oregon Green®	525	x					

* Bandpass filter for easyCyte 5HT and easyCyte 6HT

** Bandpass filter for easyCyte 6HT-2L and easyCyte 8HT

Compliance

The guava easyCyte HT System contains a Class IIIb laser operating at 488 nm in CW mode and a Class IIIb laser operating at 640 nm in CW mode. Light shields within the instrument enclose the path of laser radiation. Additionally, the instrument enclosure provides secondary protection from any laser radiation.

This product complies with:

- CFR (Code of Federal Regulations) Chapter 1, Subchapter J and installation (overvoltage) category II
- Class 1 limits for exposure to laser radiation set by the Center for Devices and Radiologic Health (CDRH)

Symbols



Attention, consult accompanying documents



Affixed in accordance with European Council Directive 73/23/EEC



Danger, laser radiation



In accordance with Canadian Standards Association



Separate collection of waste at end of life as required by European Directives. Dispose of in accordance with the applicable country regulation.



Dangerous voltage



Power on



Power off

Warranty

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Manufacturer's Declaration of Conformity

We, **EMD Millipore Corporation**
25801 Industrial Blvd.
Hayward, CA. 94545
U.S.A.

Declare under our sole responsibility that the product

Description: **Cell-based analysis system**
Product Model: **guava easyCyte™ HT System**

Is in conformance with the following European Union directives.

- **73/23/EEC**
- **89/336/EEC**

European standards used:

- Safety requirements for Electrical Equipment for measurement, Control, and Laboratory Use: **EN61010-1:2002**
- Safety of Laser Products: **EN60825-1:2007**
- Electrical Equipment for Measurement, Control and Laboratory Use-EMC Requirements- Part 1: General Requirements: **EN61326-1:2005**

Warren Hinds
Operations Manager
EMD Millipore Corporation

23 July 2012

Date

25801 Industrial Blvd., Hayward, CA. 94545 USA; 510-576-1400 (p); 510-576-1500(f)
<http://www.millipore.com>

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