

# Assessing apoptosis in cell cultures quickly and easily using the Guava<sup>®</sup> ViaCount<sup>®</sup> assay

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## TECHNICAL NOTE

**ABSTRACT** When using the Guava ViaCount reagent and the Guava PCA<sup>™</sup> instrument to count cells, cell viability is detected by differential permeation of a DNA intercalating dye. Live cells with intact membranes exclude this stain, while dead and dying cells with compromised membrane integrity allow the dye to penetrate and stain cellular DNA. We have shown that cells which take up intermediate amounts of the viability dye in ViaCount also react with Annexin V, indicating they have entered the apoptotic pathway. The apoptotic cells are distinguishable from the healthy live cells and dead cells, and can be readily quantitated using CytoSoft<sup>™</sup> version 2.0. Conventional methods for counting cells using trypan blue exclusion do not distinguish apoptotic cells from live cells. Thus, a more realistic assessment of cell culture health can be made quickly and easily, while obtaining cell count and viability data using ViaCount.



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# Assessing apoptosis in cell cultures quickly and easily using the Guava<sup>®</sup> ViaCount<sup>®</sup> assay

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## Abstract

When using the Guava ViaCount reagent and the Guava PCA<sup>™</sup> instrument to count cells, cell viability is detected by differential permeation of a DNA intercalating dye. Live cells with intact membranes exclude this stain, while dead and dying cells with compromised membrane integrity allow the dye to penetrate and stain cellular DNA. We have shown that cells which take up intermediate amounts of the viability dye in ViaCount also react with Annexin V, indicating they have entered the apoptotic pathway. The apoptotic cells are distinguishable from the healthy live cells and dead cells, and can be readily quantitated using CytoSoft<sup>™</sup> version 2.0. Conventional methods for counting cells using trypan blue exclusion do not distinguish apoptotic cells from live cells. Thus, a more realistic assessment of cell culture health can be made quickly and easily, while obtaining cell count and viability data using ViaCount.

## Introduction

The Guava ViaCount assay provides an absolute cell count and viability data on cell suspensions from a variety of cultured cell lines. The cells are drawn into a capillary flow cell of known dimensions at a precisely controlled rate for measured periods of time. Absolute cell counts are obtained on the Guava instrument systems by knowing the exact sampling volumes. Viable and non-viable cells are assessed by the differential permeability of DNA-binding dyes in the ViaCount reagent. One dye is membrane permeable and stains all nucleated cells; fluorescent signal from the nuclear staining dye is detected by photomultiplier tube 2 (PM2). The other dye will only penetrate cells with compromised membrane integrity (i.e. non-viable cells) and is detected by photomultiplier tube 1 (PM1). Events are counted if they emit a

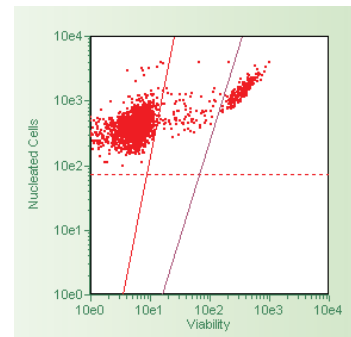
	Not Counted	Viable Cell	Non-viable Cell
Nuclear Stain (PM2)	—	+	+
Forward Light Scatter	LOW	HIGH	HIGH
Viability Stain (PM1)	—	—	+

**FIGURE 1:** Diagram and table showing how ViaCount classifies and scores events for cell count and viability assessment.

nucleated cell fluorescent signal and the forward light scatter (FSC) intensity is appropriate for that of a particle the size of a cell. Debris events with low FSC signal are not counted. Non-viable cells will emit fluorescent signal from the dead cell stain.<sup>1</sup> (See Figure 1).

In practice, three populations of cells can be distinguished by differential staining from the ViaCount dead cell stain: PM1 (-), PM1 (dim) and PM1 (+) (See Figure 2). The PM1 (-) cells would be live cells and the PM1 (+) cells would be dead cells. We thought the PM1 (dim) population might be apoptotic. One of the physiological changes seen in apoptotic cells is

**FIGURE 2:** ViaCount dot plot from a Jurkat cell sample induced to undergo apoptosis by treatment with 1 mM staurosporine for 3 hours at 37°C. Non apoptotic, live cells are in the population to the left of the left (red) marker on the dot plot [Viability stain (-), Nucleated Cell stain (+)]. The dead cells are the population to the right of the right (purple) marker [Viability stain (+), Nucleated Cell stain (+)]. Cells between the two markers are Viability stain (dim), Nucleated Cell stain (+).



loss of cell membrane integrity.<sup>2-6</sup> Degenerating membrane integrity would allow the dead cell dye to begin penetrating and staining these cells.

To show that the PM1 (dim) population was apoptotic, cells were induced to undergo apoptosis by starvation, then assayed for phosphatidylserine externalization and ViaCount staining. Translocation of phosphatidylserine from the inner side of the cell membrane to the cell surface is an early marker of apoptosis, detectable by the binding of Annexin V.<sup>7-12</sup> Annexin V binds phosphatidylserine with high affinity and specificity in the presence of calcium.<sup>13-15</sup> Dim fluorescence from the viability dye correlated with Annexin V co-staining would indicate that the population was apoptotic.

## Materials and Methods

### Cell lines and culture conditions

A Chinese hamster ovary (CHO-K1) cell line (ATCC, Catalog No. CCL-61), adapted for growth in suspension culture, was cultured in serum-free medium (Invitrogen, Catalog No. 10743). To obtain cell samples with lowered viability and to induce apoptosis, CHO-K1 cells were overgrown and cultured in a humidified 5% CO<sub>2</sub> incubator at 37°C without changing the medium for 5 to 7 days.

The human T lymphocyte cell line, Jurkat (ATCC Catalog No. TIB-152) was obtained from ATCC and cultured using the recommended medium formulation. To lower viability and induce apoptosis in cell samples, Jurkat cells were serum-starved by culturing in calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS), pH 7.2 (JRH Biosciences, Catalog No. 59321) containing 1.0% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, Catalog No. A9418) at room temperature. For some experiments, Jurkat cells were treated to undergo apoptosis by culturing in medium with 1 mM staurosporine (Calbiochem, Catalog No. 569397) for 3 hours at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### Sample preparation

**CHO-K1 cell samples.** Two CHO-K1 cell cultures were obtained: one cultured normally (typically

>85% viability) and one at lower viability (starved, <80% viable). Cell count and viability were determined by running samples on the Guava PCA using the ViaCount assay. Cell samples were adjusted to a concentration of approximately 2 to 5 x 10<sup>6</sup> cells/mL. Experimental cell samples were prepared in duplicate.

**Disaggregation of CHO-K1 cells.** The CHO-K1 cell line, adapted for growth in suspension culture, had a tendency to form cell aggregates. For better cell staining and improved counting accuracy, the CHO-K1 cells were treated with Guava ViaCount CDR™ cell dispersal reagent<sup>16</sup> (Catalog No. 4700-0050). For each sample, 50 µL of cell suspension (1 to 2.5 x 10<sup>5</sup> cells) was mixed with 50 µL of diluted 0.8X CDR and 150 µL of PBS in a 1.5 mL microcentrifuge tube. The samples were incubated on a tube rotator for 20 minutes at 37°C. At the end of the incubation period, 250 µL of PBS was added to each sample and the cells were centrifuged at 300 to 400 x g for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 500 µL of 1X Annexin V Binding Buffer (BioVision Research Products, Catalog No. 1035-100) in preparation for staining. Unstained controls were resuspended in 510 µL of 1X Annexin V Binding Buffer and held at this stage.

**Three color staining.** Dispersed CHO-K1 cells were stained with Annexin V-FITC, and ViaCount dyes. To 500 µL of cells in 1X Annexin V Binding Buffer, 5 µL of Annexin V-FITC (BioVision, Catalog No. 1001-1000), 5 µL of Guava ViaCount Flex reagent (Catalog No. 4500-0110; Part No. 4000-0130) were added. The samples were mixed well by vortexing, and then incubated for 5 minutes at room temperature. Samples were ready for data acquisition.

Appropriate positive controls (e.g. single parameter stained, ViaCount only) were prepared as described above, substituting 1X Annexin V Binding Buffer for the omitted staining reagent.

**Jurkat cell samples.** Two Jurkat cell cultures were obtained: one cultured normally (typically >95% viability) and one at lower viability (serum starved, <70% viable). Cell count and viability were determined by running samples on the Guava PCA system using the ViaCount assay. Cell samples were adjusted to a concentration of approximately 2 to 5 x 10<sup>6</sup> cells/mL.

Experimental cell samples were prepared in duplicate. For each sample, 50  $\mu\text{L}$  of cell suspension (1 to  $2.5 \times 10^5$  cells) was dispensed into a 1.5 mL microcentrifuge tube. PBS (250  $\mu\text{L}$ ) was added to each sample and the cells were centrifuged at 300 to 400  $\times g$  for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 500  $\mu\text{L}$  of 1X Annexin V Binding Buffer in preparation for staining. Unstained controls were resuspended in 510  $\mu\text{L}$  of 1X Annexin V Binding Buffer and held at this stage.

**Cell staining.** Jurkat cells were stained with Annexin V-FITC, and ViaCount Flex reagent as described for CHO-K1 cells above. Appropriate positive controls (e.g. single parameter stained, ViaCount only) were prepared as described above, substituting 1X Annexin V Binding Buffer for the omitted staining reagent.

Jurkat cells, induced to undergo apoptosis by staurosporine treatment, were stained with Guava ViaCount reagent (Catalog No. 4000-0040) by mixing 20  $\mu\text{L}$  of cell suspension (at about  $1 \times 10^6$  cells/mL) with 380  $\mu\text{L}$  of ViaCount reagent. The sample was incubated for at least 5 minutes at room temperature, protected from light, before data acquisition.

## Data Acquisition

**Guava PCA.** Proper instrument performance was verified by running the Guava Check™ application with the Guava Check Kit reagents (Catalog No. 4500-0020). Data from cell samples stained with ViaCount reagent were acquired on a Guava PCA system using CytoSoft software. Typically, 1000 events were acquired per sample, in duplicate. The ViaCount and Guava Nexin™ software applications were used to acquire and analyze the data. The viability dye fluorescence was detected in PM1 and the nucleated cell stain was detected in PM2. Cell debris (events with FSC intensity  $<10^2$ ) was gated out as required.

**Flow cytometry.** Data from triple-stained (Annexin V-FITC, ViaCount dyes) and control samples were acquired using a FACSCalibur™ flow cytometer (Becton Dickinson) using CellQuest™ software for acquisition and analysis. Negative and positive controls were used to adjust instrument settings. Three thousand (3000) events were acquired per sample.

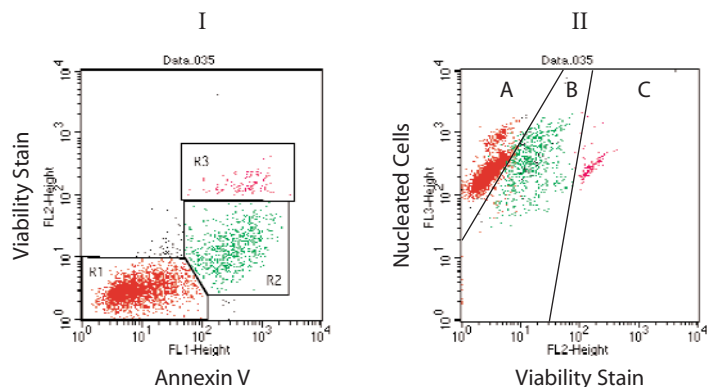
The Annexin V-FITC fluorescence was detected in FL-1, the ViaCount viability dye fluorescence was detected in FL-2, and the ViaCount nucleated cell stain was detected in FL-3. Cell debris was gated out as required, using forward and side scatter parameters to characterize particle size and morphology.

## Results and Discussion

We tested CHO-K1 and Jurkat cultures induced to undergo apoptosis by poor culture conditions. The CHO-K1 cells were overgrown and starved for several days by not replenishing the culture medium. Jurkat cells were transferred to PBS containing 1% BSA and grown at room temperature. Jurkat cells grew in a homogeneous single cell suspension and could be stained directly. The CHO-K1 culture was adapted to suspension culture, but the cells had a tendency to grow in clumps. Aggregation would hinder efficient surface staining with the Annexin V-FITC and affect counting accuracy. A clump of cells could be scored as a single event, instead of multiple cells, leading to undercounting. Also, it was likely that the % Viability could be skewed toward lower viability. A clump of cells would tend to be scored as a dead cell event, because the presence of even one PM1 (+) cell in the aggregate would identify that event as dead. To improve staining efficiency and counting accuracy, we treated the aggregated CHO-K1 cells with an enzymatic reagent, ViaCount CDR. ViaCount CDR contains a mixture of enzymatic activities (collagenase, protease, nuclease, etc.) to gently disaggregate cell clumps.

Disaggregated CHO-K1 cells and Jurkat cells were stained with Annexin V-FITC and ViaCount dyes for viability and nucleated cells. Three-color stained sample data was acquired on the FACSCalibur flow cytometer using CellQuest software. Cell debris was excluded by setting a threshold to exclude events with low forward scatter.

The results confirmed that differential staining with the ViaCount reagent can identify apoptotic cells. Nucleated cells showing dim fluorescence from the viability dye correlated with Annexin V-FITC co-staining. Similar results were seen in experiments with triple-stained Jurkat and CHO-K1 cells. An example of a CHO-K1 sample is shown in Figure 3.



**FIGURE 3:** Data analysis of CHO-K1 sample, overgrown and starved (no medium changes) to induce apoptosis. The sample was treated with ViaCount CDR to disaggregate cell clumps and washed in Annexin V Binding Buffer. Cells were stained with Annexin V-FITC (FL-1) and ViaCount dyes for viability (FL2) and nucleated cells (FL3), and data was acquired on the FACSCalibur flow cytometer using CellQuest software. See discussion below.

Two dot plots views are shown. Regional gates were set on the Annexin V-FITC (FL-1) vs. Viability Stain (FL-2) dot plot (I), with co-stained populations shown in correlating colors on the Viability Stain (FL-2) vs. Nucleated Cells stain (FL-3) dot plot (II). Based on the CellQuest identification of correlated events by color coding, marker lines were drawn on dot plot II to separate populations by color group. The CellQuest Viability Stain (FL-2) vs. Nucleated Cells Stain (FL-3) dot plot displays the data in the same way as a ViaCount dot plot.

Non-apoptotic, live cells are Annexin V-FITC (-), viability stain (-) and were gated in R1 (lower left region) on dot plot I. This population appeared in corresponding color where it would be expected on the ViaCount-equivalent dot plot II: viability stain (-), nucleated stain (+) (region A). The dead cells were Annexin V-FITC (+) and viability stain (+) and gated in R3 (upper right region) on dot plot I. As expected, the corresponding colored events were in region C on dot plot II, where the viability stain (+), nucleated cell stain (+) dead cells should appear. The Annexin V-FITC (+), viability stain (-) cells were in the early stages of apoptosis, and gated as R2 (lower right region) in dot plot I. This population correlates with the “middle” population of cells on the ViaCount-equivalent dot plot II: viability stain (dim), nucleated stain (+) (region B). These results confirm the identity of the three populations seen with ViaCount:

- PM1 (-), PM2 (+), high FSC: Live, healthy cells
- PM1 (dim), PM2 (+), high FSC: Apoptotic cells
- PM1 (+), PM2 (+), high FSC: Dead cells

Trypan blue exclusion will not provide an assessment of apoptotic status. Mascotti et al. reported that try-

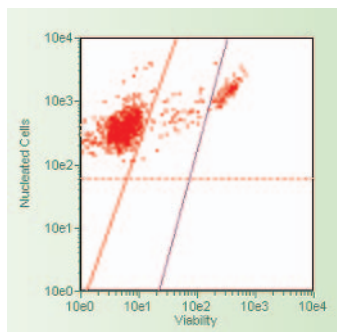
pan blue consistently overestimated the percentage of viable cells, particularly at cell viabilities below 50%, when comparing counts of bone marrow hematopoietic precursor cells done with acridine orange and propidium iodide (PI) staining vs. trypan blue.<sup>17</sup> Altman et al. also reported erroneous estimation of culture viability when comparing cells assayed by fluorescein diacetate and PI vs. trypan blue. They noted that the trypan blue dye exclusion assay significantly overestimated cell viability as the culture aged.<sup>18</sup> The inability of the trypan blue assay to discriminate apoptotic cells contributes to the overestimation of live cells seen by these laboratories. This discrepancy would be more noticeable in stressed or poorly growing cultures, where apoptosis may be induced.

The ViaCount assay provides a quick evaluation on the status of your cell culture with an accurate cell count and viability assessment. The CytoSoft version 2.0 software allows analysis of ViaCount data with the standard live/dead marker or quantitation of live, apoptotic and dead populations using the apoptosis gate feature. Results are automatically calculated and displayed on screen in the data table (see Figure 4). All data and instrument settings information are saved in a spreadsheet file that can be readily accessed, printed and analyzed using programs such as Microsoft® Excel.

Performing a ViaCount assay can help standardize or optimize cell-based experiments. A ViaCount assay requires small numbers of cells (as few as 2000) and can be done in minutes, providing an accurate cell count and a measure of apoptotic status. The level of apoptotic induction in a cell line can vary by drug treatment, dose, exposure time and culture condi-

	Cells/mL	% of Total	Cells in Original Sample
<b>Viable</b>	1.08e06	84.80%	1.08e07
<b>Apoptotic</b>	7.00e04	5.50%	7.00e05
<b>Dead</b>	1.23e05	9.70%	1.23e06
<b>Total</b>	1.27e06	100.0 %	1.27e07
<i>Dilution Factor</i>	20	<i>Original volume</i>	10 mL

**FIGURE 4:** Data analysis of sample containing Jurkat cells induced to undergo apoptosis. Data was acquired and analyzed using CytoSoft version 2.0, with the apoptosis gate activated. Non-apoptotic, live cells are in the population to the left of the left (red) marker on the dot plot.



The apoptotic cells are in the center of the dot plot, between the two (red and purple) markers. The dead cells are the population to the right of the right (purple) marker. Results are presented in the ViaCount results table and exported to a spreadsheet file.

tions, but often, experiments require the cell culture to be at a specific level of apoptotic induction or viability for suitable performance in an assay. For example, a cell line has been treated with an inducing agent for three hours. Are there enough apoptotic cells to begin the experiment? A quick ViaCount assay can show whether the percentage of apoptotic cells in the induced cell sample is too low or sufficient. This allows some optimization of the cell sample before proceeding with the experiment. For example, if the percentage of apoptotic cells is too low due to under-induction, more drug can be added or the incubation time can be extended. If the culture was over-induced and viability is too low, the cells can be discarded and a fresh culture can be prepared. In addition to saving time, effort and money, reproducibility and results may improve with better control over the condition of the cells used in experiments.

## Conclusion

When using Guava ViaCount reagent and the Guava PCA system to count cells, apoptotic cells are distin-

guishable from healthy live cells and dead cells, and can be readily quantitated using CytoSoft version 2.0. Within minutes, you can determine the percentage of cells that have entered apoptosis because cells that take up intermediate amounts of the viability dye in ViaCount reagent also react with Annexin V. Conventional methods for discriminating cell viability using trypan blue exclusion do not distinguish apoptotic cells from live cells. While obtaining cell count and viability data using the ViaCount assay, a more realistic assessment of apoptotic induction and cell culture health can be made quickly and easily, allowing better control over the condition of cells used in your experiments.

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