Luminex®
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Guava® Cell Cycle Reagent Package Insert

Technical Support
Telephone: 512-381-4397
North America Toll Free: 1-877-785-2323
International Toll Free: + 800-2939-4959
Email: *support@luminexcorp.com*
www.luminexcorp.com

RUO For Research Use Only
Not for Use in Diagnostic Procedures
4600-0790 Rev G
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Cat. No. 4500-0220 (100 Tests)

Luminex Corporation
12212 Technology Blvd.
Austin, TX 78727
U.S.A.

Description

The Guava® Cell Cycle Reagent determines the percentage of cells in G0/G1, S, and G2/M phases based on DNA content.

The cell cycle describes the process of the replication and division of chromosomes within the nucleus, which occurs prior to cell division. Cancer cells develop when the normal mechanisms for regulating 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 ways to screen for potentially therapeutic drugs, or the effects of specific genes on cell cycle, is to measure changes in cell cycle kinetics under varying conditions. For cells to divide, they must first duplicate their nuclear DNA. By labeling cellular DNA with propidium iodide (PI) you can discriminate cells in different stages of the cell cycle. Resting cells (G0/G1 phase) contain two copies of each chromosome. As cells progress toward mitosis, they synthesize DNA (S phase), allowing more PI intercalation with a resulting increase in fluorescence intensity. When all chromosomes have replicated and the DNA content has doubled (G2/M phase), the cells fluoresce with twice the intensity of the G0/G1 population. The G2/M cells eventually divide into two cells. Cells can be fixed, permeabilized and stained with PI according to the protocol below. Data from the stained cells are acquired on the Guava® System using either the assay-specific Cell Cycle software module or the InCyte™ software module. In the Cell Cycle Module, data are displayed in a single parameter histogram. Four markers are available to analyze the various populations including the optional fourth marker to discern apoptotic cells, cell aggregates, or an internal standard. Statistics for each population within the histogram include percentage of total, and RED-B mean, median, and %CV of fluorescence intensity.

The Guava Cell Cycle data for all samples within a data set are saved to a single FCS 3.0 file, and optionally to individual FCS 2.0 or 3.0 files. The data can be analyzed immediately after the sample is acquired or recalled later. In addition to the saved FCS data file, all results and the acquisition information are exported to a comma separated values (CSV) spreadsheet file.

The Cell Cycle software module does not apply any sophisticated "curve-fitting" algorithms to the data. In addition to their other benefits, curve-fitting algorithms can compensate for the presence of aggregates which include doublets of G0/G1 cells that fluoresce as brightly as G2/M cells. If desired, a third-party curve-fitting software package such as ModFit™ or MultiCycle can apply more sophisticated analysis algorithms to the data, and thus provide a more accurate assessment of the percentage of cells in each phase and their relevant statistics. However, for most applications, the Cell Cycle module is sufficient for assessment of the number of cells in each phase.

Summary of Protocol

Prepare cellular samples for staining as follows:
Transfer 1 x 10^5 to 1 x 10^6 cells to each tube.

↓

Centrifuge cells at 300 x g for 5 minutes and wash once with 1X PBS.

↓

While mixing/resuspending cells, slowly add enough ice cold 70% ethanol*
so the cell concentration is 5 x 10^5 to 1 x 10^6 cells/mL.

↓

Incubate for at least 3 hours at –20°C.

↓

Add 200 µL of fixed cells to a new tube.

↓

Centrifuge the cells at 300 x g for 5 minutes and wash once with 1X PBS.

↓

Add 200 µL of Guava® Cell Cycle reagent to each tube and incubate for 30 minutes at room temperature in the dark.

Materials Provided

- Guava® Cell Cycle Reagent (Catalog No. 4500-0220, 100 tests)

Materials Required but Not Provided

- Guava® Instrument with the Cell Cycle software module
- Cell suspension(s)
- Ethanol 70%
- 1X PBS
- Medium appropriate for your cells
- Micropipettors, single, 8 or 12 channel (the latter two for 96-well plate assays)
- Disposable micropipettor tips, sterile preferred
- 15- or 50-mL conical tubes, sterile preferred
- 12 x 75 mm polystyrene tubes (VWR Cat. No. 60818-270)
- Vortex mixer
- Centrifuge
- Disposable gloves
- Guava ICF Instrument Cleaning Fluid (Catalog No. 4200-0140)
- 100% Bleach solution containing between 5-6% hypochlorite
- Deionized water
- Guava Cell Dispersal Reagent (Cat No. 4700-0050) (for adherent cells, optional)

For High-Throughput (HT) Guava® Systems

- V-shaped, 25-mL and/or 55-mL troughs (25 mL, Apogent Discoveries Cat. No. 8093 and 55 mL VWR Cat. No. 210070-970)
- 96-well microplate plates, round bottom or equivalent. Refer to the appropriate Guava® System user's guide for other compatible microplates.
- 1.5-mL microcentrifuge tubes without screw caps or with screw caps, 1.5 mL or equivalent for cleaning. Refer to the appropriate Guava® easyCyte™ System User's Guide for other compatible tubes.
- 0.5-mL microcentrifuge tubes Refer to the appropriate Guava® easyCyte™ System User's Guide for other compatible tubes.
- Centrifuge with 96-well microplate holders
- Guava® ViaCount™ Flex Reagent (Cat. No. 4700-0060), optional

- Guava® ViaCount™ Reagent (Cat No. 4000-0040), optional

For Single-Loader (SL) Guava® Systems

- 1.5-mL microcentrifuge tubes or 1.2-mL titer tubes for sample acquisition. Refer to the appropriate Guava® easyCyte™ System User's Guide for other compatible tubes.
- Guava® ViaCount™ Reagent (Cat No. 4000-0040), optional

Warnings and Precautions

1. For Research Use Only. Not for use in diagnostic procedures.
2. Wear appropriate personal protective equipment (PPE), including a lab coat and disposable gloves, when performing procedures. Wash your hands thoroughly after performing the test.
3. The Guava Cell Cycle Reagent contains dyes that may be carcinogenic and/or mutagenic. Exercise standard precautions when obtaining, handling, and disposing of potentially carcinogenic and mutagenic reagents. Refer to the SDS for specific information on hazardous materials.
4. The Guava Cell Cycle Reagent contains sodium azide, which is toxic if ingested. Reagents containing sodium azide should be considered a poison. If products containing sodium azide are swallowed, seek medical advice immediately and show product container or label. (Refer to NIOSH, National Institute for Occupational Safety and Health; CAS#: 2628-22-8; and also to GHS, The Globally Harmonized System of Classification and Labeling of Chemicals.) Aqueous solutions of sodium azide, when mixed with acids, may liberate toxic gas. Any reagents containing sodium azide should be evaluated for proper disposal. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal, flush with large volumes of water to prevent build-up in plumbing. Check with regulatory agencies to determine at what concentration sodium azide may cause a product to be regulated as hazardous.
5. Avoid microbial contamination of the solution, which may cause erroneous results.
6. Handle all samples as if infectious using safe laboratory procedures such as those outlined in CDC/ NIH B*iosafety in Microbiological and Biomedical Laboratories, and in the CLSI Document M29 Protection of Laboratory Workers from Occupationally Acquired Infections*.
7. Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
8. Do not pipette by mouth.
9. Perform the procedure given in this package insert as described. Any deviation from the outlined protocols may result in assay failure or cause erroneous results.
10. Do not use the kit or any kit components past the expiration date indicated on the kit carton label. Do not interchange kit components from different kit lots. Lot numbers are identified on the kit label.
11. Follow your institution's safety procedures for working with chemicals and handling biological samples.
12. In the event of damage to the protective packaging, consult the Safety Data Sheet (SDS) for instructions.
13. Safety Data Sheets (SDS) for kit reagents are available by contacting Luminex Corporation or visiting our website at *www.luminexcorp.com*

Reagent Storage, Handling, and Stability

- The Guava® Cell Cycle Reagent should be stored refrigerated (2 to 8°C). Do not freeze. Refer to the expiration date on the package label.
- Do not use the reagent after the expiration date.
- The Guava Cell Cycle Reagent contains light-sensitive dyes. Shield from excessive exposure to light.

Before You Begin

Assay Considerations

Staining cycling cells using the recommended protocol and data acquisition on the Guava® Instruments can usually be completed within one to two hours, depending on the number of samples and the cell concentration. However, prior to the staining, cell fixation requires at least one to twelve additional hours. Additional time (hours to days of culture or pre-treatment with test compounds) may be required as well to prepare the cells, depending on the particular test conditions.

It is important to remove the ethanol used for fixation before adding the Guava Cell Cycle Reagent. However, in some cases, depending on the cell line, the PBS washing step to completely remove the ethanol can be eliminated. Removal of ethanol and/or washing in PBS should be done in either 12 x 75 mm tubes or 96-well round bottom plates but not in 1.5-mL microcentrifuge tubes because of substantial cell loss due to cells sticking to the tube. If cells are cultured in 96-well flat bottom plates, they should be transferred into 96-well round bottom plates for ethanol fixation, washing and staining.

A typical assay uses 200 µL of Guava Cell Cycle Reagent to stain 1 x 10^5 to 2 x 10^5 fixed cells per assay condition. When acquiring samples on the Guava instrument, if the cell count is higher than 1200 particles per µL, the software will display a high particle concentration warning. The sample should then be diluted with the Guava Cell Cycle Reagent until the cell concentration is under 1200 particles per µL.

Control Cell Preparation

Prepare a negative control sample. The negative control should be a sample from your cell culture in log phase growth and not treated with any drug. If desired, prepare a positive control sample that has been treated with a compound known to arrest your cells in a particular phase of the cell cycle. Prepare both the negative and positive control samples as described below for the test samples.

Cell Fixation

NOTE: It is important to have a single cell suspension prior to fixation. Otherwise the fixation process will result in a high percentage of doublet cells that will decrease the accuracy of the results. See “Cell Sample Preparation” on page 24 for information on preparing cell suspensions.

Fix Cells in a 96-well Plate

1. Transfer the cell sample from the 96-well flat bottom or 24-well plate to a 96-well round bottom plate if the cells are not already in a round bottom plate.
2. Centrifuge the cells at 450 x g for 5 minutes with the brake on low and at room temperature.
3. Remove and discard the supernatant, being careful not to touch the pellet.
4. Add 200 µL of 1X PBS to each well using a multi-channel pipettor.
5. Mix the cells in the well by pipetting up-and-down several times.
6. Centrifuge the cells in the round bottom plate at 450 x g for 5 minutes with the brake on low and at room temperature.

7. Remove and discard the supernatant.
8. Using a multi-channel pipettor, thoroughly resuspend the cells by repeat pipetting in the residual PBS, pipetting up and down several times.
9. Place the round bottom plate containing the resuspended cells on a lab shaker.
10. Add 200 µL of ice-cold 70% ethanol dropwise into the wells while shaking at low speed (speed 3).
11. Seal the plate with a microplate sealer and refrigerate cells for at least one and up to 12 hours prior to staining. Fixed cells are stable for several weeks at 4°C and for 2 to 3 months at –20°C.
12. Proceed to "Cell Staining Protocol" on page 5.

Fix Cells in a 15 or 50-mL Tube

NOTE: For smaller cell numbers fixation can also occur in 12x75mm tubes.

1. Centrifuge the tube at 450 x g for 5 minutes with the brake on low.
2. Remove and discard the supernatant.
3. Add 1 mL of 1X PBS for every 1 x 10^6 cells.
4. Mix the cell sample by vortexing or pipetting repeatedly to ensure a homogenous suspension.
5. Centrifuge the tube at 450 x g for 5 minutes with the brake on low.
6. Remove and discard the supernatant, leaving approximately 200 µL of 1X PBS.
7. Resuspend the cells thoroughly in the residual PBS by vortexing or repeated pipetting.
8. Add the resuspended cells dropwise into an appropriately sized tube containing 1 mL of ice-cold 70% ethanol per 1 x 10^6 cells, while vortexing at medium speed.
9. Refrigerate the cell preparation for at least one and up to 12 hours prior to staining. Fixed cells are stable for several weeks at 4°C and for two to three months at –20°C.
10. Proceed to "Cell Staining Protocol" on page 5.

Cell Staining Protocol

Stain Cells in 96-Well Format

1. Warm Guava® Cell Cycle Reagent to room temperature; shield from excessive light exposure. Warm 1X PBS to room temperature.
2. Transfer 200 µL of the samples into the wells of a 96-well round bottom plate if the samples have not yet been transferred.
3. Centrifuge the 96-well round bottom plate containing the samples at 450 x g for 5 minutes with the brake on low and at room temperature.
4. Remove and discard the supernatant, being careful not to touch the pellet. After centrifugation, the well should contain a visible pellet or a white film on the bottom of the plate.
5. Using a multi-channel pipettor, add 200 µL of 1X PBS to each well. Mix cells in the wells by pipetting up and down several times. Let the plate stand at room temperature for 1 minute.
6. Centrifuge the 96-well round bottom plate at 450 x g for 5 minutes with the brake on low and at room temperature.
7. Remove and discard the supernatant being careful not to touch the pellet.

   NOTE: The PBS washing step (steps 5 to 7) can be omitted if the user has first shown that this wash step is not necessary to minimize the %CV of the G0/G1 peak. However, the cells should still be centrifuged once to remove the ethanol. The Guava Cell Cycle Reagent should not be added to cells still in ethanol.

8. Add 200 µL of Guava Cell Cycle Reagent to each well.
9. Mix by pipetting up and down several times.

10. Incubate the 96-well round bottom plate at room temperature, shielding away from light for 30 minutes.
11. Acquire the samples on a high-throughput (HT) instrument.

Stain Cells in Tubes

1. Add 1 x 10^5 to 2 x 10^5 cells in 200 µL to 1 mL volume of ethanol-fixed cells to a 12 x 75 mm polystyrene test tube.

   NOTE: Staining in polystyrene will reduce cell loss. Cell loss with ethanol fixed cells can be seen using 1.5 mL polypropylene tubes.

2. Centrifuge ethanol-fixed cells at 450 x g for 5 minutes with the brake on low.
3. Remove and discard the supernatant.
4. Resuspend the cells in 1 mL 1X PBS.
5. Vortex cells and incubate for 1 minute.
6. Centrifuge at 450 x g for 5 minutes with the brake on low at room temperature.
7. Remove and discard the supernatant.

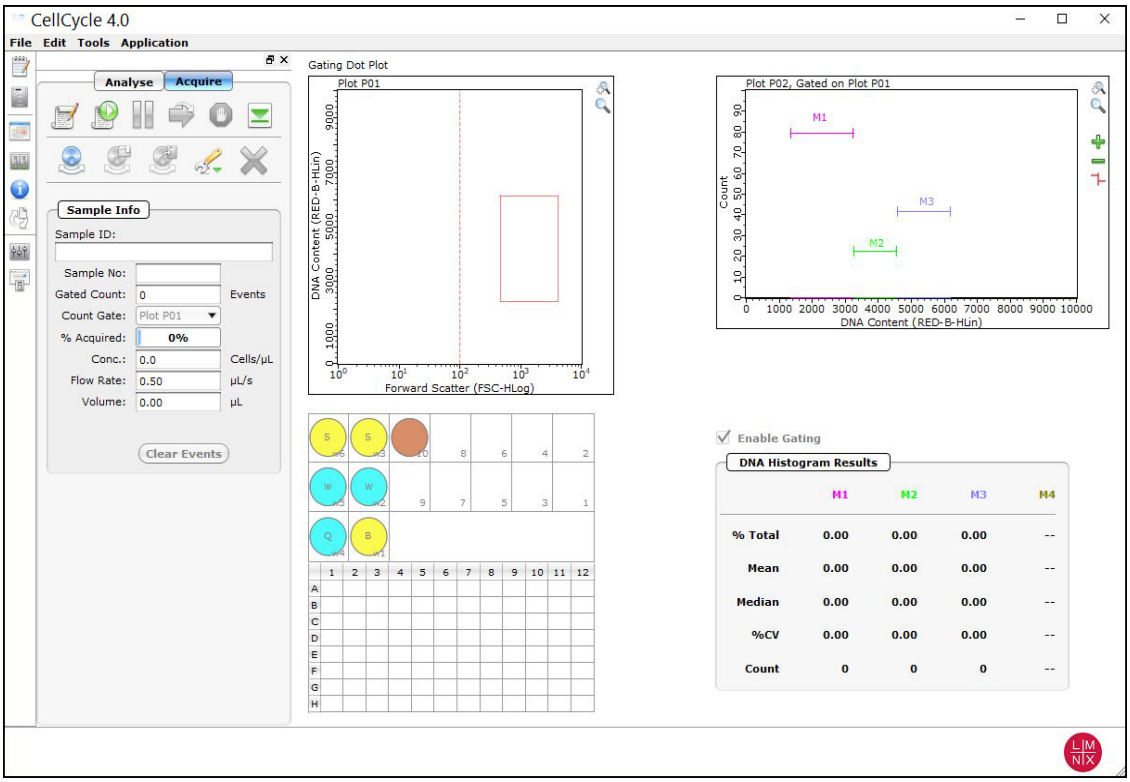
   NOTE: The PBS washing step (steps 4 to 7) can be omitted if the user has first shown that this wash step is not necessary to minimize the %CV of the G0/G1 peak. However, the cells should still be centrifuged once to remove the ethanol. The Guava Cell Cycle Reagent should not be added to cells still in ethanol.

8. Resuspend the cells in 200 µL Guava Cell Cycle Reagent.
9. Incubate at room temperature for 30 minutes, shielded from light.
10. Transfer all samples to 1.5-mL microcentrifuge tubes or wells and acquire the samples on a Guava single-loader (SL) or high throughput (HT) instrument.
11. Acquire the data on the Guava instrument.

Run the Assay on a High-throughput HT System

For details on software screen buttons and control panels, see the appropriate Guava® easyCyte™ User's Guide.

1. Open GuavaSoft™ Software and click **CellCycle** from the Favorites list. If you do not see the assay listed in the Favorites list, click the **Program Search** drop-down menu, choose the assay, and click **Launch**,

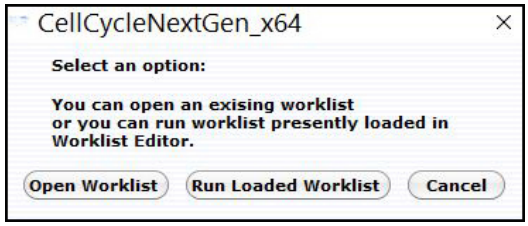


2. Prepare samples for analysis in a microplate or sample tubes.
3. Click the **Edit Worklist** button, the first button in the Button panel, or select **Application > Worklist Editor** to open Worklist Editor and define the worklist parameters.

   NOTE: You can also open an existing worklist.

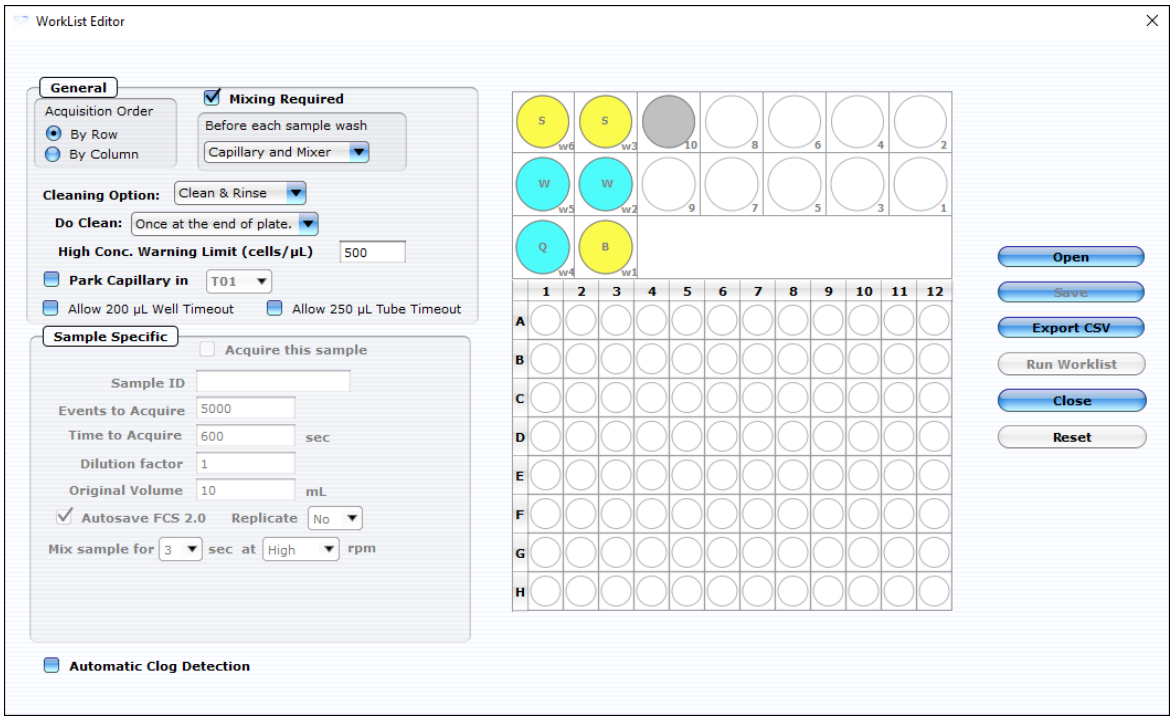
   a. To open an existing worklist, click the **Start Worklist** button from the Button panel. The **Open WorkList File** dialog box displays.

   b. Choose between opening a saved worklist or running the currently loaded worklist. Worklists are .xml files.

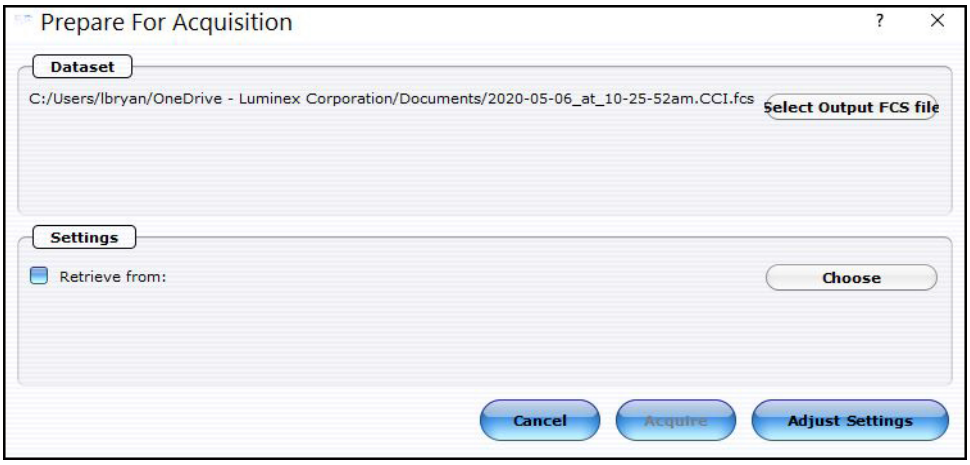


   c. Or, from the WorkList Editor, click **Open**.

d. Choose the WorkList file and click **Run Worklist**. The tray ejects and a dialog box displays prompting you to enter a data set file name.



4. Define the worklist parameters in WorkList Editor software and click **Run Worklist** . When you start a worklist, the sample tray ejects. The **Prepare For Acquisition** dialog box displays allowing you to select a data file name and location and instrument settings.



a. Click **Select Output FCS file** to save the file. The **Create New Session File** dialog box displays. Navigate to the location you want to save the file to, and click **Save**.

NOTE: The default storage location is in My Documents. The default file name is the date and time.

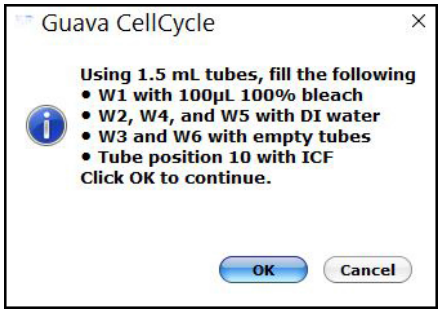
b. For **Settings**, to retrieve the settings file, click **Choose**. The **Read Settings from** dialog box displays. Navigate to the .gst or .fcs file you want to open, and click **Open**.

NOTE: The FCS or instrument settings file must be from InCyte, version 3.0, or later.

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.

5. Click **Adjust Settings**. If you retrieved instrument settings and you want to skip the adjust settings step, click **Acquire** to begin acquiring the first sample.

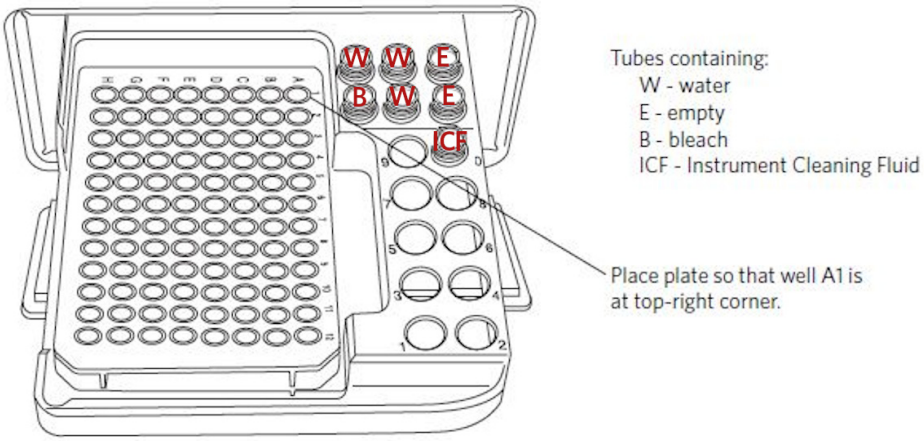
NOTE: Whether you are ready to acquire or adjust settings, a dialog box displays prompting you to load your samples.



6. Place the microplate or sample 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).

     NOTE: 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 100% 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.
   - Load a tube containing 1.5 mL of water for parking the capillary.

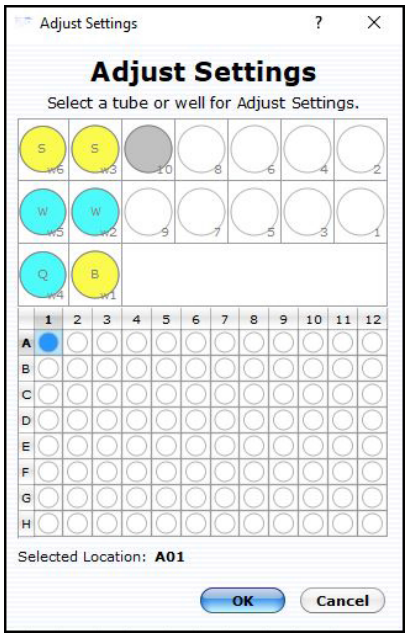


7. Click **OK** in the dialog box after you are finished loading samples and cleaning tubes to load the sample tray.

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

Adjust the Instrument Settings and Acquire Data

1. A dialog box displays prompting you to select the sample for adjusting settings. Click to select the well or tube (1–9) used to adjust settings, then click **OK**. Luminex recommends using a stained negative or isotype control sample for the initial adjustments. You can choose to skip the adjust settings step even if you did not retrieve settings, however Luminex recommends always performing the adjust settings step.



2. Check the **Conc** (Cells/ µL) value in the Sample Info pane and ensure that it is less than or equal to 1200.

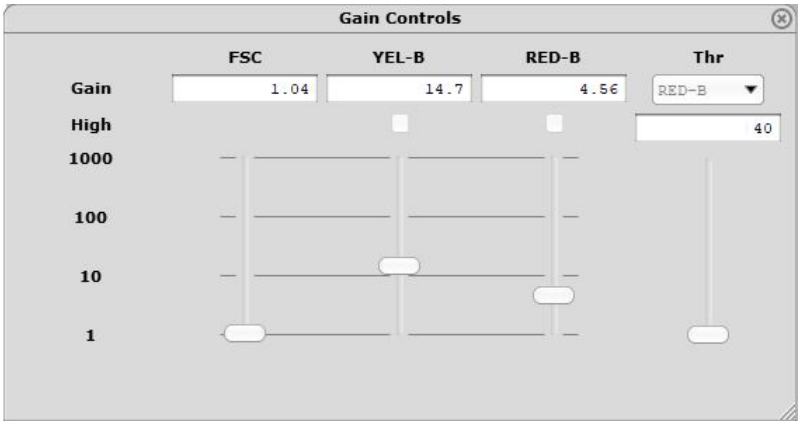
   NOTE: If the value is greater than the high limit for the corresponding flow rate, dilute the sample with the appropriate buffer to lower the concentration and minimize the risk of coincident events.

3. To fine tune the settings, you can make the following adjustments using the Gain Controls. Open each instrument adjustment window using the Tools menu or the 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.

a. Click the **Gain Controls** icon. The **Gain Controls** dialog box will display.
   - Set the **Refresh Rate** to the number of events you want to display.
   - Set the **Flow Rate** to Very Low (0.12 µL/s) or Low (0.24 µL/s). The default flow rate is very low (0.12uL/s). Optimal Cell Cycle results are shown at the very low flow rate.
   - 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 RED-B dot plot until the desired amount of debris is eliminated below the marker.
   - Adjust the gain (using the RED-B slider or the arrow keys on the keyboard) so that the G0/G1 population is positioned at approximately 2000 for 4.0 CC SW on the RED-B histogram.
   - 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: Use the **Clear Events** button in the Sample Info panel to clear the display..



4. 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, by clicking **Adjust Settings**, loading the sample, and clicking **OK**.
   - If you want 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.

   NOTE: Once the worklist is complete, you can no longer save the instrument settings.

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

NOTE: 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.

NOTE: If you want 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**.

NOTE: The % Acquired progress bar provides an estimate of the target event count during the acquisition period.

NOTE: 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 the worklist, a copy of the files are automatically loaded into the Analysis control panel. Guava Cell Cycle saves the data for all samples as a single FCS 3.0 file to the specified location.

The FCS file contains:

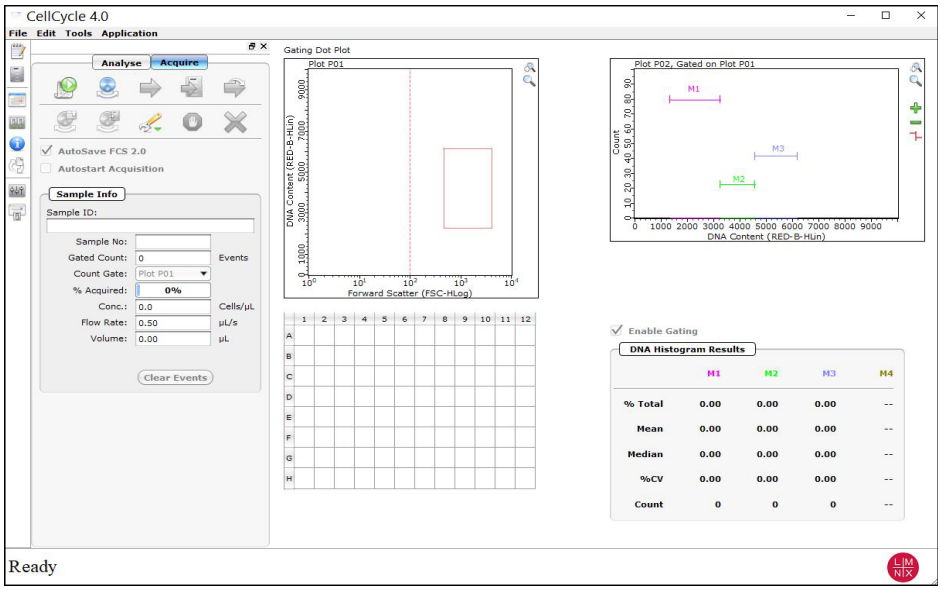
- the acquired data for all tubes in the run
- instrument settings (gains, miscellaneous settings)

6. If necessary, back up data files, to free up hard disc space.

Run the Assay on a Single-loader (SL) System

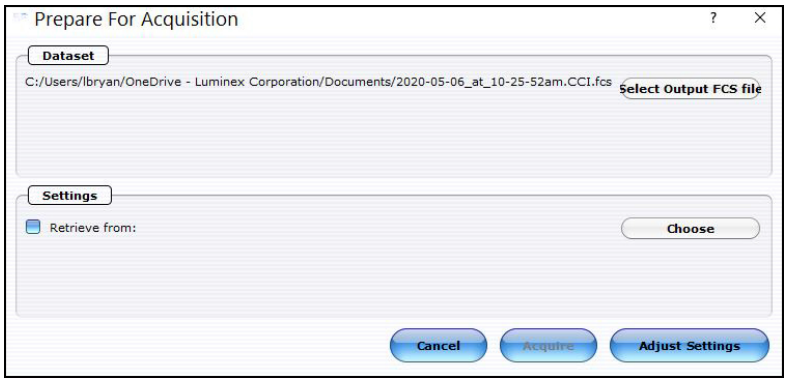
NOTE: The Guava® Cell Cycle screen opens in Acquisition mode, if the easyCyte™ System is turned on.

1. Open GuavaSoft™ Software and click **CellCycle** from the Favorites list. If you do not see the assay listed in the Favorites list, click the **Program Search** drop-down menu, choose the assay, and click **Launch**.



2. Prepare samples for acquisition in sample tubes.

3. Click the **Start New Session File** icon. A **Prepare For Acquisition** dialog box displays allowing you to choose a data file name and location and instrument settings.



   a. Click **Select Output FCS** file to save the file. The **Create New Session File** dialog box displays. Navigate to the location you want to save the file to, and click **Save**.

      NOTE: The default storage location is in My Documents. The default file name is the date and time.

   b. For **Settings**, to retrieve the settings file, click **Choose**. The **Read Settings from** dialog box displays. Navigate to the .gst or .fcs file you want to open, and click **Open**.

      NOTE: The FCS or instrument settings file must be from InCyte, version 3.0, or later.

   NOTE: Always save GuavaSoft Software data files directly to the laptop's hard drive during acquisition. Saving data files to a network or location other than the laptop's hard drive may result in data loss. You may copy the file(s) to another location when acquisition is complete.

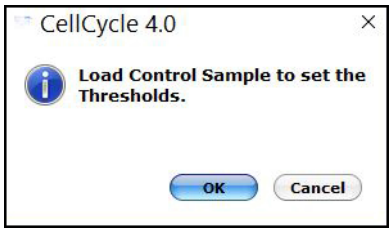
   NOTE: Your system administrator may have configured GuavaSoft Software to disable the retrieval of Methods and instrument settings from the Method files and individual instrument settings files. If the Settings options are disabled, you will not be able to adjust instrument settings. However, you can retrieve settings and Methods from a single FCS file.

4. Click **Adjust Settings**.

   NOTE: If you have already retrieved instrument settings and you want to skip the Adjust Settings step, click **Acquire** in the **Prepare for Acquisition** dialog box to proceed to acquisition, however, Luminex recommends always performing the adjust settings step. A dialog box displays prompting you to load the control sample into the instrument.

5. Mix a stained cell sample and load the sample tube onto the sample loader of the Guava easyCyte System. Luminex recommends using a stained negative control sample for the initial adjustments.

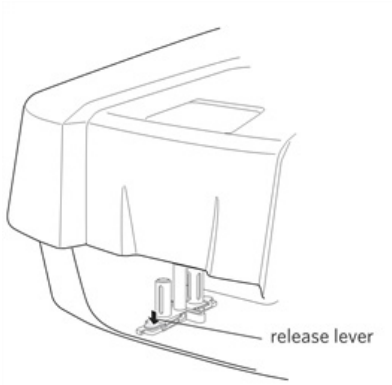
6. Click **OK** in the CellCycle 4.0 dialog box.



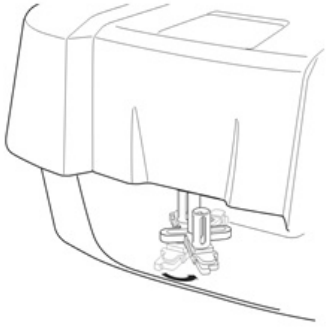
7. Adjust the appropriate settings for your experiment and once you are satisfied and ready to acquire data, click **Next Step**.

Adjust the Instrument Settings

1. Mix a stained cell sample and load the sample tube on the sample loader of the Guava® easyCyte™ System. Luminex recommends using a stained negative control sample for the initial adjustments.
2. From the Guava ViaCount Acquisition screen, click the **Adjust Setting** icon from the Button panel.
   a. To load sample tubes, pinch the release lever with your thumb and forefinger and gently lower the arm assembly (1st image).
   b. Once lowered, rotate the assembly to change the sample tube (2nd image), then push up on the assembly until you hear a click.
   c. If prompted, click **OK** in the **Messages** dialog box.



1. Pinch release lever and gently lower sample holder.



2. Rotate loader assembly to change tubes.

3. Check the **Conc** (Cells/µL) value in the **Sample Info** pane and ensure that it is less than or equal to 1200.

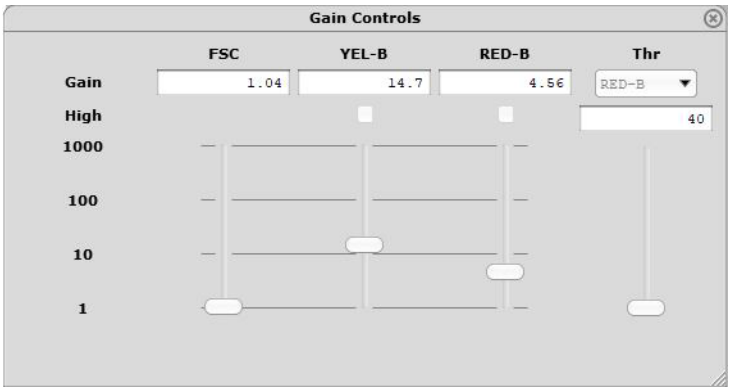
   NOTE: If the value is greater than the high limit for the corresponding flow rate, dilute the sample with the appropriate buffer to lower the concentration and minimize the risk of coincident events. For optimal performance, Luminex recommends a concentration of 250 cells/µL or lower.

4. To fine tune the settings, you can make the following adjustments using the Gain Controls. Open each instrument adjustment window using the Tools menu or the 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.

   a. Click the **Gain Controls** icon. The **Gain Controls** dialog box will display.
      - Set the **Refresh Rate** to the number of events you want to display.
      - Set the **Flow Rate** to Very Low (0.12 µL/s) or Low (0.24 µL/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 RED-B dot plot until the desired amount of debris is eliminated below the marker.
      - Adjust the gain (using the RED-B slider or the arrow keys on the keyboard) so that the G0/G1 population is positioned at approximately 2000 for 4.0 CC SW on the DNA Content (RED-B-Lin) histogram.

NOTE: If necessary, use the Count Scale field to the left of the histogram to adjust the y-axis scale. 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: Use the **Clear Events** button in the Sample Info panel to clear the display.



5. 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, by clicking **Adjust Settings**, loading the sample, and clicking **OK**.
   - If you want 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.

     NOTE: Once the worklist is complete, you can no longer save the instrument settings.
6. Vortex the first sample and load it on the instrument.
7. Enter the number of events to acquire in the Sample Info panel. The default number of events to acquire is 5000.
8. If you want to identify individual samples or sets of samples, enter an optional ID in the Sample ID field. The sample ID may be any text up to 40 characters long. If you do not enter a sample ID, the default sample number will be used as the sample ID, for example, Sample #1, Sample #2, etc.

   NOTE: If you select **Autostart Acquisition**, acquisition automatically starts when you load each tube. You do not need to click **Acquire Next Sample**. Autostart Acquisition automatically turns off if you click any of the following: **Adjust Settings**, **Retrieve Settings**, **Quick Clean**, or **Backflush**. You must recheck the box to continue using the feature. If you click **Autostart Acquisition** and want to enter a sample ID, type the ID into the Sample ID field after acquisition is complete and before loading the next sample.
9. Click the **Acquire Next Sample** button. The system acquires the first sample.

   NOTE: The progress bar provides an estimate of the target event count during the acquisition period.

   NOTE: If a clog or total loss of signal is detected, we recommend removing the clog by running a Quick Clean with water, then a Quick Clean with ICF, followed by Backflush into an empty tube, then another Quick Clean with ICF, and a final Quick Clean with water.

   NOTE: If the acquisition rate appears to slow dramatically, the fluid pathway may be blocked. Click the **Abort** button, then click **Capillary Cleaning Tools** and select **Backflush**. Load a tube of 20% bleach and click **OK**. When the backflush is complete, select **Quick Clean**. Load a tube of DI water and click **OK**. Click **Acquire Next Sample** to continue.
10. Click **Save and Close Current Sample**. You may still enter or change the Sample ID for the current sample before clicking **Save and Close Current Sample**.

11. Repeat steps above for the remaining samples.
12. Click the **Stop and Close Session File** after the last sample.
13. When you are finished, click the **Capillary Cleaning Tools** icon in the Button panel and select **Quick Clean**. Load a tube of deionized water and click **OK**.

    NOTE: If you ran blood samples, run two Quick Clean cycles with water, followed by one cycle using ICF, and finally one cycle using water to rinse.

At the completion of the run, a copy of the files are automatically loaded into the Analysis control panel. Guava Cell Cycle saves the data for all samples as a single FCS 3.0 file to the specified location.

The FCS file contains:

- the acquired data for all tubes in the run
- instrument settings (gains, miscellaneous settings)

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.

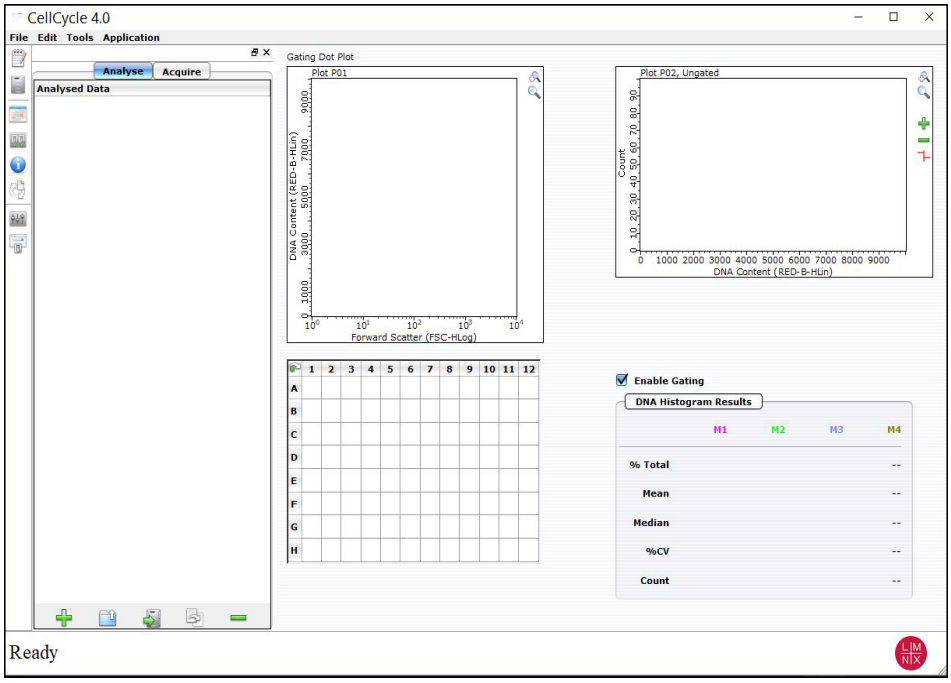
You can save changes made to the sample ID, gate, or markers within Analysis by overwriting the existing file or saving a new file.

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.

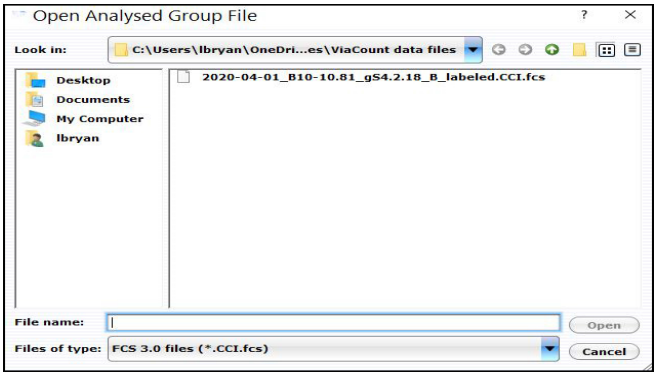
If you access the Analysis screen during data acquisition you can view or print data for any samples already acquired. You may also view the event log. However, you cannot change analysis settings (gates and markers) from the analysis screen during acquisition. Any analysis settings you wish to change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

1. Open the GuavaSoft Software and click **CellCycle** from the Favorites list. If you do not see the assay listed in the Favorites list, click the **Program Search** drop-down menu, choose the assay, and click **Launch.**

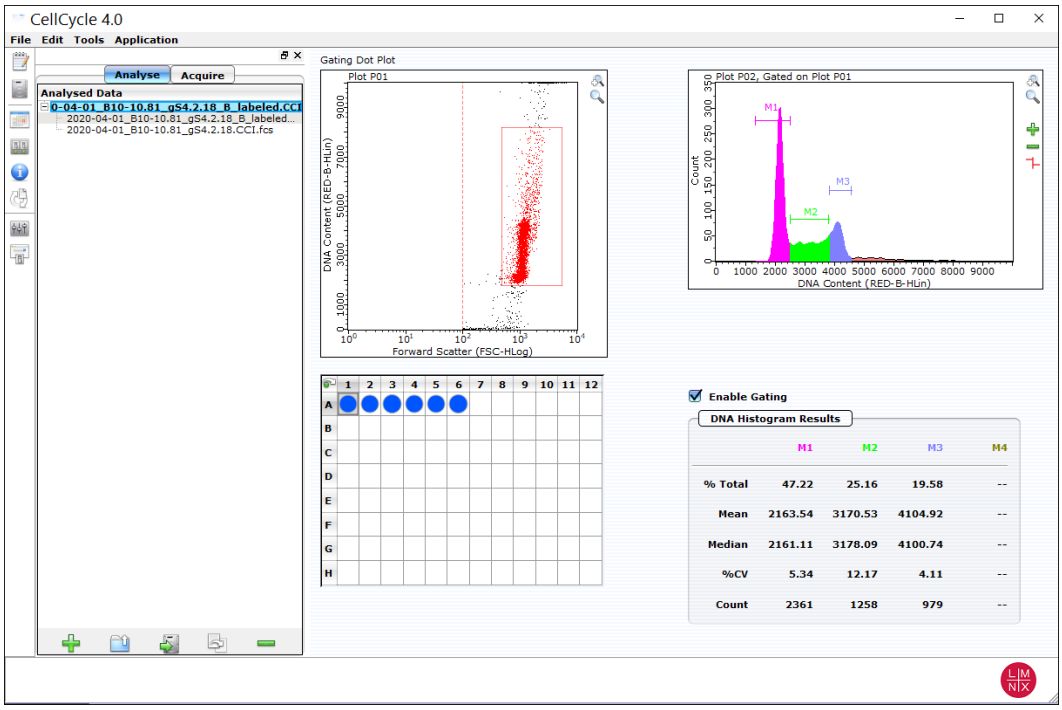
2. Click the **Analyse** button to switch from the Acquisition screen to the Analyse screen.



3. Click **Open Analysed Group**. Select an FCS file for analysis and click **Open**.



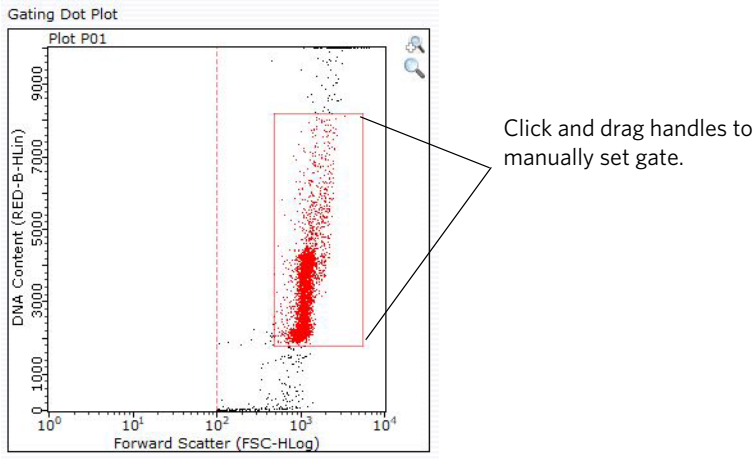
The data and results for the first sample in the data set display. The marker setting displays as it was when the sample was acquired. To see a list of all samples in the data set, click the title bar of the Analysis Sample List control panel.



Set the Dot Plot Gate

Clicking on Enable Gating either in adjust settings or analysis will allow you to remove excess debris from the FSC vs RED-B plot. The Cell Cycle Results are updated each time you adjust the gate.

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

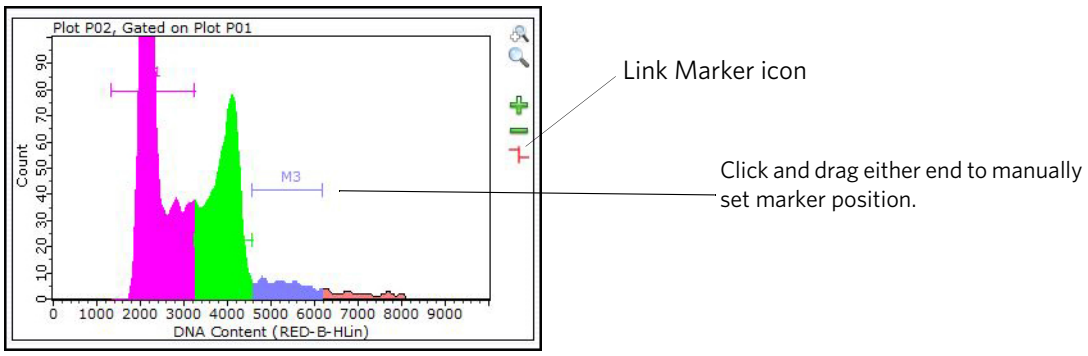


Adjust the Histogram Markers

The histogram markers allows you to get statistics on any population within the RED-B parameter. You can set up to four markers to identify and generate statistics on the G0/G1, S-phase, G2/M, and 4th populations. The RED-B Results are

automatically updated each time you move the markers. To analyze the data within the dot plot gate, click **Enable Gating** under the histogram plot.

1. To set the RED-B 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 allowing for non-overlapping markers, when you move the left side of marker 2, the right side of marker 1 will move automatically.
   You may overlap the markers by de-selecting the **Link Marker** icon. Be aware that events falling within two markers will be counted twice. Marker 4 can overlap even if the Link Marker is selected.



RED-B histogram markers 1, 2, and 3 selecting diploid G0/G1 cell (pink), S phase (light green), and G2/M cells (blue).

2. When you have finished analyzing the samples in the current file, you can save any analysis changes you made by clicking the **Save Analyzed Group** icon. A dialog box displays 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.

3. If you want to view the event log, click **Event 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 as appropriate.
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	61.10	30.82	5.32	--
Mean	2324.38	3897.36	5297.34	--
Median	2198.36	3946.83	5282.88	--
%CV	14.22	8.19	8.29	--
Count	3055	1541	266	--

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.

Expected Results

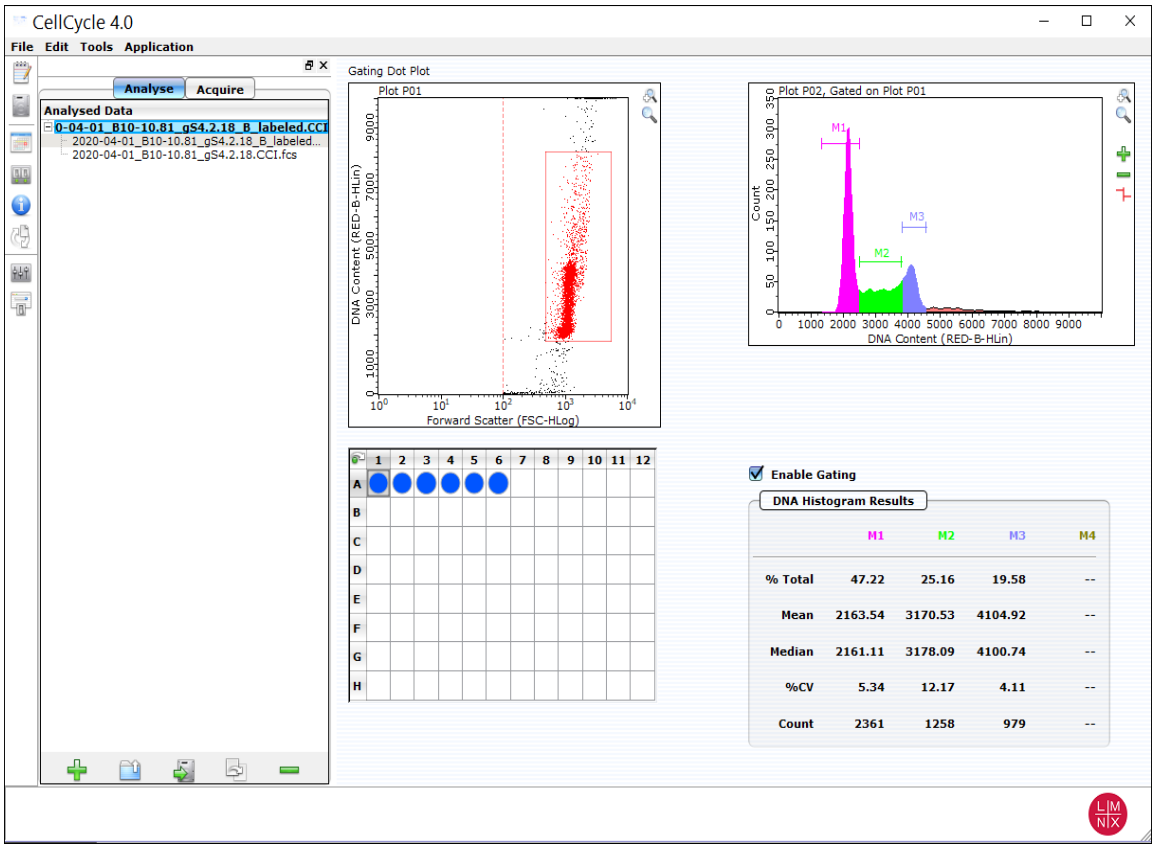
Figures 1 shows typical results obtained with the Guava® Cell Cycle Reagent. Log-phase Jurkat T cells were serum ethanol fixed overnight, and stained in 96-well plates according to the protocol described above.

The histogram plot shows the distribution of the cell cycle phases (G0/G1, S and G2/M). The DNA Histogram Results show the result for the percentage of cells in G0/G1 (M1), S (M2) and G2/M (M3) under % Total. The dot plot shows the Forward Scatter (FSC) versus DNA content of the cell sample.

Untreated Jurkat cells had 47.22%, 25.16%, and 19.58% of cells in G0/G1 (M1), S (M2) and G2/M (M3), respectively. The Guava Cell Cycle Reagent and Assay can be used to detect different phases of cycling cells.

Figure 1: Examples of cycling cells analyzed using the Guava Cell Cycle Reagent and Assay. Figure 1 Jurkat cells were prepared according to the above protocol and acquired on a Guava system. The histograms show the cells in G0/G1 (pink peak on left), S (green center peak) and G2/M (blue peak on right). In the dot plot, the cycling cells are shown within the rectangular gate and are in red. The cells excluded from the gate are shown in black and are either debris or sub-G0 cells (below the gate) or aggregates or G4 cells (above the gate).

Figure 1: Jurkat cells



Troubleshooting Tips

- Setting the FSC threshold too low may affect your results because cell debris will be included. Additionally, noise may appear in the RED-B histogram as a result. Try adjusting the threshold upward in order to reduce noise and debris or enable gating in analysis to exclude unwanted debris or other events.
- Avoid excessive exposure of the stained samples to light.
- If the concentration of the stained cell sample for data acquisition is low (<7 x 10^4 cells/mL), the Guava® Instruments will not be able to acquire 5,000 events in the allotted time for sample collection (10 min). Centrifuge the sample at 400 x g for 7-10 minutes and remove a sufficient amount of the supernatant to increase the cell concentration to >7 x 104 cells/mL.
- If there an unexpected high %CV or double G0/G1 peak, repeat staining, and ensure that the ethanol has been properly removed.
- The default number of events to acquire is 5000. You may input a different number, however, your statistical error may increase as you decrease the number of events for acquisition. You should not collect below 2000 gated events. Collecting below 2000 gated events may yield erroneous results.
- Run Guava easyCheck™ (Catalog No. 4500-0025) to verify proper instrument function and accuracy.
- Be sure that samples are properly resuspended prior to acquisition. For the single-loader (SL) instruments, vortex samples just prior to acquiring. For the high-throughput (HT) instruments, check that the mixing option has been selected in the Worklist file used to collect the data in the Guava Cell Cycle software application. Cells in the sample will settle quickly and your Guava Cell Cycle results (percent of cells in G0/G1, S and G2/M, etc.) will be inaccurate unless each sample is mixed prior to acquisition.
- A Quick Clean will be performed at the end of every Worklist when using the high-throughput (HT) instruments. If your samples contain significant amounts of cellular debris that might build up in the flow system and cause a clog, you might want to select more frequent Quick Cleans after every 12 to 24 sample acquisitions. Alternately, if your samples contain significant amounts of cellular debris, run Quick Clean with Guava ICF followed by water, to prevent clogs or blockage. If you are acquiring samples on the single-loader (SL) instrument, you should perform manual Quick Cleans as described above.
- If you are acquiring data from a sample, but the Cell Count number is not increasing and the "Events to Acquire" bar is not moving, there is probably either insufficient volume to continue to acquire sample, or a blockage of the flow system. Check first for the lack of sufficient sample volume (on an high-throughput (HT) instrument, you must first pause the software application and eject the tray). If the sample volume is below 50 µL, there is not enough sample for the instrument to acquire. Either add additional Guava Cell Cycle Reagent to bring the sample volume up to greater than 50 µL, or proceed to the next sample. If the sample volume is more than 50 µL, then the lack of events acquired is probably due to a clog. A clog or blockage of the flow system can be caused by cell aggregates, cell debris, bleach crystals, or other particulates. Click **Backflush** to flush out the clog into a tube containing 100 µL bleach. Then run Quick Clean to remove bleach residue from the outside of the flow cell. If this procedure does not alleviate the problem, refer to the appropriate Guava instrument user's guide, or contact Technical Service.

For more troubleshooting tips, refer to the user's guide for the appropriate Guava instrument.

Guava® Cell Cycle Assay Troubleshooting

Problem	Possible Cause	Solutions
Message: *This file already exists. You must pick a new name.*	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: *This file exists with read-only attributes. Please use a different file name.*	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.
Guava® Cell Cycle Software Module starts in Analysis mode. Acquisition mode is not available.	1. Communication problem between instrument and laptop. 2. A registration code was not entered or was entered incorrectly.	1. Ensure USB cable is connected between instrument and laptop. 2. Enter the registration code. The code is case sensitive.
Message: *Less than 10 particles/uL. sample is too dilute. Accuracy may be compromised* 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 for HT systems, and 90 µL for single-loader systems. 3. Ensure sample mixing option was selected in WorkEdit Software, or click Abort, remove sample, mix, and reload.

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 µL for round-bottom wells, 150 µL for 0.5-mL tubes, and 900 µL for 1.5-mL tubes for HT systems, and 90 µL for single-loader systems. 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 Luminex Technical Support. 7. Ensure tubing connector is secure.
Message: *More than 500 particles/uL. sample is too concentrated. Please dilute or accuracy may be compromised*	Sample is too concentrated	Dilute sample with appropriate buffer or cell cycle reagents. Recommended concentration range for accurate results is 1 x 10^5 to 1.2 x 10^6 cells/mL
Debris appearing in the RED-B 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.
Events appear in FSC vs. RED-B dot plot but not in RED-B histogram.	FSC vs. RED-B gate excludes events.	Ensure FSC vs. RED-B gate is set to include population of interest.
Events appear off scale in dot plot	FSC or RED-B voltages set incorrectly, or sample staining brightly	Adjust settings to increase or decrease FSC or RED-B gain so cellular populations appear on sale.

Cell Sample Preparation

Prepare Non-Adherent Cells

1. Set up initial culture conditions, such that after culture and treatment, cells are at a concentration of 1 x 10^5 to 1 x 10^7 cells/mL in serum or albumin containing medium.
2. Proceed to "Cell Staining Protocol" on page 5.

Prepare Adherent Cells

For harvesting adherent cells, use your method of removal. Reagents such as EDTA or trypsin can be used to dissociate the cells from the flask and should create single-cell suspensions. If using mechanical means to dislodge the cells, additional reagents such as Guava® Cell Dispersal Reagent (Cat No. 4700-0050) may be used to dissociate clumps.

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

Limitations

1. The results of the assay are dependent upon proper handling of samples and reagents.
2. For accuracy, at least 2000 gated events should be collected.
3. If the cell per µL (p/µL) is greater than 1200, dilute the sample with Guava Cell Cycle Reagent.
4. Cell fixation, cell washing and cell staining should be done on 96-well round bottom plates, 12 x 75 mm tubes, or 15- or 50-mL Falcon tubes. We do not recommend using 96-well flat bottom plates or 1.5 microcentrifuge tubes for cell fixation, cell washing or cell staining.

References

1. Gupta RS. Cross-resistance of nocodazole-resistant mutants of CHO cells toward other microtubule inhibitors: Similar mode of action of benzimidazole cabamate derivatives and NSC 181928 and TN-16. Mol Pharmacol. 1986;30:142–148.
2. Ho J, Gillis K, Fishwild D. A simple and robust system for determining cell cycle distribution: The Guava cell cycle assay. Guava Application Note. 2004.