Luminex
complexity simplified.

Guava® Cell Cycle Reagent

Cat. No.4500-0220 (100 Tests)

To determine the percentage of cells in G0/G1, S and G2/M phases based on DNA content

Research Use Only
Not for Use in Diagnostic Procedures

PRODUCT DESCRIPTION AND INTENDED USE

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/G1phase) 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 PM2 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.

MATERIALS PROVIDED

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

HANDLING AND STORAGE

1. 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.
2. Do not use the reagent after the expiration date.
3. The Guava Cell Cycle Reagent contains light-sensitive dyes. Shield from excessive exposure to light.

WARNINGS AND PRECAUTIONS

1. The Guava® Cell Cycle Reagent is intended for research use only and not for use in diagnostic procedures.
2. Wear proper laboratory attire (lab coat, gloves, safety glasses) when handling or using this reagent.
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. All biological specimens and materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Avoid specimen contact with skin and mucous membranes.
7. Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
8. A Safety Data Sheet (SDS) is available from our website (www.luminexcorp.com) or by contacting Technical Support.

EQUIPMENT AND MATERIALS REQUIRED

- Guava® Instrument with the Cell Cycle software module
- Guava Cell Cycle Reagent (Cat. No. 4500-0220)
- 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)
- 20% bleach solution
- 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 (Falcon #353910, #353918), or equivalent. Refer to the Guava instrument's user's guides for compatible microplates.
- 1.5-mL microcentrifuge tubes without screw caps (VWR Cat. No. 20170-201) or with screw caps, 1.5 mL (VWR Cat. No. 16466-030), or equivalent for cleaning
- 0.5-mL microcentrifuge tubes (VWR Cat. No. 16466-036) for sample acquisition
- Centrifuge with 96-well microplate holders
- Guava® ViaCount™ Flex Reagent (Cat. No. 4700-0060), optional

For Single-loader (SL) Guava® Systems

- 1.5-mL microcentrifuge tubes (VWR Cat. No. 16466-030 or equivalent), or 1.2-mL titer tubes (E&K Scientific, Cat. No. 604508-RC or equivalent) for sample acquisition
- Guava® ViaCount™ Reagent (Cat No. 4000-0040), optional

REAGENT AND SAMPLE PREPARATION

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 lose 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 *Appendix A: Cell Sample Preparation* on page 3 for information on preparing cell suspensions.

A. Cell fixation 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 2.

B. Cell fixation in a 50-mL tube

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 appropriate 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* in the following section.

Cell Staining Protocol

A. Cell staining 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.

B. Cell staining 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: You must use a 12 x 75 mm tube instead of a 1.5 mL microcentrifuge tube due to a large amount of cell loss with the fixed Jurkat cells (and perhaps other cell lines) over time when stored in 1.5 mL microcentrifuge 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 and acquire the samples on a Guava single-loader (SL) instrument.
11. Acquire the data on the Guava instrument.

EXPECTED RESULTS

Figures 1a and 1b show typical results obtained with the Guava® Cell Cycle Reagent. Log-phase Jurkat T cells were serum starved for 24 hours and treated with (Figure 1b) and without (Figure 1a) 0.029 µg/mL of nocodazole for an additional 24 hours in medium, ethanol fixed overnight and stained in 96-well plates according to the protocol described above.

The upper plot shows the distribution of the cell cycle phases (G0/G1, S and G2/M) in histogram format. 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 bottom plot shows the Forward Scatter (FSC) versus DNA content of the cell sample.

Untreated Jurkat cells had 48.9%, 19.4% and 32.1% of cells in G0/G1 (M1), S (M2) and G2/M (M3), respectively. Jurkat cells treated with nocodazole for 24 hours had fewer cells in G0/G1 and S phases and many more in the G2/M phase (11.0%, 11.0% and 76.9% in G0/G1, S and G2/M, respectively). As expected, nocodazole, an anticancer drug that interferes with the structure and function of microtubules in interphase and mitotic cells, arrested cells in the G2/M phase. Thus, the Guava Cell Cycle Reagent and Assay can be used to detect differences between cycling and arrested cells.

Figure 1a and 1b. Examples of cycling and arrested cells analyzed using the Guava Cell Cycle Reagent and Assay. Untreated (Figure 1a) and nocodazole-treated (Figure 1b) 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 green and are either debris or sub-G0 cells (below the gate) or aggregates or G4 cells (above the gate).

Figure 1a. Untreated Jurat cells

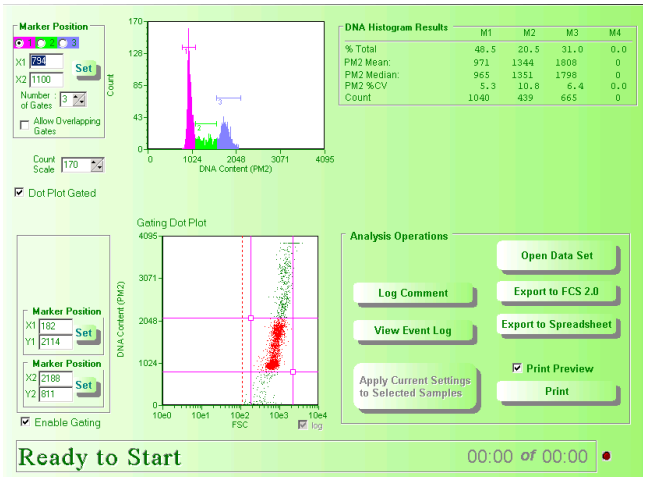
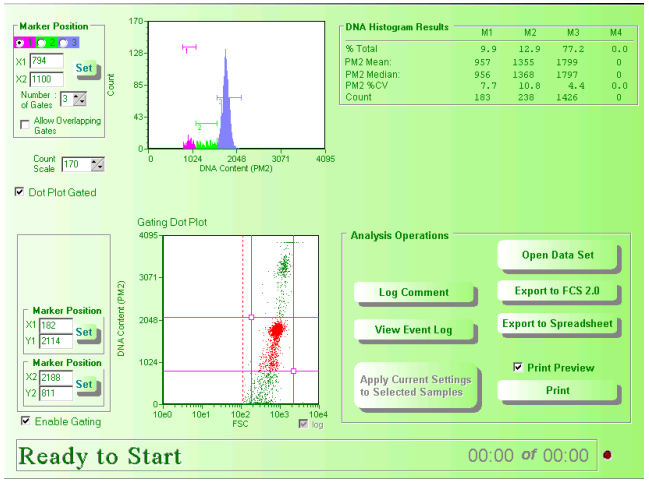


Figure 1b. Nocodazole-treated Jurkat cells



TROUBLESHOOTING TIPS

1. Setting the FSC threshold too low may affect your results because cell debris will be included. Additionally, noise may appear in the PM2 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.
2. Avoid excessive exposure of the stained samples to light.
3. 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 10^4 cells/mL.
4. If there an unexpected high %CV or double G0/G1 peak, repeat staining, and ensure that the ethanol has been properly removed.
5. 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.
6. Run Guava easyCheck™ (Catalog No. 4500-0025) to verify proper instrument function and accuracy.
7. 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.
8. 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.
9. 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.

APPENDIX A: CELL SAMPLE PREPARATION

Preparing Non-Adherent and Adherent Cells

The following protocols describe how to harvest non-adherent or adherent cells cultured in 96-well plates, flasks, or other tissue culture vessels. Each of the culturing conditions requires different protocols to harvest the cells.

Preparing 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 2.

Preparing 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 2.

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 particle 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.

TRADEMARKS

Order Information

For ordering information or technical support contact Luminex Technical Support.

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

Revision Date: July 2019
4600-0790 rev F