# Guava® PCA-96™ Cell Cycle

ASSAY PROTOCOL

Guava® technologies inc

## Table of Contents

Guava Technologies

# Guava® PCA-96™ Cell Cycle

Authors: Jestine Ho, Katherine Gillis, Dianne Fishwild

## 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/G1phases) 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 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. Data are displayed in a single parameter histogram. Up to four markers may be set to analyze the various populations. Statistics for each population within the histogram in CytoSoft include a percentage of total, plus the PM1 mean, median and %CV fluorescence intensity. If desired, a third party “curve-fitting” software package such as ModFit™ or MultiCycle can apply more sophisticated analysis model algorithms to identify the three phases of the cell cycle and to calculate the relevant statistics. However, CytoSoft also can be used to identify the populations and to estimate the number of cells within each phase. While CytoSoft does not employ any sophisticated “pulse-processing” technology to eliminate potentially contaminating G0/G1 phase cell doublets from within the apparent G2/M population, these particular aggregates can be excluded from the G2/M peak using curve fitting software such as ModFit or MultiCycle. The Guava Cell Cycle 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 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.

## Equipment and materials required

### Equipment and consumables

- Guava PCA-96™ with Cell Cycle Option
- Guava CytoSoft version 2.5 with the Guava Cell Cycle software module
- ModFit LT™, MultiCycle or comparable software (optional, for cell cycle analysis using various software

algorithms)

- Centrifuge
- Vortex mixer
- Refrigerator
- 37°C $CO_2$ incubator
- -20°C freezer
- Ice
- Tissue culture flasks
- Pipettes
- Pipettor
- Micropipette tips (200 and 20µL)
- Micropipettors, single and multi-channel (200 and 20µL)
- 50mL conical tubes
- 12x75mm polystyrene tubes
- 1.5mL microcentrifuge tubes with lids
- 96 well round bottom plate (BD Falcon #353075, #353915)
- 96 flat bottom plate (BD Falcon 353910 or 353918)
- V-shaped 25 ml and/or 55 mL trough (25ml, Apogent Discoveries #8093 and 55 mL VWR 210070-970)
- Sterile, serological pipets
- Stirrer bar
- Corning filtration unit, 0.45 micron 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, (1XPBS), pH 7.4
- Triton X-100 detergent (Sigma, X100)
- DNase-free RNase (Sigma, R6513)
- Propidium iodide at 1mg/ml in H2O (v/v) (Molecular Probes, P-3566)
- Distilled water
- Bleach
- Guava ICF™ Instrument Cleaning Fluid, Cat. No. 4200-0140
- Ice cold 70% Ethanol
- Guava PCA-96 ViaCount Flex reagent, Cat. No. 4700-0060 (optional)
- Guava Check Kit, Cat. No. 4500-0020

## 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 twelve 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/µL 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 0.2mL of cell staining solution (see below) to stain 1 x $10^5$ to 2 x $10^5$ 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**
   a. Add 9 mL of 1X PBS to a 15 mL Falcon tube.
   b. 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.
   c. Mix the tube by pipetting up and down several times or vortexing until you get a homogenous mixture.
   d. Store the 10% Triton X-100 at room temperature or 4°C.
2. **Preparing 10 mg/mL of DNAse-free RNase in water**
   a. Using an analytical balance, weight out 25 mg of DNAse-free RNAse.
   b. Add the RNase to a 15 mL conical tube.
   c. Add 2.5 mL of deionized water to the tube.
   d. Vortex the tube until the RNAse is completely dissolved.
3. **Making up 0.1% Triton X-100**
   a. Add 118.8 mL of 1X PBS to a 200 mL container.
   b. Add 1.2 mL of 10% Triton X-100 Working Stock to the container.
   c. Using a stirrer bar, mix the solution until it is homogenous.
   d. Filter the 0.1% Triton X-100 in 1X PBS using the Corning Filtration Unit.
4. **Preparing Cell Cycle Staining Reagent**
   a. Cover a sterilized 100 mL bottle with aluminum foil to shield the reagent away from light.
   b. Add 95.5 mL of 0.1% Triton X-100 in 1X PBS to the sterile 100 mL bottle.
   c. Add 2 mL of 10mg/mL stock DNAse-free RNAse to the bottle.
   d. Add 2.5 mL of 1 mg/mL Propidium Iodide Stock to the sterile bottle.
   e. 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

1. Avoid exposure of the Cell Cycle staining reagent to light.
2. 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 96-well microplate.**
   a. 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.
   b. Add 1 x $10^5$ to 2 x $10^5$ cells in a 200 µL volume (between 5 x $10^5$ cells/mL and 1 x $10^6$ 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 µL of inducing reagent to the appropriate wells.
   c. Culture cells, if necessary, as per. your own protocols.
   d. 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 adherent cells**

   a. Transfer the cells from the culture vessels to tubes.

   b. Determine the concentration of the cell sample using Guava PCA-96 ViaCount Flex reagent. If necessary, adjhust the cell sample to betewen 5 x $10^5$ to 1x$10^6$ cells/mL, and with a multi-channel micropipettor, add 200 mL cell sample to the well of a round bottom plate. Proceed to the protocol for Cell Fixation in a 96-well plate or refer to Cell Fixation in Tube to fix the cells.

   c. Rinse the flask once with 5-10 mL of 1X PBS. Add the PBS into the same conical tube above.

   d. Dilute the Guava® CDR 1:3 with 1X PBS.

   e. For a T-75 flask, add 3 mL of diluted Guava® CDR and incubate for 3-5 minutes in a 37°C incubator.

   f. Add 7 mL of media and pipet repeatedly to release cells from the flask bottom.

   g. Pipet the Guava® CDR and media into the same tube.

   h. Mix the cell sample by vortexing or pipetting repeatedly to ensure a homogenous suspension.

   i. Determine the concentration of the using Guava PCA-96 ViaCount Flex reagent. If necessary, adjust the cell sample to between 5 x $10^5$ and 1 x $10^6$ cells/mL, and with a multi-channel micropipettor, add 200 µL cell sample to the well of a Round Bottom plate. 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.

1. **Cell fixation in 96 well plate**

   a. 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.

   b. Centrifuge the cells at 450 x g for 5 minutes with the brake on low and at room temperature.

   c. Remove and discard the supernatant being careful not to touch the pellet.

   d. Add 200 µL of 1X PBS to each well using a multi-channel pipettor.

   e. Mix the cells in the well by pipetting up-and-down several times.

   f. Centrifuge the cells in the round bottom plate at 450 x g for 5 minutes with the brake on low and at room temperature.

   g. Remove and discard the supernatant.

   h. Place the round bottom plate containing the pellet cells with residual PBS on a lab shaker.

   i. Add 200 µL of 70% ice cold ethanol drop-wise into the wells while shaking at low speed (speed 3).

   j. 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.

   i. 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 x $10^6$ 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 µL 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 concnentration 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.

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

## Cell staining protocol (in 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 µL 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 µL 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 using CytoSoft

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

## 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.
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 1**.)
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.

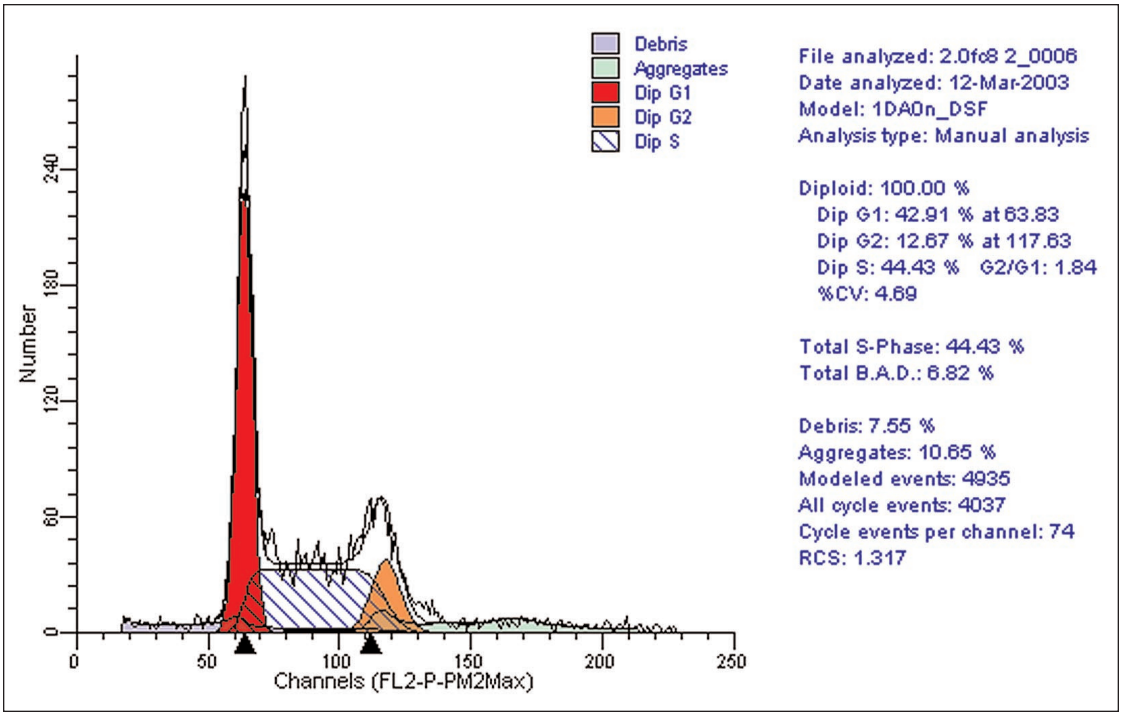



**FIGURE 1:** ModFit LT™ analysis display.

18. Repeat steps 3-17 for remaining samples.

**NOTE:** This data does 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 X-Parameter 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.

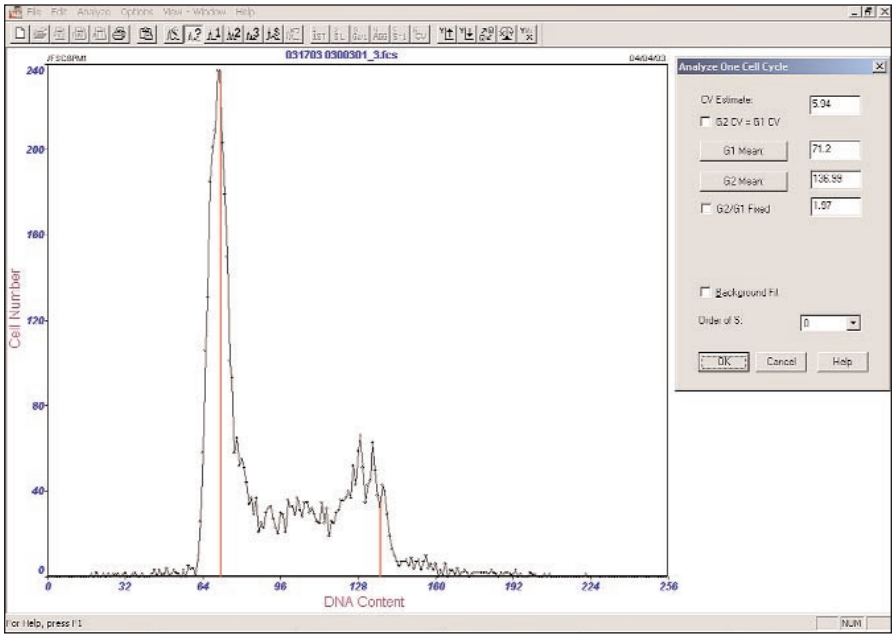



**FIGURE 2:** Peak positioning in MultiCycle.

c. Double Click the Left Mouse Button to end the polygon.

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

7. Click the checkbox next to **FL2-P** under the To Mcycle column
8. Click **OK**.
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**. (See **Figure 2**.)
12. The software will automatically fit the curve and give you the %G1, %S and %G2 phase cells. (See **Figure 3**.)
13. To Exclude Aggregates and Debris click on the **Agg** Button on the Toolbar or under the Edit pull down menu. (See **Figure 4**.)
14. To save or print the analyzed file, choose those options from the File menu.

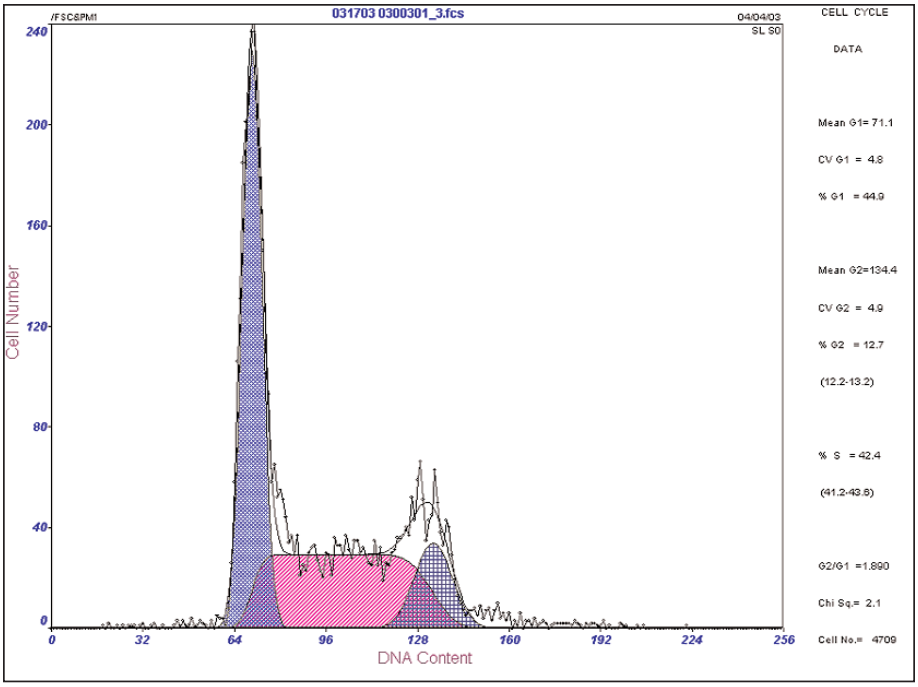



**FIGURE 3:** MultiCycle analysis display.

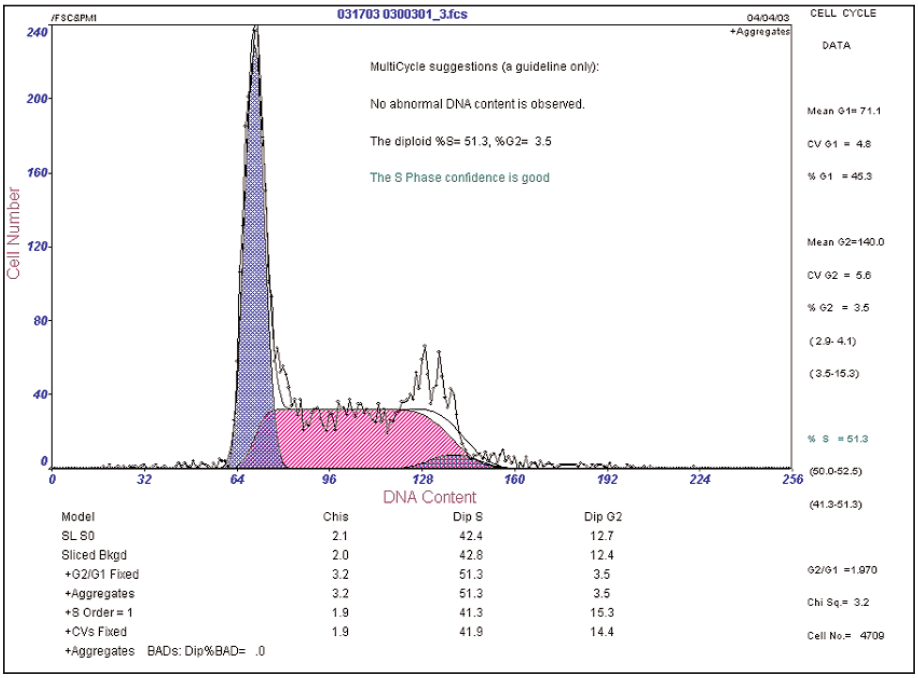



**FIGURE 4:** MultiCycle analysis display with aggregate and debris excluded.

15. Repeat steps 2-14 for remaining samples.

**NOTE:** This data does not get exported to a CSV (comma separated values) spreadsheet file.

## Expected results

The results of your cell 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 the instrument and analysis settings are stored in the CSV file. If desired, a third party "curve-fitting" software package such as ModFit LT or MultiCycle can apply more sophisticated analysis model algorithms to identify the three phases of the cell cycle and to calculate the relevant statistics. However, CytoSoft can be used to identify the populations and to estimate the number of cells within each phase. CytoSoft also does not employ any sophisticated "pulse-processing" technology to eliminate potentially contaminating G0/G1 phase cell doublets from within the apparent G2/M population, which may be a minor source of error. The Guava Cell Cycle data for all samples within a data set are saved to an FCS 3.0 file, and optionally to FCS 2.0 files.

### Screen result

Figure 5a shows a typical CytoSoft histogram and dot plot from a typical PC3 cell 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 can be analyzed immediately using ModFit LT. Modfit LT employs more sophisticated analysis model algorithms to identify the three phase of cell cycle. Figure 5b shows a typical result from Modfit. Unlike CytoSoft, ModFit LT uses mathematical curve fitting algorithms, so that the percentages for the three phases of the cell cycle provided by ModFit are more accurate and smalle ModFit uses mathematical curve fitting algorithms, unlike CytoSoft, the percentages for the 3 phases of the cell cycle are more accurate when using ModFit and smaller changes in the percentages of cells in the different phases of the cell cycle can be better determined. All data shown here were processed and plotted using ModFit LT.

### Drug induction

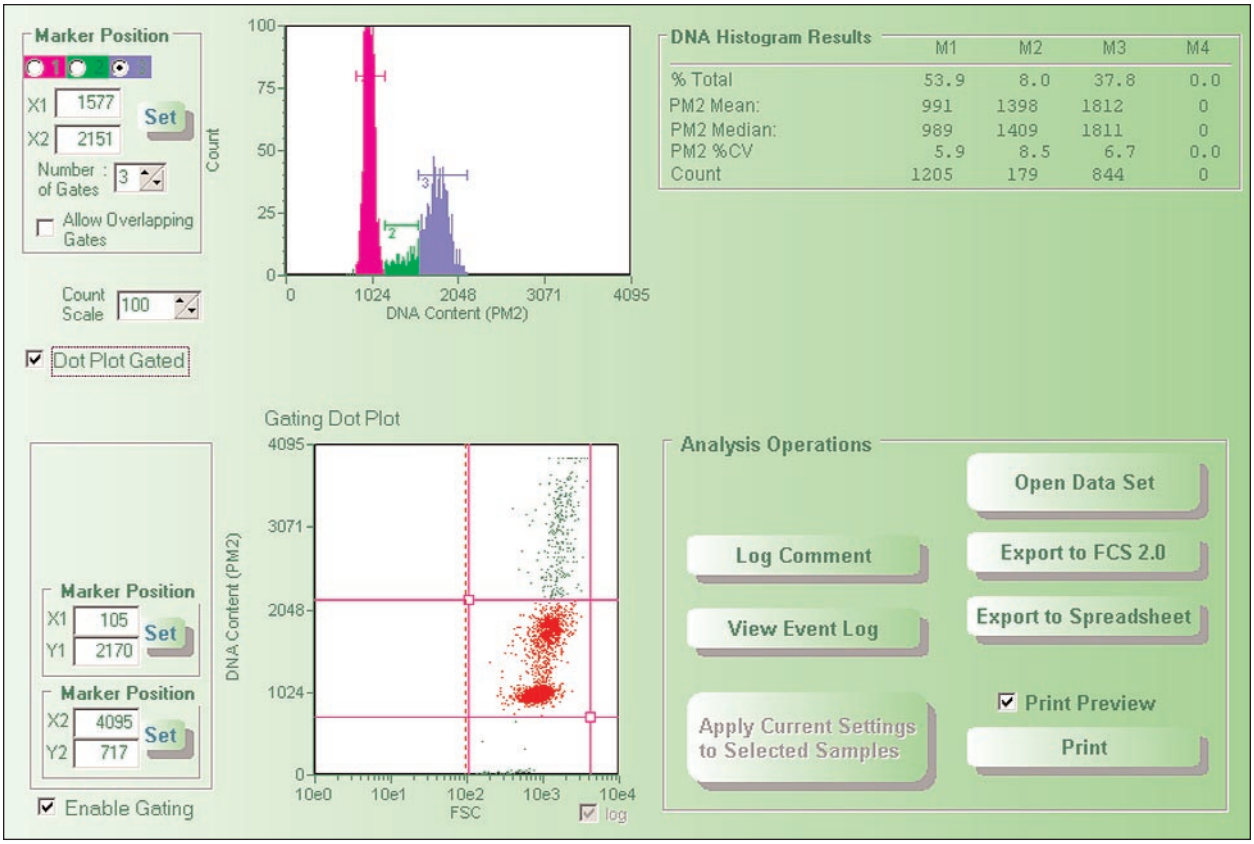
Jurkat cells were serum starved for 24 hours and treated with 0.05 µg/mL of Aphidicolin (S phase arrest) and 0.029 µg/mL of Nocadozole (G2/M phase arrest) for 24 hours in a flat bottom 96 well microplate. A control sample, with no drug, was prepared at the same time. After drug induction, cells were transferred to a round bottom 96 well microplate, washed and fixed with 70% ethanol. The cells were kept in 4°C overnight and stained according to the Cell Staining (in a 96 well format) protocol above.

Figure 6a shows a ModFit plot of Jurkat cells with no drug induction while 6b shows Jurkat cells treated with Aphidicolin and 6c shows Jurkat cells treated with Nocadozole. 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 Nocadozole 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 Nocadozole) were similar on the Guava PCA-96 system, the Guava PCA and the FACSCalibur (Figure 7), 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 show the expected results. Cells treated with Aphidicolin had decreased numbers of cells in G1 and increased numbers in S. Cells treated with Nocadozole had substantially decreased numbers of cells in G1, 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.

### 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



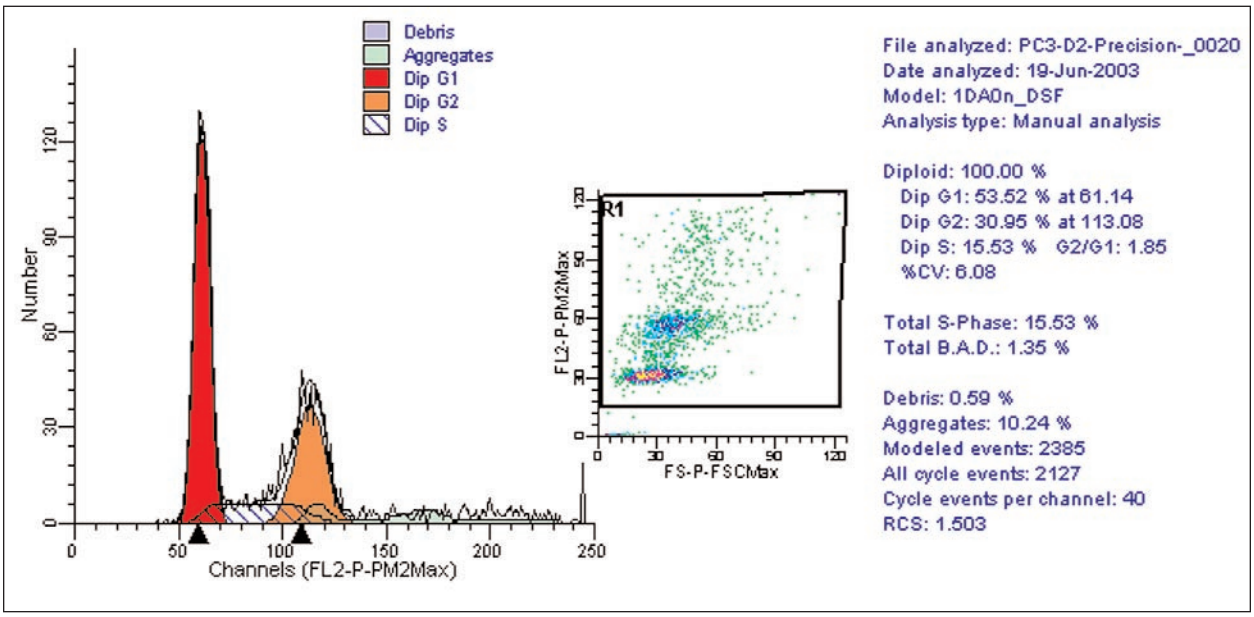




**FIGURE 5.** (A) An example of results obtained using the Cell Cycle protocol for PC3 cells cultured in a flask. PC3 cells were split 1:3 and cultured in a T-75 flask for 2 days. Guava® CDR was used to remove the cells from the flask. Cells were fixed with 70% ethanol in the tube and then stained in 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.

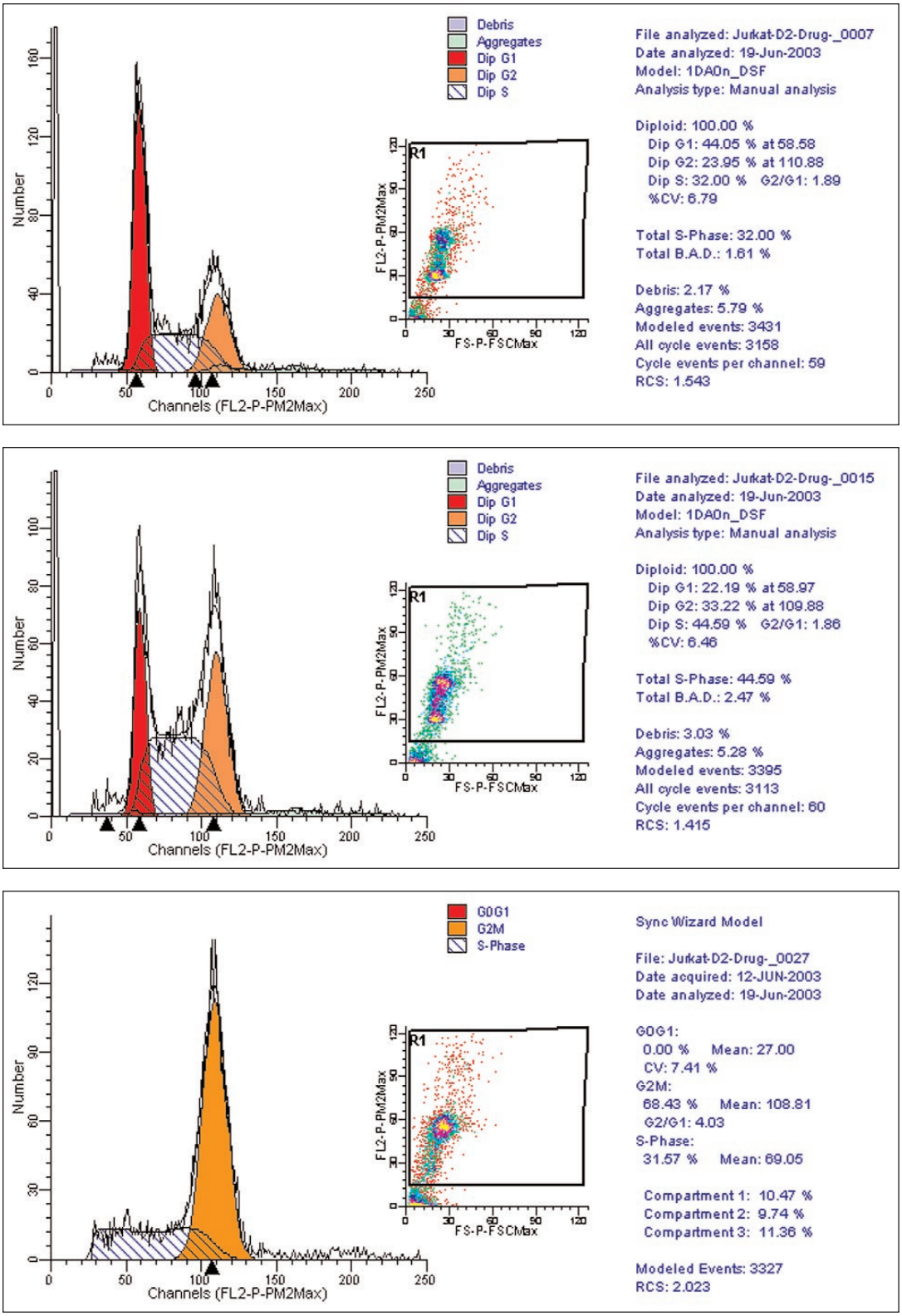



**FIGURE 6:** Jurkat cells were cultured with Aphidicolin to arrest cells in S phase or with Nocadozole to arrest cells in G2/M phase. These treated cells plus cells not exposed to drug were then fixed, stained and acquired on the Guava PCA-96 system. The perrcentage of cells in each phase of the cell cycle was determined using ModFit for each replicate (n=10). Representative data from each of the treatment groups ((A) no drug, (B) Aphidicolin, (C) Nocadozole) is shown here.

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.

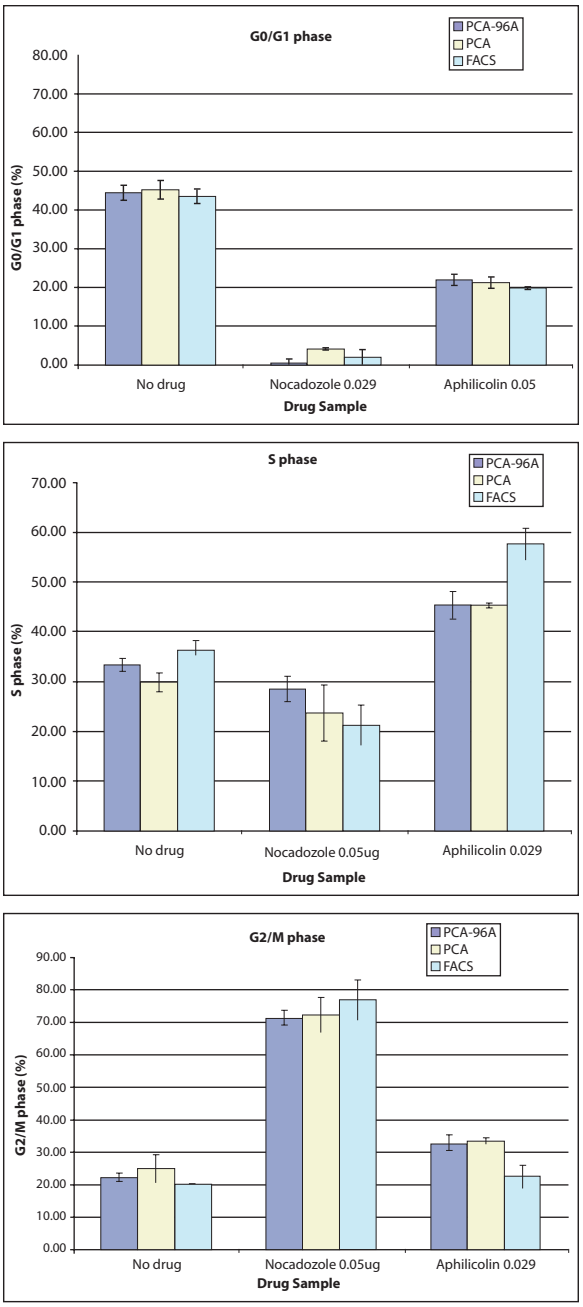



**FIGURE 7:** Comparison of Cell Cycle analysis of drug induced cells acquired on various instruments. This figure shows the percentages of G0/G1 phase (A), S phase (B) and G2/M phase (C) of Cell Cycle analysis of drug treated Jurkat cells on the Guava PCA-96 System (n=10 per condition), the Guava PCA (n=2) and the FACSCalibur (n=2).

## Non-adherent cell line

Results show that post staining of Jurkat suspension cell line is stable at least 5.5 hours (Figure 8). The percentages of G0/G1 phase, S phase and G2/M phase remain constant from time 0 to 5.5 hours.

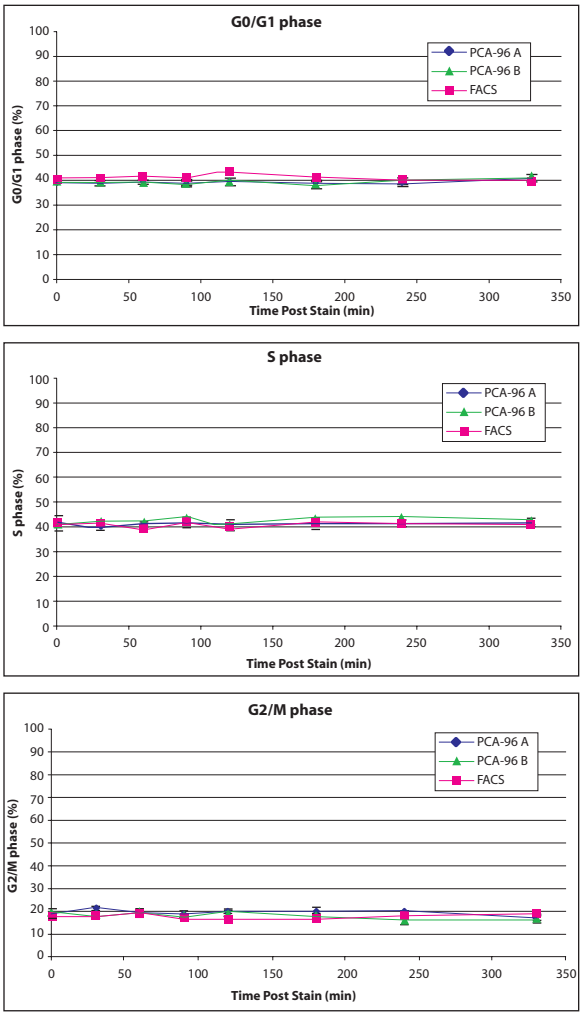



**FIGURE 8:** Post stain stability of a non-adherent cell line. Jurkat suspension cells were cultured overnight in a flat bottom 96 well plate at a cell concentration of 1.5 x $10^5$ cells per well. The cell samples were transferred to a round bottom 96 well plate, fixed with 70% ethanol and stained 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), 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.

## Adherent cell line

The percentages of G0/G1, S and G2/M phases of PC3 adherent cells remained consistent to at least 5 hours post stain (Figure 9). The percent of S phase for PC3 cells determined on the Guava PCA-96 was higher and the percent of G0/G1 phase correspondingly lower compared to the FACSCalibur result. This may be explained by PC3 having a low percentage of S phase cells when grown under log phase conditions than Jurkat cells.

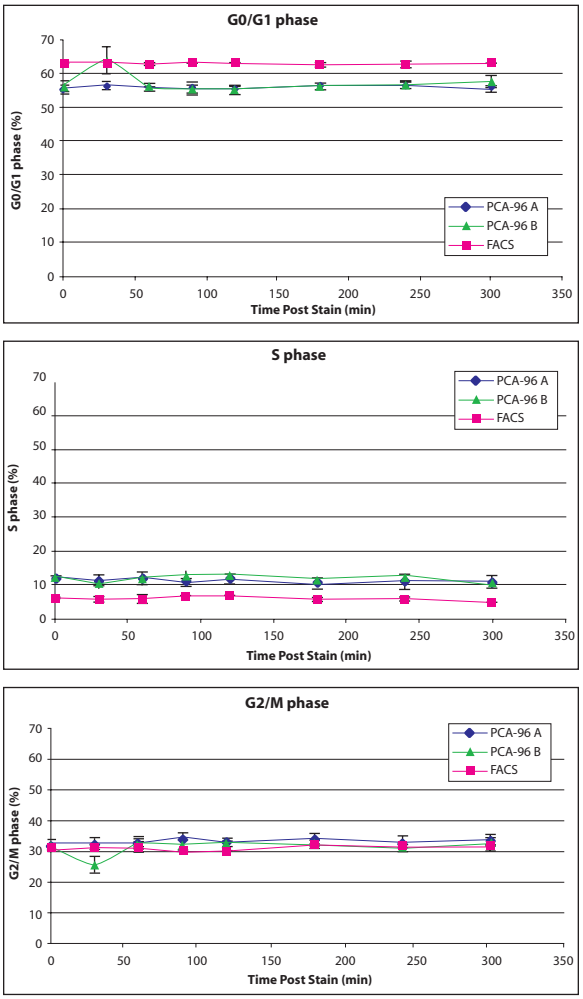



**FIGURE 9:** Post-stain stability of an adherent cell line. PC3 adherent cells were cultured for 2 days in a flask at a cell concentration of 1 x $10^6$ cells/mL. The cell samples were transferred to round bottom 96 well plates, fixed with 70% ethanol and stained the following day. The cells were acquired 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), 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.

## 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 data %CV for cultures with different distributions of G0/G1, S and G2/M phases.

**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

As expected, the data %CVs decreased as the % of cells in that phase increased. When 20% of cells were in a particular phase, the %CV was <10; when 50% of cells were in a particular phase, the %CV was <5.

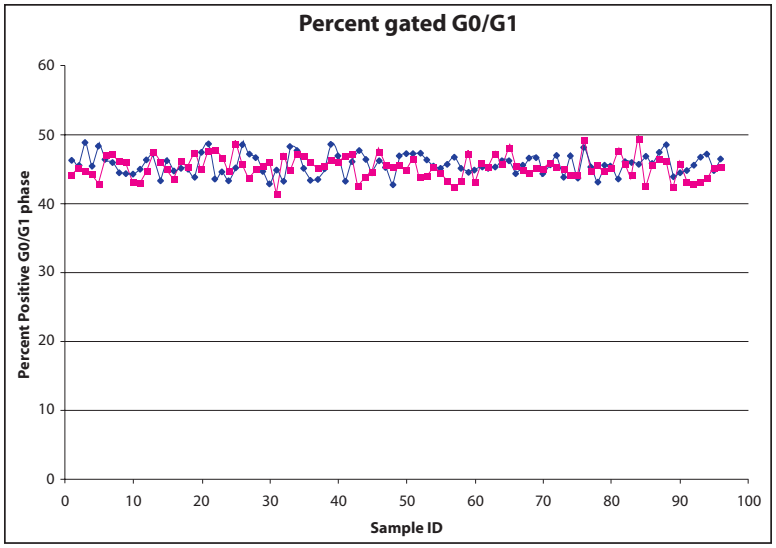
The precision of data acquired across an entire 96-well plate is also important. To assess this, PC3 cells were cultured in tubes and stained in a round bottom 96 well plate are shown below. The same sample was run on two different Guava PCA-96 Systems at the same time using a worklist containing automatic 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 10).

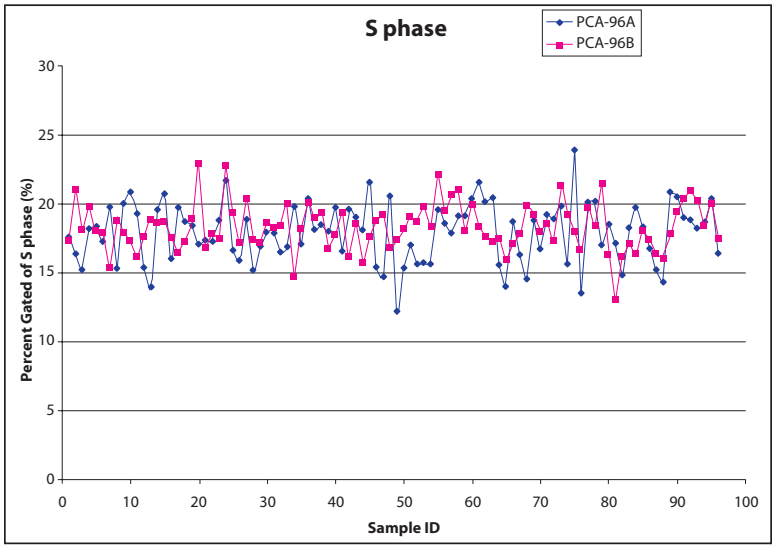
## Accuracy of Guava PCA-96 vs Guava PCA vs FACS

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 aquired on the Guava PCA-96 Sys-

tem, the Guava PCA and BD FACSCalibur (FACS) and compared, as shown in Figure 11. The percent differences of G0/G1, S and G2/M phases were calculated and are presented below.

The Guava PCA-96 shows no trend of overestimating or underestimating of G0/G1 phase compared to FACS or Guava PCA (Figure 11a). 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 (Figure11b). 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 to the FACS and Guava PCA is -1.5% and 2.3% respectively.



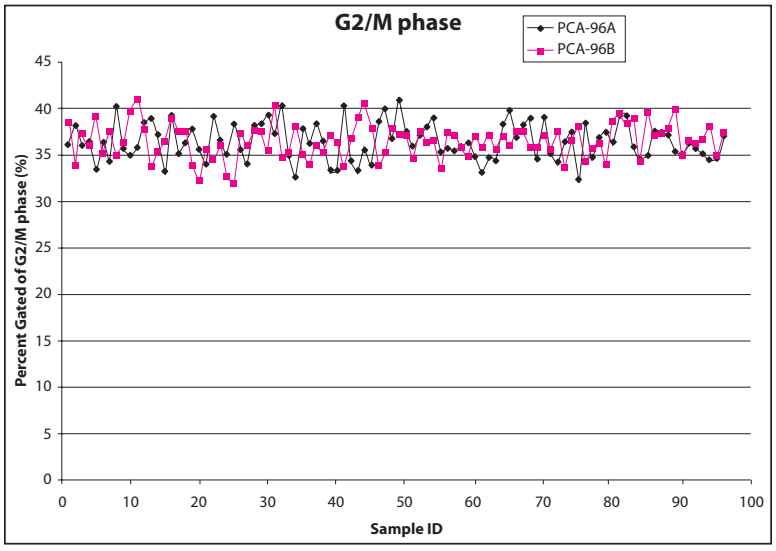
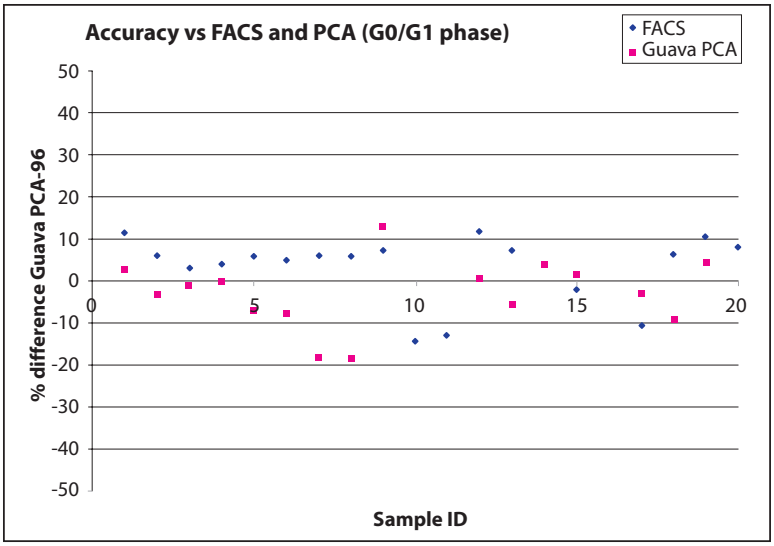
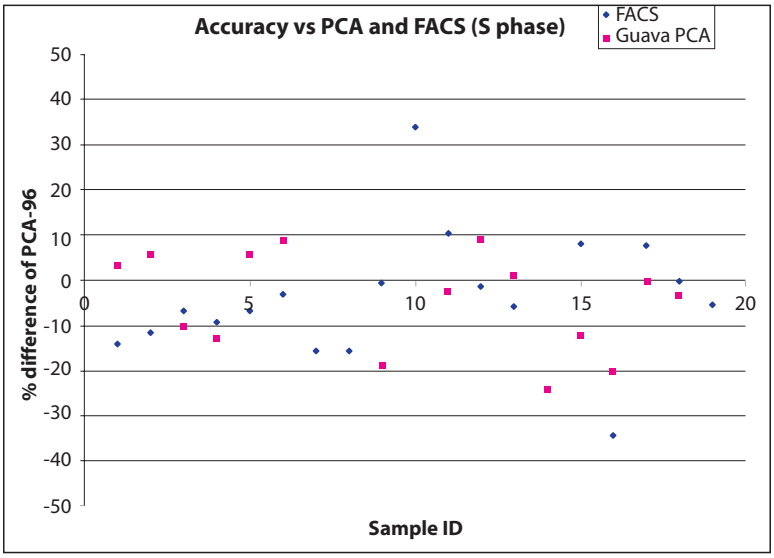




**FIGURE 10:** Precision of results across 96-well microplates. The percentages of the G0/G1 phase (10a), S phase (10b) and G2/M (10c) phase over an entire 96 well plate of PC3 adherent cells acquired on two different Guava PCA-96 Systems are shown here. The average percentage of G0/G1 phase is 45%, 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 high data % CV for S phase on both Guava-PCA 96 is due to a low percentage of cells in the S phase.



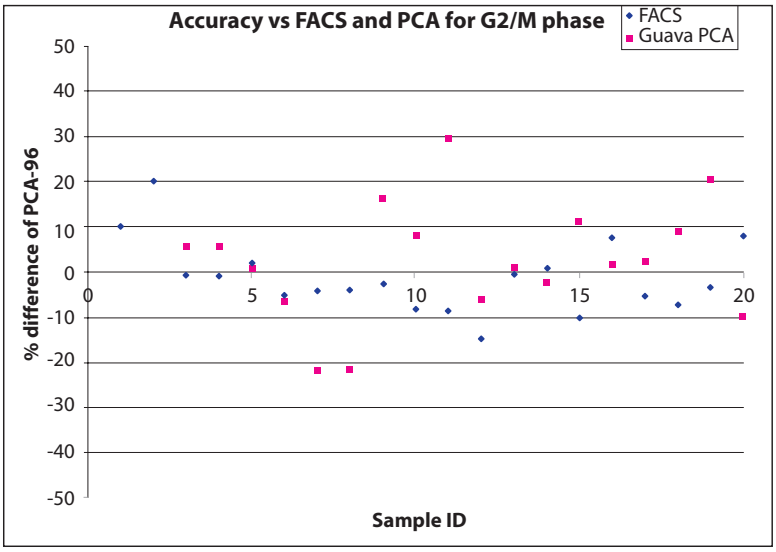




**FIGURE 11:** Comparing results obtained on the Guava PCA-96 System to those from the Guava PCA and the BD FACSCalibur. The comparison of results obtained on the Guava PCA-96 System for G0/G1 phase (A), S phase (B) and G2/M phase (C) to those from the Guava PCA and the BD FACSCalibur are shown here. 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.

## 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 x $10^4$ 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 400xg 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. 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.
6. 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.
7. 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.
8. 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 uL, dilute the sample using the Cell Cycle reagent to at least 100 µL or press "Next" to go to the next well.
9. 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.

Guava and ViaCount are registered trademarks of Guava Technologies, Inc.

PCA, Guava Cell Cycle, Guava Check, CytoSoft, and CytoAnalysis are trademarks of Guava Technologies, Inc.

ModFIT LT is a trademark of Verity Software House, Inc. FACSCalibur is a trademark of Becton, Dickinson and Company..


Part No. NEEDS PART NUMBER Revised 23-Sept-2003

**Guava Technologies, Inc.**
25801 Industrial Blvd., Hayward, CA 94545
**TOLL FREE IN US** (866) 448-2827
**PHONE** (510) 576-1400
**FAX** (510) 576-1500
**EMAIL** info@guavatechnologies.com
**WEBSITE** www.guavatechnologies.com