

# Guava® PCA™-96 Cell Cycle

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## OBJECTIVE

The objective of this assay protocol is to guide the user from cell cycle sample preparation through data analysis to obtain relative G0/G1, S, and G2/M cell cycle phase percentages for samples collected using the Guava PCA-96 System. Jurkat (human T cell leukemia) suspension and PC3 (human prostate cancer cell) adherent cell lines were analyzed using the protocol below and the results are presented in the section "Expected Results."

## INTRODUCTION

The cell cycle describes the process of the replication and division of chromosomes within the nucleus, which occurs prior to a cell dividing. Cancerous 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) it is possible to discriminate cells in different stages of the cell cycle. Resting cells (G0/G1 phases) contain two copies of each chromosome. As cells progress toward mitosis, they synthesize DNA (S phase), allow-

ing more PI intercalation with a resulting increase in fluorescence intensity. When all chromosomes have replicated and the DNA content has doubled (G2/M phases), 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 PCA-96 using CytoSoft™ software. CytoSoft is used to identify the three phases of the cell cycle and to calculate the relevant statistics, including percentage of total, PM1 mean, median and %CV fluorescence intensity, for each population. Data are displayed in a single parameter histogram with up to four markers to analyze the different populations. The first three markers are used to delineate the G0/G1, S and G2/M phases. The fourth marker can be used to demarcate either apoptotic cells or an internal standard. The data for all samples within a data set are saved to an FCS 3.0 file, and optionally to individual FCS 2.0 files. The data are analyzed immediately after the data are 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.

CytoSoft can be used to monitor changes between the different phases of the cell cycle. To analyze the data further for more subtle changes between these populations or to eliminate aggregates including G0/G1 phase cell doublets from within the apparent G2/μ population, we recommend third party "curve fitting" software. These software programs apply sophisticated curve fitting algorithms to the Guava Cell Cycle data that more accurately calculate the percentages of the cell populations and their relevant statistics.

## EQUIPMENT AND MATERIALS REQUIRED

### Equipment and consumables

- Guava PCA™-96 with Cell Cycle software option
- Guava CytoSoft version 2.5 or higher with the Guava Cell Cycle software module
- Third party DNA cell cycle analysis software (optional, for cell cycle analysis using various software algorithms)
- Centrifuge
- Vortex mixer
- Refrigerator
- 37 °C CO<sub>2</sub> incubator
- -20 °C freezer
- Ice
- Tissue culture flasks
- Pipettes
- Pipettor
- Micropipette tips (200 and 20 µL)
- Micropipettors, single and multi-channel (200 and 20 µL)
- 50 mL conical tubes
- 12 x 75 mm polystyrene tubes
- 1.5 mL microcentrifuge tubes with lids
- 96-well round bottom plate (BD Falcon #353075, #353915)
- 96-well flat bottom plate (BD Falcon 353910 or 353918)
- V-shaped 25 mL and/or 55 mL trough (25 mL, Apogent Discoveries #8093 and 55 mL VWR 210070-970)
- Sterile, serological pipets
- Stirrer bar
- Corning filtration unit, 0.22 µm pore size, 250 mL (Corning Cat. No. 431096)
- Lab shaker
- Microplate sealers
- Analytical balance
- Disposable gloves
- Plate shaker

### Biological and chemical reagents

- Cell sample
- Appropriate cell culture media (including serum)
- Phosphate buffered saline 1X, (1X PBS), pH 7.4
- Triton X-100 detergent (Sigma, X100)
- DNase-free RNase (Sigma, R6513)
- Propidium iodide at 1 mg/mL in H<sub>2</sub>O (v/v) (Molecular Probes, P-3566)
- Distilled water
- Bleach
- Guava ICF™ Instrument Cleaning Fluid, Cat. No. 4200-0140
- Ice-cold 70% ethanol
- Guava CDR™ Cell Dispersal Reagent, Cat. No. 4700-0050 (optional)
- Guava PCA-96 ViaCount® Flex reagent, Cat. No. 4700-0060 (optional)
- Guava Check™ Kit, Cat. No. 4500-0020 (optional)

## HANDLING AND STORAGE

1. Store the Triton X-100 at room temperature.
2. Store propidium iodide and PBS at 2–8 °C.
3. Protect the propidium iodide reagent from light at all times.
4. Store RNase in -20 °C freezer.

NOTE: Refer to specific manufacturer's documentation for details on each specific product.

## BEFORE YOU BEGIN

Please review this Assay Protocol in its entirety, prior to cell cycle data collection and analysis.

## SAMPLE AND REAGENT PREPARATION

### Time considerations

Staining cycling cells using the recommended protocol usually can be completed within 1 hour. However, prior to the staining, cell fixation requires at least 2 to 12 additional hours. Finally, data acquisition of an entire 96-well plate on the Guava PCA-96 may take several hours but the actual elapsed time depends on the cell concentration and the number of events to be acquired, as well as the number of washing and

mixing steps. Acquisition of a typical 96-well plate with a cell concentration of 500 cells/ $\mu\text{L}$  and acquisition of 5000 events takes about 3.5 hours. 2500 events is also sufficient for a representative analysis and the time for acquisition is less than 2 hours.

### *Recommended staining conditions*

A typical assay test uses 0.2 mL of cell staining solution (see below) to stain  $1 \times 10^5$  to  $2 \times 10^5$  fixed cells per well in a 96-well microplate.

### *Preparation of working and cell staining solution*

#### **1. Preparing 10% Triton X-100 in 1X PBS**

- Add 9 mL of 1X PBS to a 15 mL Falcon tube.
- Using a 1 mL pipettor, slowly pipette 1 mL of Triton X-100 from the bottle, wipe the tip of the pipette and add the detergent to the Falcon tube containing the 1X PBS.
- Mix the tube by pipetting up and down several times or vortexing until you get a homogenous mixture.
- Store the 10% Triton X-100 at room temperature or 4 °C.

#### **2. Preparing 10 mg/mL of DNase-free RNase in water**

- Using an analytical balance, weigh out 25 mg of DNase-free RNase.
- Add the RNase to a 15-mL conical tube.
- Add 2.5 mL of deionized water to the tube.
- Vortex the tube until the RNase is completely dissolved.

#### **3. Making up 0.1% Triton X-100**

- Add 118.8 mL of 1X PBS to a 200 mL container.
- Add 1.2 mL of 10% Triton X-100 to the container.
- Using a stirrer bar, mix the solution until it is homogenous.
- Filter the 0.1% Triton X-100 in 1X PBS using the Corning Filtration Unit.

#### **4. Preparing Cell Cycle Staining Reagent**

- Cover a sterilized 100-mL bottle with aluminum foil to shield the reagent away from light.
- Add 95.5 mL of 0.1% Triton X-100 in 1X PBS to the sterile 100-mL bottle.
- Add 2 mL of 10 mg/mL stock DNase-free RNase to the bottle.
- Add 2.5 mL of 1 mg/mL Propidium Iodide Stock to the sterile bottle.
- Cap the bottle and mix the Cell Cycle Staining Reagent by inverting it up and down several times.

### *Storage and handling for the cell staining reagent solution*

- Avoid exposure of the Cell Cycle staining reagent to light.
- Store Cell Cycle Staining reagent at 4–8 °C for up to one month.

NOTE: Propidium iodide may be carcinogenic and/or mutagenic. Exercise standard precautions when obtaining, handling, and disposing of potentially carcinogenic and mutagenic reagents.

## **CELL PREPARATIONS**

### **1. Assaying non-adherent samples grown in a 96-well microplate**

- Prior to adding cells to the microplate, determine the cell concentration of the stock using Guava PCA-96 ViaCount Flex reagent. Please see the package inserts for instructions in how to use those products to determine cell concentrations.
- Add  $1 \times 10^5$  to  $2 \times 10^5$  cells in a 200  $\mu\text{L}$  volume (between  $5 \times 10^5$  cells/mL and  $1 \times 10^6$  cells/mL) to each well of a microplate with or without experimental treatments. If adding inducing agents after addition of cells to the microplate, make stocks and add 20  $\mu\text{L}$  of inducing reagent to the appropriate wells.
- Culture cells, if necessary, as per your own protocols.
- Proceed to Cell Fixation in a 96-well plate protocol.

## 2. Assaying samples that have been grown in tissue culture vessels other than 96-well microplate

*For non-adherent cells:*

- Transfer the cells from the culture vessels to tubes.
- Determine the concentration of the cell sample using Guava PCA-96 ViaCount Flex reagent. If necessary, adjust the cell sample to between  $5 \times 10^5$  to  $1 \times 10^6$  cells/mL. With a multi-channel micropipettor, add a 200  $\mu$ L cell sample to the well of a round bottom plate.

NOTE: Cells may be fixed for as little as 2 hours, however, the quality of the results obtained with shorter fixations may vary with each cell line and will not be as good as those from overnight fixations.

- Proceed to the protocol for "Cell fixation in a 96-well plate" or refer to "Cell fixation in tube" to fix the cells.

*For adherent cells:*

- Rinse the flask once with 5–10 mL of 1X PBS. Add the PBS into the same conical tube above.
- Dilute the Guava CDR 1:3 with 1X PBS.
- For a T-75 flask, add 3 mL of diluted Guava CDR and incubate for 3–5 minutes in a 37 °C incubator.
- Add 7 mL of media containing serum and pipet repeatedly to release cells from the flask bottom.
- Pipet the Guava CDR and media into the same tube.
- Mix the cell sample by vortexing or pipetting repeatedly to ensure a homogenous suspension.
- Determine the concentration of the using Guava PCA-96 ViaCount Flex reagent. If necessary, adjust the cell sample to between  $5 \times 10^5$  and  $1 \times 10^6$  cells/mL. Then, either leave the cells in the tube, or, with a multichannel micropipettor, add 200  $\mu$ L cell sample to the well of a Round Bottom plate.

NOTE: Cells may be fixed for as little as 2 hours, however, the quality of the results obtained with shorter fixations may vary with each cell line and will not be as good as those from overnight fixations.

- Proceed to "Cell Fixation in a 96-well plate Protocol" or refer to "Cell Fixation in Tube" to fix the cells.

## CELL FIXATION

NOTE: It is important to achieve a single cell suspension prior to fixation. It is strongly advised that users perform cell fixation in round bottom plates so as to not lose cells with washing. Cells may be fixed for as little as 2 hours, however, the quality of the results obtained with shorter fixations may vary with each cell line and will not be as good as those from overnight fixations.

### 1. Cell fixation in a 96-well plate

- Transfer the cell sample from the flat-bottom plate to a round-bottom plate if the cells are not already in a round-bottom plate.
- Centrifuge the cells at 450 x g for 5 minutes with the brake on low and at room temperature.
- Remove and discard the supernatant being careful not to touch the pellet.
- Add 200  $\mu$ L of 1X PBS to each well using a multi-channel pipettor.
- Mix the cells in the well by pipetting up and down several times.
- Centrifuge the cells in the round bottom plate at 450 x g for 5 minutes with the brake on low and at room temperature.
- Remove and discard the supernatant.
- Place the round bottom plate containing the pellet cells with residual PBS on a lab shaker.
- Add 200  $\mu$ L of 70% ice cold ethanol dropwise into the wells while shaking at low speed (speed 3).
- Seal the plate with a microplate sealer and refrigerate cells for at least 12 hours prior to staining. Fixed cells are stable for several weeks at 4 °C and for two to three months at –20 °C.

NOTE: Cells may be fixed for as little as 2 hours, however, the quality of the results obtained with shorter fixations may vary with each cell line and will not be as good as those from overnight fixations.

- Proceed to Cell Staining Protocol.

**2. Cell fixation in tube**

- a. Centrifuge the tube at 450 x g for 5 minutes with the brake on low.
- b. Remove and discard the supernatant.
- c. Add the same volume of 1X PBS to each tube as was in the original culture (or to get approximately  $1 \times 10^6$  cells/mL).
- d. Mix the cell sample by vortexing or pipetting repeatedly to ensure a homogenous suspension.
- e. Centrifuge the tube at 450 x g for 5 minutes with the brake on low.
- f. Remove and discard the supernatant leaving approximately 500  $\mu$ L of 1X PBS.
- g. Resuspend the cells in the residual 1X PBS and transfer the suspension drop-wise while vortexing on medium speed (setting at 5) into a 50 mL conical tube containing enough 70% ethanol to make the final concentration approximately  $10^6$  cells/mL.
- h. Refrigerate the cell preparation for at least 12 hours prior to staining. Fixed cells are stable for several weeks at 4 °C and for two to three months at -20 °C.

NOTE: Cells may be fixed for as little as 2 hours, however, the quality of the results obtained with shorter fixations may vary with each cell line and will not be as good as those from overnight fixations.

- i. Proceed to Cell Staining Protocol (in 96-well format).

**CELL STAINING PROTOCOL (96-WELL FORMAT)**

1. Warm Cell Cycle staining reagent to room temperature; shield from excessive light exposure. Warm 1X PBS to room temperature.
2. Transfer 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  $\mu$ L of 1X PBS to each well and mix the wells by pipetting up and down several times. Let the plate stand at room temperature for one 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.
8. Add 200  $\mu$ L of Cell Cycle Staining 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 sample on the Guava PCA-96 system.

**DATA ACQUISITION**

Refer to the *Guava PCA-96 System User's Manual* for detailed protocols describing data acquisition.

**DATA ANALYSIS**

Refer to the *Guava PCA-96 System User's Manual* for detailed protocols describing data analysis using CytoSoft.

The results of your cell cycle assays are automatically exported from CytoSoft to a spreadsheet file. Statistics for each population within the histogram in CytoSoft include percent total, PM1 mean, median and %CV fluorescence intensity for all events and for gated events. In addition, all of the instrument and analysis settings are stored in the CSV file. All samples within a data set are saved to an FCS 3.0 file, and optionally, to FCS 2.0 files.

CytoSoft does not employ sophisticated curve fitting algorithms to process the data. Curve fitting algorithms more accurately partition the cells into the 3 phases of the cell cycle and hence yield better percentages and other relevant statistics. Curve fitting algorithms can also be employed to eliminate the G0/G1 doublet cells that are counted as G2/M cells based on fluorescence, because both have 4N DNA content. To get a more accurate assessment of your data, we

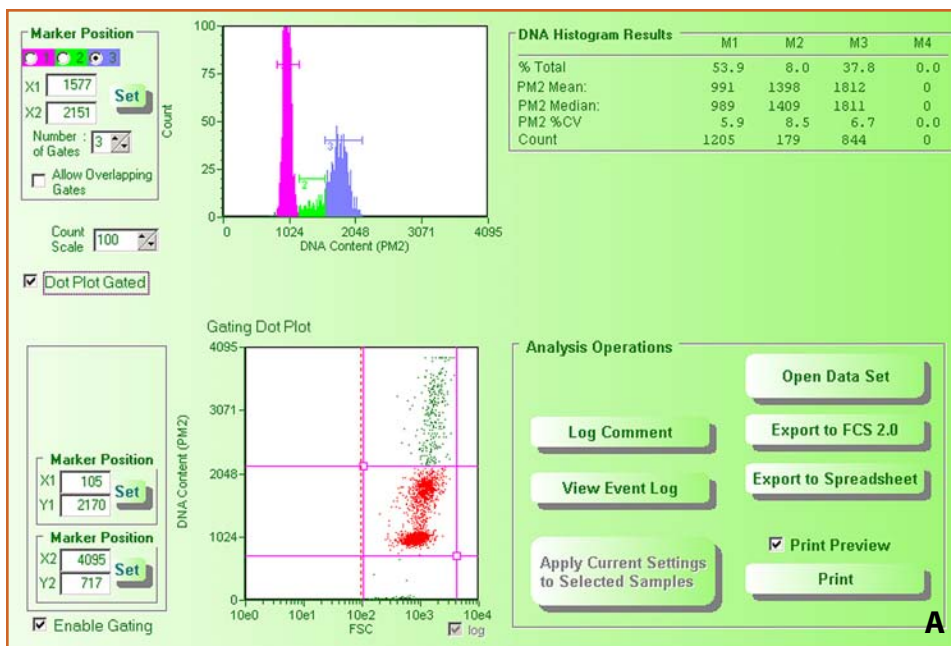
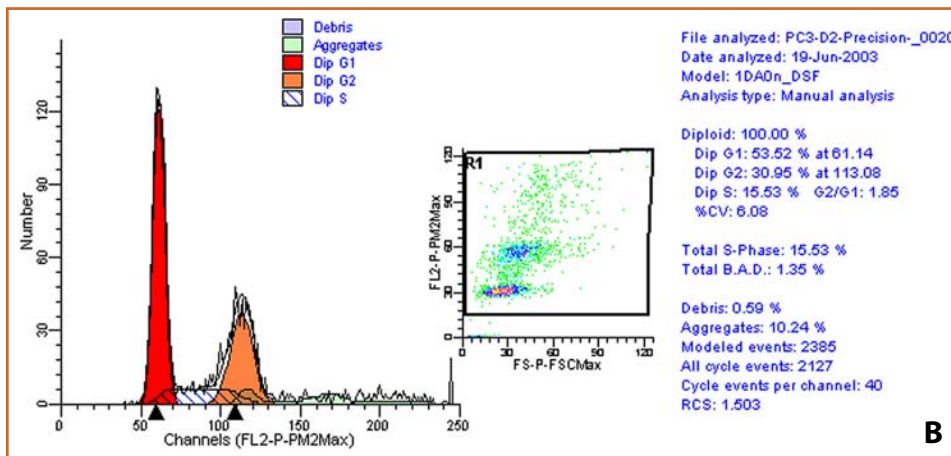


FIGURE 1. (A) An example of results obtained using the Cell Cycle protocol for PC3 cells cultured in a flask. PC3 cells were split 1:3 and cultured in a T-75 flask for 2 days. Guava CDR was used to remove the cells from the flask. Cells were fixed with 70% ethanol in the tube and then stained in the 96-well round bottom plate as described above. Results show percentages for the G0/G1 phase, S phase, and G2/M phase. (B) The same sample as in (A) analyzed with Modfit LT.



recommend using third party “curve-fitting” software programs, such as ModFit LT or MultiCycle. Please see the Guava application note, “Use of Third Party DNA Analysis Software for processing Guava Cell Cycle Data” for more detailed information.

**EXPECTED RESULTS**

*Control cells*

Figure 1a shows a typical CytoSoft histogram and dot plot from a healthy PC3 cell (human prostate) sample acquired on the Guava PCA-96 system. The DNA histogram results table presents the percentages of each phase by markers: M1 (G0/G1), M2 (S), M3 (G2/M), and M4 (if enabled). The PM2 mean and median fluorescent intensities as well as the PM2 % CV are also shown in the histogram results table. These results can be

directly used to screen for large changes in particular phases of the cell cycle.

After acquisition on the Guava PCA-96 system, the data was analyzed immediately using Guava Cell Cycle software or ModFit LT™. Figure 1b shows a typical result from ModFit LT. All data shown here were processed and plotted using ModFit LT.

*Drug induction*

Jurkat cells (human T leukemia) were serum starved for 24 hours and treated with 0.05 µg/mL of Aphidicolin (S phase arrest) and 0.029 µg/mL of Nocodazole (G2/M phase arrest) for 24 hours in a flat bottom 96-well microplate. A control sample, with no drug, was prepared at the same time. After drug induction, cells were transferred to a round bottom 96 well microplate, washed and fixed with 70% ethanol. The cells were

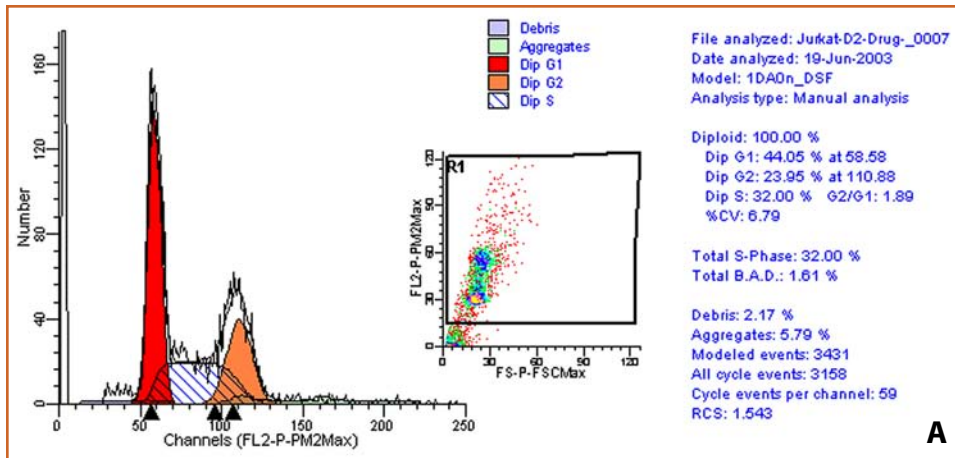
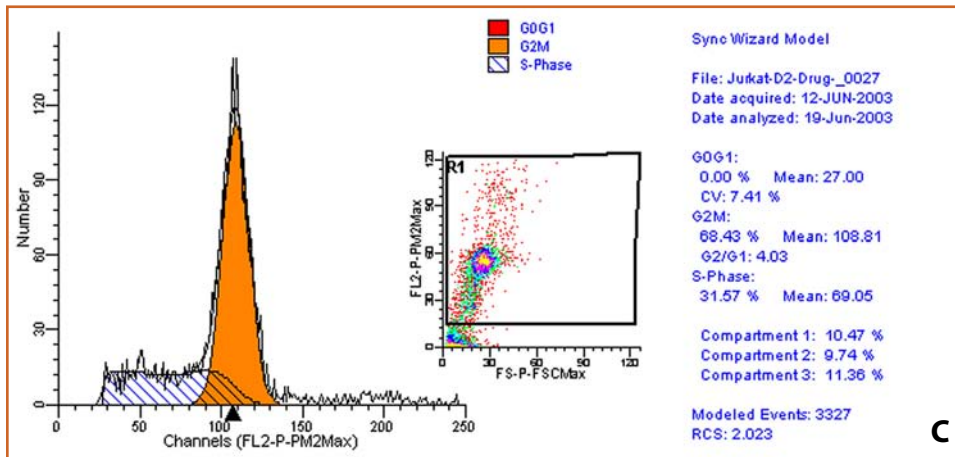
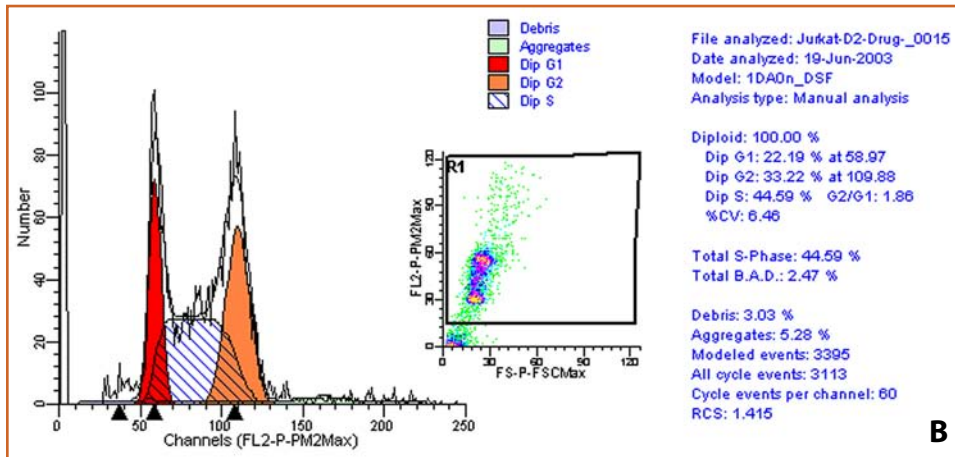


FIGURE 2. Jurkat cells were cultured with Aphidicolin to arrest cells in S phase or with Nocodazole to arrest cells in G2/M phase. These treated cells plus cells not exposed to drug were then fixed, stained and acquired on the Guava PCA-96 system. The percentage of cells in each phase of the cell cycle was determined using ModFit for each replicate (n=10). Representative data from each of the treatment groups [(A) no drug, (B) Aphidicolin, (C) Nocodazole] are shown here.



kept in 4 °C overnight and stained according to the Cell Staining (in a 96-well format) protocol above.

Figure 2a shows a plot of Jurkat cells with no drug induction while 2b shows Jurkat cells treated with Aphidicolin and 2c shows Jurkat cells treated with Nocodazole. As expected, there is a lower percentage of cells in the G0/G1 phase and a higher percentage in the S phase when Jurkat cells were arrested with Aphidicolin compared to

Jurkat cells with no drug treatment. Jurkat cells exposed to Nocodazole showed a significant increase in cells in the G2/M phase and essentially no Jurkat cells in the G0/G1 phase.

The percentages of cells in the G0/G1, S and G2/M phases for all three treatment conditions of Jurkat cells (no drug, Aphidicolin and Nocodazole) were similar on the Guava PCA-96 system, the Guava PCA and the

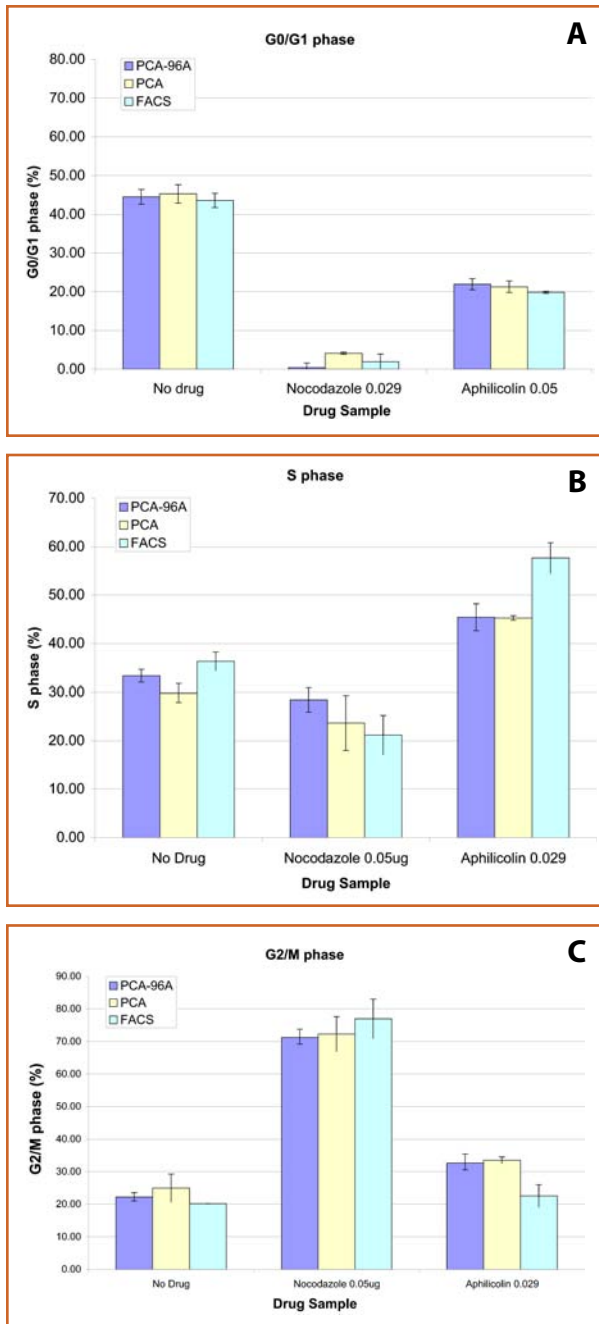


FIGURE 3. Comparison of Cell Cycle analysis of drug induced cells acquired on various instruments. This figure shows the percentages of G0/G1 phase (A), S phase (B) and G2/M phase (C) of Cell Cycle analysis of drug treated Jurkat cells on the Guava PCA-96 System (n=10 per condition), the Guava PCA (n=2) and the FACSCalibur (n=2).

FACSCalibur (Figure 3), indicating that the Guava PCA-96 yields accurate results for cells actively in cycle as well as in arrest. Results from the Guava PCA-96 system clearly showed the expected results. Cells treated with Aphidicolin had decreased numbers of cells in G1 and increased numbers in S. Cells treated with Nocodazole had substantially decreased numbers of cells in G1,

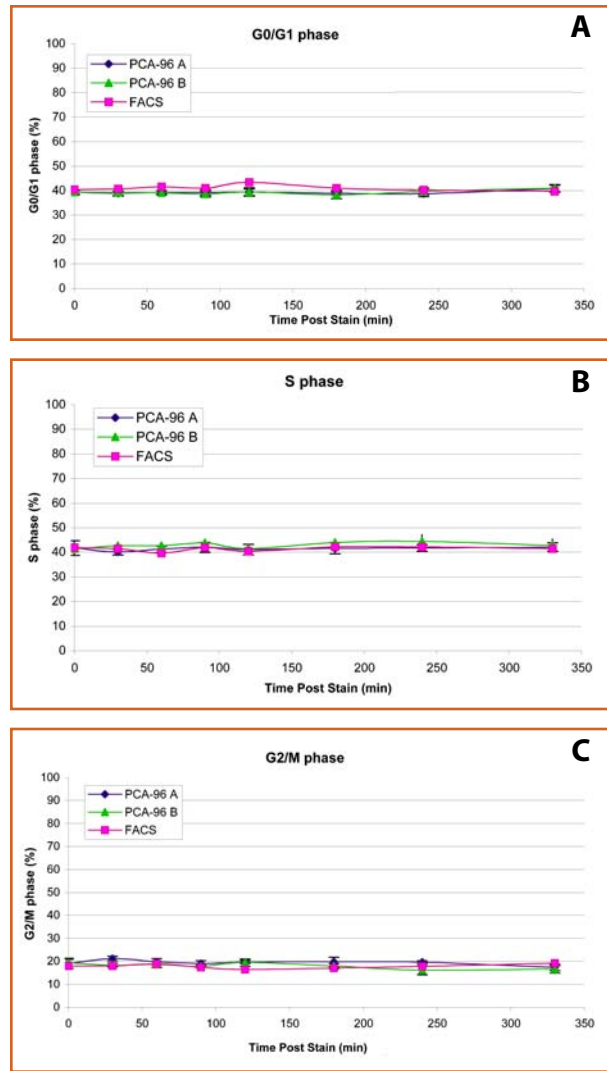


FIGURE 4. Post-stain stability of a non-adherent cell line. Jurkat suspension cells were cultured overnight in a flat bottom 96-well plate at a cell concentration of  $1.5 \times 10^5$  cells per well. The cell samples were transferred to a round bottom 96-well plate, fixed with 70% ethanol and stained the following day. The cells were read on two Guava PCA-96 instruments (n=6 per time point) and one FACSCalibur (FACS; n=2) at the following time points: immediately after 30 minute incubation time (t=0), then at 30, 60, 90, 120, 180, 240, and 330 minutes. The average percentages with standard deviations of G0/G1 phase (A), S phase (B) and G2/M phase (C) are shown here.

slightly decreased cell numbers in S and significantly increased cell numbers in G2/M. Note also that the replicate samples acquired on all instruments yielded very similar results as indicated by the small error bars.

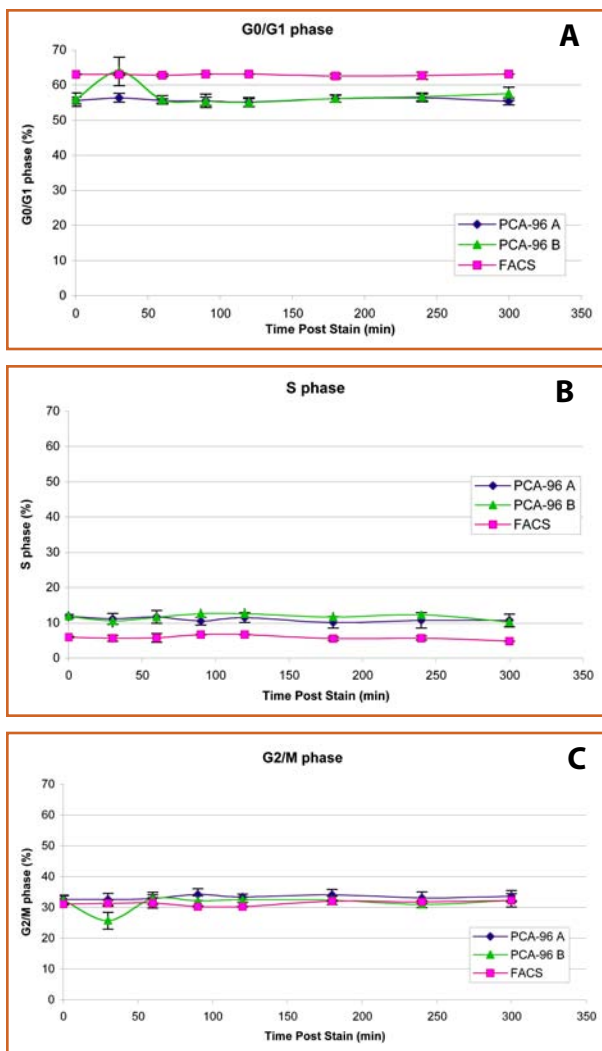


FIGURE 5. Post-stain stability of an adherent cell line. PC3 adherent cells were cultured for 2 days in a flask at a cell concentration of  $1 \times 10^5$  cells/mL. The cell samples were transferred to round bottom 96-well plates, fixed with 70% ethanol and stained the following day. The cells were acquired on two Guava PCA-96 instruments (n=6 per time point) and one FACSCalibur (FACS; n=2) at the following time points: immediately after a 30 minute incubation time (t=0), then at 30, 60, 90, 120, 180, 240, and 300 minutes. The average percentages with standard deviations of G0/G1 phase (A), S phase (B) and G2/M phase (C) are shown here.

**Post-stain stability on the 96-well plate**

The stability of cells after staining was assessed at various time points out to 5.5 hours. Stability of staining is crucial because to ensure the best results, a large number of events (~5000) should be acquired at the slowest flow rate and this may take up to 3.5 hours per plate.

**Non-adherent cell line.** Results show that post-staining of Jurkat suspension cell line is stable at least 5.5 hours (Figure 4). The percentages of G0/G1 phase, S phase and G2/M phase remain constant from time 0 to 5.5 hours.

**Adherent cell line.** The percentages of G0/G1, S and G2/M phases of the PC3 adherent cells remained consistent to at least 5 hours post stain (Figure 5). The percent of S phase for PC3 cells determined on the Guava PCA-96 was higher and the percent of the G0/G1 phase correspondingly lower compared to the FACSCalibur result. This may be explained by PC3 cells having a low percentage of S phase cells when grown under log phase conditions compared with Jurkat cells. The Guava PCA-96 has slightly broader G0/G1 peaks than the FACSCalibur and if the percentage of S phase is low ( $\leq 10\%$ ) then curve fitting algorithms will overestimate the S phase and concomitantly underestimate the G0/G1 phase for data acquired on the Guava PCA-96.

**Precision**

Jurkat suspension cells and PC3 adherent cells were cultured in flasks or in 96 well plates, fixed and stained in tubes or 96-well plates. The average percent positive, standard deviation and percent CV of the data were calculated for the G0/G1, M and G2/M phases. Table 1 shows the summary of the %CV for cultures with different distributions of G0/G1, S, and G2/M phases. As expected, the %CVs decreased as the % of cells in that phase increased. When 20% of cells were in a particular phase, the %CV was  $<16$ ; when 50% of cells were in a particular phase, the %CV was  $<5$ .

The precision of data acquired across an entire 96-well plate is also important. To assess this, PC3 cells were cultured in tubes and stained in a round bottom 96-well plate. The same sample was run on two different Guava PCA-96 Systems at the same time using

TABLE 1. Precision of cell cycle results acquired on a Guava PCA-96 System\*.

ID	% Positive	CV of % G0/G1 phase	CV of % S phase	CV of % G2/M phase
5	>50	<3	<5	<3
4	41–50	<5	<8	<5
3	21–40	<10	<10	<12
2	11–20	<10	<16	<15
1	<10	<45	<25	<30

\*data include sample sizes varying from n=6 to n=96

\*Guava Technologies cannot provide specific technical support on the use of the third party software mentioned in this paper.

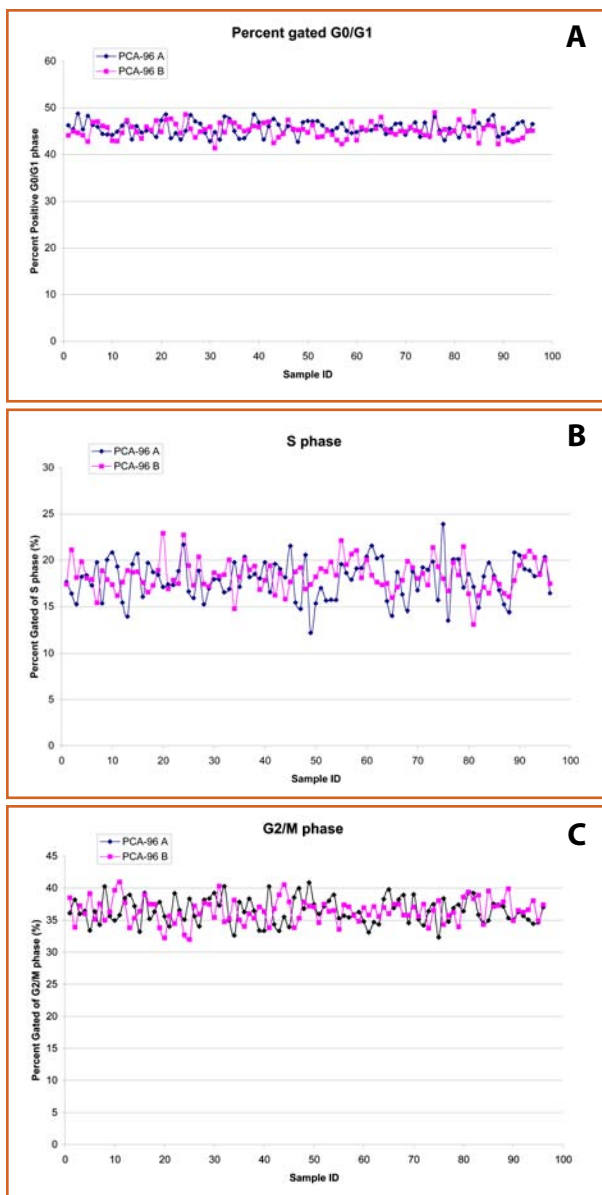


FIGURE 6. Precision of results across 96-well microplates. The percentages of the G0/G1 phase (A), S phase (B) and G2/M (C) phase over an entire 96-well plate of PC3 adherent cells acquired on two different Guava PCA-96 Systems are shown here. The average percentage of G0/G1 phase is 45%, S phase is 18% and G2/M phase is 37%. The percent CV of G0/G1, S and G2/M phase for all 96 samples for Guava PCA-96-A is 3.2%, 12.4%, and 5.4%, respectively. The percent CV of G0/G1, S and G2/M for all 96 samples for Guava-PCA-96-B is 3.5%, 9.1%, and 5.1%, respectively. The higher % CV for S phase on both Guava-PCA-96 is due to a low percentage of cells in the S phase.

a worklist containing automatic quick clean after every 12th sample. The instrument setting for flow rate was Very Low and the events collected was 2500 events. The entire 96-well plate took approximately 2 hours to finish. Note that even over this long acquisition time, the results obtained from the end of the plate were essentially identical to those at the beginning (Figure 6).

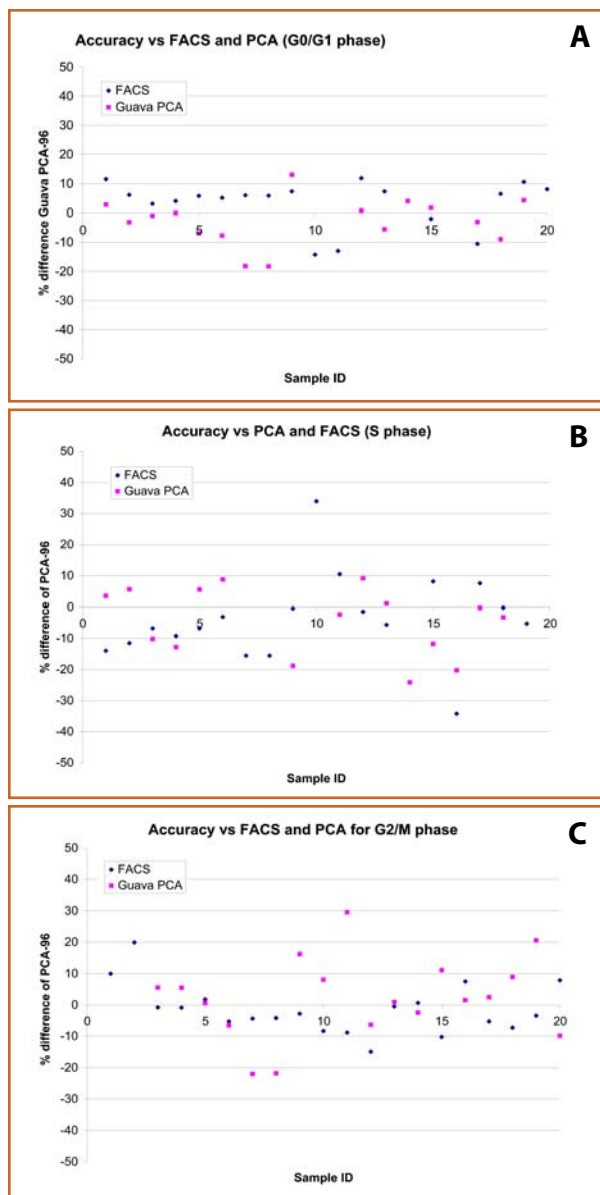


FIGURE 7. Comparing results obtained on the Guava PCA-96 System to those from the Guava PCA and the BD FACSCalibur. The comparison of results obtained on the Guava PCA-96 System for G0/G1 phase (A), S phase (B) and G2/M phase (C) to those from the Guava PCA and the BD FACSCalibur are shown here. The formula used to determine the percent difference between the Guava PCA-96 vs. Guava PCA or FACS is the percent positive of PCA-96 minus percent positive of predicate method (Guava PCA or FACS) divided by percent of predicate method.

### Accuracy of Guava PCA-96 vs. Guava PCA vs. FACS

Twenty different cell samples, including Jurkat suspension cells and PC3 adherent cells, were assayed according to the Cell Cycle staining protocol. Data on these cells were acquired on the Guava PCA-96 System, the Guava PCA and BD FACSCalibur (FACS) and compared, as shown in Figure 7; percent differences of G0/G1, S, and G2/M phases were also calculated.

The Guava PCA-96 shows no trend of overestimating or underestimating of G0/G1 phase compared to FACS or Guava PCA (Figure 7a). The average percent difference between the Guava PCA-96 and the FACS and Guava PCA for G0/G1 phase of the twenty samples is 3.3% and 2.9%, respectively. There is no trend of underestimating or overestimating of the percent of S phase on the Guava PCA-96 or FACS, regardless of cell type (Figure 7b). The S phase data generated on the PCA-96 are generally more variable when compared to the data from the FACSCalibur mostly because the S phase contains the fewest numbers of cells. While the highest percent difference was 33% because of the low percentage of PC3 cells in the S phase, the average differences were less than 5% compared to the FACS and Guava PCA. There is no trend overestimating or underestimating the percent of G2/M phase of the Guava PCA-96 compared to FACS or Guava PCA. The average difference of the twenty samples acquired on the Guava PCA-96 to the FACS and Guava PCA is -1.5% and 2.3% respectively.

### ANALYZING GUAVA CELL CYCLE DATA WITH THIRD PARTY SOFTWARE

CytoSoft can be used to monitor large shifts between the different phases of the cell cycle. To further analyze the data for more subtle changes between these populations, we recommend third party “curve fitting” software. These software programs apply sophisticated curve fitting algorithms to the Guava Cell Cycle data that more accurately calculate the percentages of the cell populations and their relevant statistics. This section describes the use of two such programs, ModFit LT and MultiCycle for analyzing Guava Cell Cycle data.

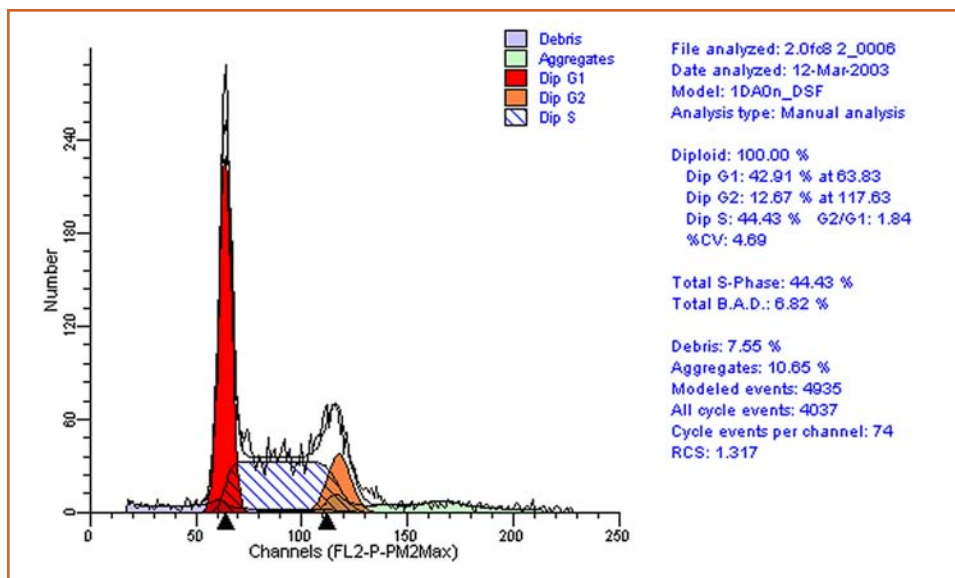
### Data Acquisition

Guava CytoSoft automatically exports all Cell Cycle data into FCS 2.0 format. This format can be read directly with either ModFit LT or MultiCycle.

### Data analysis using ModFit

1. Open the ModFit software application.
2. Log in as an advanced user.
3. Open a file by clicking the **Open File** box to the left of the shortcut bar or by using the pull-down menu under **File**.
4. Choose the cell cycle file you want to analyze.
5. Choose FL2-PM2Max from the Choose Parameter for Analysis dialog and click **OK**.
6. Under the Define Gate dialog click checkbox next to the Gate 1 On and click **Define Gate 1**.
7. Select P1: FS-P-FSCMax for the X Parameter and P5: FL2-P-PM2Max for the Y Parameter and click **OK**.
8. Position gate to include events of interest and click **OK**.
9. Click **OK** in Define Gate dialog.
10. Click Choose Model (Mod) button from the tool bar or using the pull-down menu under Analysis.
11. Set Properties for Manual Analysis. NOTE:Guava recommends Enabling the Auto Debris and AutoAggregates features, as well as setting the linearity to 1.8.
12. Click **OK** when complete.

FIGURE 8. ModFit LT analysis display.



13. The software will automatically look for the G1 & G2 peaks and display results. If the peak assignment requires manual adjustment, select the peak label or either side of the cursor and move the peak position until it is in the appropriate location.
14. Click the Fit Data (Fit) button on the tool bar or under the Analysis pull-down menu.
15. The software will automatically fit the curve and give you the %G1, %S and %G2 phase cells. (See Figure 8.)
16. If desired, the quality of the data fit can be determined by selecting Fit Diagnostics under the Tools menu.
17. To save or print the analyzed file, choose those options from the File menu.
18. Repeat steps 3 through 17 for remaining samples. NOTE: These data do not get exported to a CSV (comma separated values) spreadsheet file.
9. The software will automatically try to locate the G0/G1 and G2/M peaks. If the peak assignment requires manual adjustment, click either the G1 Mean or G2 Mean boxes and click the red line and move to the appropriate location.
10. If adjustments to the G2/G1 ratio need to be made, enter the appropriate value.
11. When peak positioning looks correct, click **OK**.
12. The software will automatically fit the curve and give you the %G1, %S and %G2 phase cells.
13. To exclude aggregates and debris, click on the **Agg** button on the Toolbar or under the Edit pull-down menu. (See Figure 9.)
14. To save or print the analyzed file, choose those options from the File menu.
15. Repeat steps 2 through 14 for remaining samples. NOTE: These data do not get exported to a CSV (comma separated values) spreadsheet file.

### Analysis using MultiCycle

1. Open the MultiCycle software application.
2. Open a file by clicking the **Open File** box to the left of the shortcut bar or by using the pull-down menu under File.
3. Choose the cell cycle file you want to analyze.
4. Under the 2P Gating, select **FS-P** for the XParameter and **FL2-P** for the Y Parameter. Click the check box next to Gate #1. When the check box is clicked, the gating dot plot will come up automatically.
5. Click **OK** when description dialog come up.
6. Gate around the major population by:
  - a. Using the left mouse button to start the polygon.
  - b. Point & click the left mouse button at next vertex.
  - c. Double click the left mouse button to end the polygon.

**NOTE:** DO NOT DRAG THE MOUSE.

7. Click the check box next to **FL2-P** under the To Mcycle column
8. Click **OK**.

### Troubleshooting Tips

1. Setting the FSC threshold too low may affect your results, because cell debris will be included and affect analysis values. 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 during data analysis to exclude unwanted debris 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 ( $<7 \times 10^4$  cells/mL), the Guava PCA-96 will not be able to acquire 5,000 events in the allotted time for sample collection (10 min). Centrifuge the sample at  $400 \times g$  for 7–10 minutes and remove a sufficient amount of the supernatant to increase the cell concentration to  $>7 \times 10^4$  cells/mL.
4. 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.
5. Run **Guava Check** (Catalog No. 4500-0020) to verify proper instrument function and accuracy.

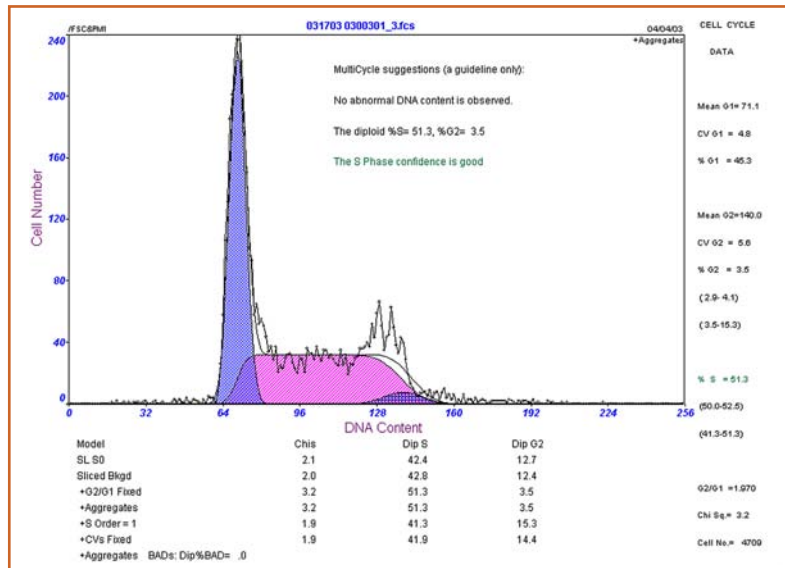


FIGURE 9. MultiCycle analysis display with aggregate and debris excluded.

- Periodically run **Quick Clean** using a deionized water tube (at least after every 12 to 24 sample acquisitions) to prevent a buildup of cell debris in the flow system. If your samples contain significant amounts of cellular debris, run Quick Clean with Guava ICF followed by water, to help prevent clogs or blockage.
- A clog or blockage of the flow system can be caused by cell aggregates, cell debris, free DNA strands, bleach crystals, or other particulates. 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 a blockage of the flow system. Click on "Pause" and then Click **Backflush** to flush out the clog follow by a Quick Clean using ICF fluid and then a final Quick clean using water. Load a tube of deionized water and run Quick Clean to remove bleach residue. If this procedure does not alleviate the problem, consult the *Guava PCA-96 System User's Guide* or contact Technical Service for additional help.
- If there are no events being acquired and there is no clog in the fluidics, then check the volume of sample in the well. If the sample in the well is less than 100  $\mu$ L, dilute the sample using the Cell Cycle reagent to at least 100  $\mu$ L or press "Next" to go to the next well.
- For more troubleshooting tips, refer to the *Guava PCA-96 System User's Guide*.

#### Trademarks and Patents

Microsoft is a registered trademark of Microsoft Corporation.  
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 ModFit LT is a trademark of Verity House Software.  
 MultiCycle is a product of Phoenix Flow Systems.



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