

# Monitoring apoptosis using four different assays on the Guava<sup>®</sup> PCA<sup>™</sup> system



## TECHNICAL NOTE

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## Guava Technologies

# Monitoring apoptosis using four different assays on the Guava<sup>®</sup> PCA<sup>™</sup> system

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## Abstract

The Guava PCA system allows you to perform multiple assays to examine cells at various stages in the progression of apoptosis. The Guava ViaCount<sup>®</sup> assay can identify cells which have entered the apoptotic pathway by differential permeability of nucleic acid staining dyes, while providing an accurate cell count and viability assessment. The Guava Nexin<sup>™</sup> assay measures membrane changes associated with apoptosis using Annexin V-PE, and 7-AAD to identify dead cells. The Guava MultiCaspase assay tags activated caspases within cells with a fluorescent inhibitor (SR-VAD-FMK) and uses 7-AAD to identify dead cells. The Guava TUNEL assay detects the fragmentation of nuclear DNA to analyze cells committed to the apoptotic pathway. We performed the Guava ViaCount, Guava MultiCaspase, Guava Nexin and Guava TUNEL assays on CHO-K1 cells prepared from a single induction treatment to look at the differences in quantifying apoptotic cells by the different methods. Each assay is distinguishing apoptotic from non-apoptotic cells; however, they are identifying cells at different stages through the progression of apoptosis with different markers of apoptosis.

## Introduction

Apoptosis, or programmed cell death, is an important and active regulatory pathway of cell growth and proliferation. Cells respond to specific apoptotic induction signals by initiating intracellular processes that result in characteristic physiological changes. Among these changes are externalization of phosphatidylserine to the cell surface, depolarization of mitochondrial membranes, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, loss of cell

membrane integrity, and cellular shrinkage.<sup>1-6</sup> Apoptosis is a kinetic event, and the time period during which cells exhibit the “markers” of apoptosis is variable and short. The manifestations of apoptotic progression are affected by several factors: 1) the cell type, 2) cell condition, 3) the treatment to induce apoptosis, 4) the method used to characterize the physiological change.

The Guava PCA system currently offers four different ways to categorize cells as they progress through apoptosis. Guava ViaCount and Guava Nexin detect membrane changes associated with apoptosis. ViaCount identifies apoptotic cells by differential permeability of nucleic acid intercalating dyes. The compromised membrane integrity of an apoptotic cell allows penetration of a previously impermeant nucleic acid dye.<sup>7</sup> The Guava Nexin assay uses Annexin V-PE to bind phosphatidylserine,<sup>8-10</sup> which has translocated from the internal to external side of the cell membrane upon induction of apoptosis.<sup>11-16</sup> The cell impermeant dye, 7-AAD, is included in the assay as an indicator of membrane structural integrity. 7-AAD is excluded from live, healthy cells and early apoptotic cells, but permeates later stage apoptotic and dead cells.<sup>17</sup>

Caspase enzymes play a central role in the apoptotic death process.<sup>18,19</sup> Caspases (cysteinyll-directed aspartate-specific proteases) form a family of enzymes<sup>20</sup> that initiate the apoptotic cascade (caspase-2, -8, -9, -10), carry out cellular breakdown (-3, -6, -7) and process cytokines (-1, -4, -5, -11 to 14). The activation of these caspases commits a cell to death by the apoptotic pathway. The Guava MultiCaspase Detection Kit uses a fluorochrome-conjugated inhibitor of caspases called sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK). This inhibitor is cell permeable and non-cytotoxic. Once inside the cell, the SR-VAD-

FMK inhibitor binds covalently to multiple caspases that have been activated in apoptosis.<sup>21</sup> Because the SR-VAD-FMK is covalently coupled to the enzyme, it is retained in the cell, while unbound SR-VAD-FMK reagent can diffuse out of the cell and be washed away. The resulting red fluorescent signal is proportional to the number of active caspase enzymes that were present in the cell when the reagent was added. Cells showing positive SR-VAD-FMK staining correspond to early through late stage apoptotic cells in a population, because this reagent binds to most of the activated caspase enzymes. The addition of 7-AAD allows discrimination of cells in late stage apoptosis and those that are dead from non-apoptotic (live) and early apoptotic cells.

A distinctive marker along the apoptotic pathway to cell death is the degradation of chromatin structure and nuclear DNA by activated nucleases. Chromatin is degraded into fragments of 50 to 300 kb, and subsequently into smaller 200 bp pieces of DNA. Traditionally, these fragments are extracted from apoptotic cells and analyzed by agarose gel electrophoresis. The characteristic “DNA ladder” of fragments is seen from apoptotic cell extracts, whereas viable cell DNA remains intact.<sup>22</sup> The fragmentation of DNA during apoptosis generates exposed 3'-hydroxyl ends in the nuclear DNA. In the Guava TUNEL assay, terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of Br-dU residues into the fragmenting nuclear DNA at the 3'-hydroxyl ends by nicked end labeling. TRITC-conjugated anti-Br-dU antibody binds to the incorporated Br-dU residues, labeling the apoptotic cells.<sup>23,24</sup> In contrast to the other three assays, cells must be fixed and permeabilized to allow intracellular labeling reactions for the Guava TUNEL assay.

Cell types respond differently to treatments to induce apoptosis, and exhibit varying levels of the markers of apoptosis. For example, Del Bino et al. report a rapid 4 to 6 hours progression from apoptotic induction to cell disintegration for HL-60 leukemia cells when triggered by treatment with topoisomerase inhibitors and DNA damage; however, MCF-7 breast cancer cells begin apoptosis after a 24 hour delay and exhibit a progression of much longer duration when induced by the same treatment.<sup>25</sup> In our lab, we have observed changes in the responsiveness of Jurkat cells

to apoptotic induction agents as the passage number increases. For some clonal Jurkat cultures, “older” cells at high passage number resist induction.

There can be dissociation of characteristic features of apoptosis with the extent or progression of programmed cell death. Several laboratories have reported the delay or absence of internucleosomal DNA fragmentation in cells exhibiting the other markers of apoptosis.<sup>26-29</sup> Grigoriev et al. reported expression of caspase-3 and caspase-7 did not correlate with the extent of apoptosis in primary breast carcinomas, whereas DNA fragmentation measured by the TUNEL assay showed concordance.<sup>30</sup> Frey studied six different cell systems induced to undergo apoptosis by six different treatments and found different patterns of changes in Annexin V binding. MOLT-4 cells treated with etoposide, thymocytes treated with dexamethasone and aged neutrophils exhibited characteristic increases in Annexin V binding with apoptotic progression. For Raji cells cultured in low serum, U937 cells treated with anti-Fas antibody and HL-60 cells treated with camptothecin, this change could not be routinely detected; however, other markers of apoptosis (mitochondrial changes, acidification of the cytoplasm) could be detected.<sup>31</sup>

In addition, cells can exhibit certain early features of apoptosis under adverse conditions, yet not be fully committed to the apoptotic pathway. Hammill et al. found that a B cell lymphoma bound Annexin V upon crosslinking of the membrane immunoglobulin (mIg) receptor. Upon removal of the crosslinking anti-mIg, some of the early apoptotic cells reestablished phospholipid asymmetry and resumed growth.<sup>32</sup> We have seen ViaCount staining and Annexin V binding increase if cell cultures are slightly over-digested with trypsin. However, if the cells are incubated in complete culture medium for a short period (15-20 minutes), the cells will recover and staining results will reflect the expected viability. Thus, it is important to handle the cells carefully and perform a variety of assays when characterizing apoptotic cells.

We have developed a number of assays that can detect and quantitate cells in various stages of apoptosis on the Guava PCA system. We attempted to

capture a “snap shot” of the apoptotic status of an induced cell culture by performing the four assays on samples from the same culture.

## Materials and Methods

### Experimental procedures

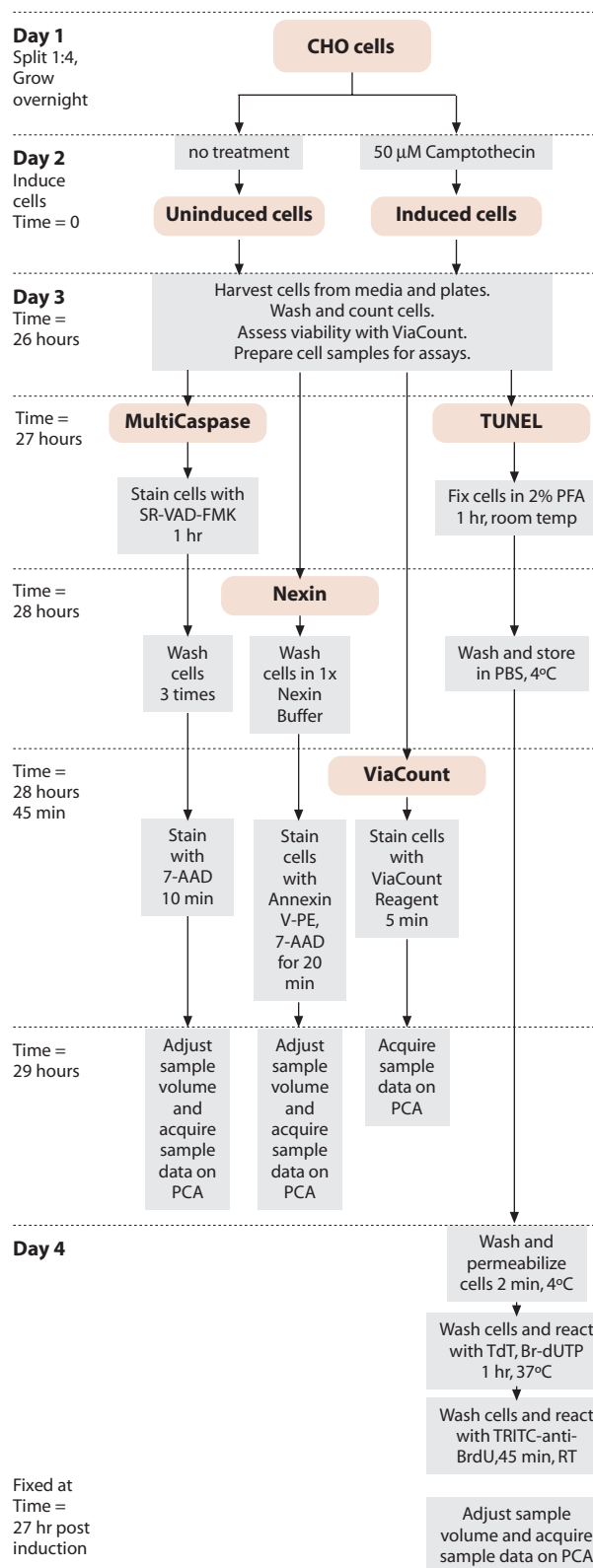
A flow chart summarizing the experimental workflow is shown in Figure 1. On assay day, two people were required to perform this experiment because much of the sample preparation and data acquisition required concurrent processing to meet timing considerations.

### Cell lines and culture conditions

An adherent Chinese hamster ovary cell line (CHO-K1; ATCC Catalog No. CCL-61) was grown in culture flasks using Ham’s F12 medium (F12K) supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Cells were grown in a humidified 5% CO<sub>2</sub> incubator at 37°C. An excess amount of cells (approximately  $2 \times 10^7$ ) were cultured for the experiment.

Two days before the assays were to be done, a 90-95% confluent T75 flask of CHO-K1 cells was split 1:4 (1 part cells, 3 parts medium) into two T75 flasks. The CHO-K1 cells in each flask were cultured to 50-70% confluence (typically achieved overnight). The day before the experiment, cells in one T75 flask were induced to undergo apoptosis by treatment with medium containing 50 μM camptothecin. The induction medium was prepared by using a 50 mM stock solution of camptothecin (Calbiochem, Catalog No. 208925) in DMSO; 25 μL of 50 mM camptothecin was added to 25 mL of culture medium. The other T75 flask was left untreated to provide uninduced control cells.

About 26 hours after addition of camptothecin, induced and uninduced CHO-K1 cells were harvested from their flasks for assay. The culture medium was removed and reserved from each flask. Apoptotic and dead cells tend to adhere less strongly to the culture substrate or float in the medium, so the culture medium was centrifuged to recover these cells. The adherent cells were washed



**FIGURE 1:** Experimental Procedure Flow Chart

with calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS), then trypsinized to remove the cells from the culture flask. Cells were collected by using complete culture medium (with serum) to stop the digestion of the cells. The cells were centrifuged and the supernatant removed. The adherent cells were pooled with the corresponding apoptotic and dead cells recovered from the culture medium. Cells were washed with PBS, then resuspended in culture medium. Cell count and viability was assessed for each cell suspension using Guava ViaCount (Catalog No. 4000-0040). Aliquots of cells were taken to prepare samples for each of the four assays. The timing of the preparation steps outlined on the flow chart was followed as closely as possible.

## Sample Preparation

### Guava MultiCaspase assay

Eight samples were prepared in duplicate: 1) Uninduced, unstained; 2) Induced, unstained; 3) Uninduced, SR-VAD-FMK only; 4) Induced, SR-VAD-FMK only; 5) Uninduced 7-AAD only; 6) Induced, 7-AAD only; 7) Uninduced, SR-VAD-FMK + 7-AAD; 8) Induced SR-VAD-FMK + 7-AAD. One aliquot of induced cells and one of uninduced cells (containing  $5 \times 10^5$  cells each) was used. Each cell stock was adjusted to a final concentration of  $5 \times 10^5$  cells/mL. Test samples were prepared as described in the package insert for the Guava MultiCaspase kit (Catalog No. 4500-0101).<sup>33</sup> Briefly, 100  $\mu$ L of cells ( $5 \times 10^4$ ) was used per test sample and stained with 5  $\mu$ L of 20X SR-VAD-FMK solution. Buffer was substituted as appropriate for unstained and single stain controls. Cells were stained for 1 hour at 37°C in the tissue culture incubator, protected from light exposure. After three washes, 5  $\mu$ L of Caspase 7-AAD was added to appropriate tubes containing cells in 100  $\mu$ L of Wash Buffer. Samples were stained for 10 minutes at room temperature, and then resuspended in 500  $\mu$ L of Wash Buffer. Cell samples were ready for data acquisition on the Guava PCA system.

### Guava Nexin assay

The Guava Nexin assay was started one hour after the start of the MultiCaspase assay. Eight samples

were prepared in duplicate: 1) Uninduced, unstained; 2) Induced, unstained; 3) Uninduced, Annexin V-PE only; 4) Induced, Annexin V-PE only; 5) Uninduced 7-AAD only; 6) Induced, 7-AAD only; 7) Uninduced, Annexin V-PE + 7-AAD; 8) Induced Annexin V-PE + 7-AAD. An aliquot containing  $1 \times 10^6$  induced cells and one of uninduced cells was used. Each cell sample was centrifuged and the medium was removed. The cell pellets were resuspended in 0.5 mL of 1X Nexin Buffer to a final concentration of  $2 \times 10^6$  cells/mL. Test samples were prepared as described in the package insert for the Guava Nexin kit (Catalog No. 4500-0010).<sup>34</sup> Briefly, 40  $\mu$ L of cells ( $8 \times 10^4$ ) was used per test sample and stained with 5  $\mu$ L of Annexin V-PE and 5  $\mu$ L of Nexin 7-AAD. 1X Nexin Buffer was substituted as appropriate for unstained and single stain controls. Cells were stained for 20 minutes on ice, shielded from light exposure. At the end of the incubation period, 450  $\mu$ L of 1X Nexin Buffer was added to each tube. Cell samples were ready for data acquisition on the Guava PCA system.

### Guava ViaCount assay

The Guava ViaCount assay was performed on duplicate samples from the induced and uninduced cultures about 1 hour 45 minutes after the start of the MultiCaspase assay (i.e. 15 minutes before the end of the MultiCaspase and Nexin assay sample preparation). The cell samples were at a concentration of  $10^6$  to  $10^7$  cells/mL; therefore, 20  $\mu$ L of cell suspension was mixed with 380  $\mu$ L of Guava ViaCount reagent (20 fold dilution) in a 1.5 mL microcentrifuge tube, as described in the package insert for Guava ViaCount reagent.<sup>35</sup> Samples were mixed well and incubated for about 5 minutes at room temperature, protected from light. Test samples were ready for data acquisition on the Guava PCA system.

### Guava TUNEL assay

The Guava TUNEL Assay uses cells that must be fixed and permeabilized before staining. For this assay, duplicate samples of induced and uninduced CHO-K1 cells were fixed, permeabilized and stained. A positive control and negative control sample from the Guava TUNEL kit (Catalog No. 4500-0120) was also prepared as described in the package insert.<sup>36</sup>

**CHO-K1 cell fixation.** Aliquots of CHO-K1 cells, containing  $2 \times 10^6$  induced cells and  $2 \times 10^6$  uninduced cells, were taken for fixation 27 hours post-induction, at the same time as the start of the MultiCaspase assay. Each cell suspension was transferred to an appropriate sized centrifuge tube with cap. The cells were centrifuged at  $300 \times g$  for 5 minutes with the brake on low. The culture medium was removed; each pellet was vortexed to disperse the cells in residual medium, then resuspended in 1 mL of PBS. Cells were centrifuged again, and the supernatant was removed. Cell pellets were resuspended in 1 mL of PBS, then 1 mL of freshly prepared Paraformaldehyde Fixative was added while vortexing to prevent the formation of fixed cell aggregates. Paraformaldehyde Fixative was 4% paraformaldehyde in PBS, pH 7.4. This yielded a final concentration of CHO-K1 cells at  $1 \times 10^6$  cells/mL in 2% paraformaldehyde for fixation. The cells were fixed for 1 hour at room temperature on a tube rocker. After fixation, cells were centrifuged and the supernatant was aspirated and discarded. The cells were washed twice with 2 mL of PBS each time. The cells were centrifuged and the cell pellet was resuspended in 2 mL of PBS (final cell concentration of  $1 \times 10^6$  cells/mL), and stored at 4 to 8°C overnight.

**Cell permeabilization and staining.** For each test and kit control sample,  $5 \times 10^5$  fixed cells were transferred into a 12 x 75 mm polystyrene tube with cap (VWR, Catalog No. 60819-310), and 1 mL of Wash Buffer was added. (NOTE: For best results, it is important to use polystyrene tubes for sample preparation.) The tubes were vortexed and centrifuged at  $300 \times g$  for 5 minutes with the brake on low. The supernatant was aspirated and removed. The control cells supplied in the Guava TUNEL kit do not require permeabilization. To the CHO-K1 cell pellets only, 100  $\mu$ L of cold Permeabilization Buffer was added while vortexing. Permeabilization Buffer was composed of 0.1% v/v Triton X-100 (Sigma, Cat. No. T9284), 0.1% w/v sodium citrate (Sigma, Cat. No. S4641). The cells were incubated on ice for 2 minutes, and then 1 mL of Wash Buffer was added to all tubes (including positive and negative controls). The samples were centrifuged and supernatant removed. The cells were washed again with 1 mL Wash Buffer and centrifuged. After removal of the supernatant, cells were ready for staining.

All samples were stained as described in the package insert. Briefly, the cell pellet ( $5 \times 10^5$  cells) was resuspended in 50  $\mu$ L of DNA labeling solution, containing TdT Enzyme and Br-dUTP in TdT Reaction Buffer. After a 60 minute incubation at 37°C, cells were washed with Rinsing Buffer and centrifuged. The cell pellets were resuspended in 0.1 mL of antibody solution containing anti-BrdU-TRITC antibody and incubated for 45 minutes at room temperature, shielded from light. Cells were washed with Rinsing Buffer and transferred to 1.5 mL microcentrifuge tubes for data acquisition on the Guava PCA system.

## Data Acquisition

Proper instrument performance was verified by running the Guava Check application with Guava Check reagents (Catalog No. 4500-0020). Data was acquired on the Guava PCA system using CytoSoft software as described in the Guava PCA User's Guide and respective package inserts. For Guava MultiCaspase, Guava Nexin and Guava TUNEL assays, 2000 events were usually acquired. For Guava ViaCount, 1000 events were acquired.

Negative, positive and single parameter staining controls were used to verify reagent performance and set analysis markers delineating the negative and positive populations.

### Cell Populations Identified Using Guava Markers

#### Guava MultiCaspase

- **Live & non-committed apoptotic cells:** SR-VAD-FMK (-), 7-AAD (-)
- **Early & Mid-stage apoptotic cells:** SR-VAD-FMK (+), 7-AAD (-)
- **Late stage apoptotic & dying cells:** SR-VAD-FMK (+), 7-AAD (+)
- **Dead cells:** SR-VAD-FMK (- or dim), 7-AAD (+)

#### Guava TUNEL

- **Non-apoptotic, live cells:** TUNEL (-) or TRITC (-)
- **Apoptotic cells:** TUNEL (+) or TRITC (+)

#### Guava Nexin

- **Live & non-committed apoptotic cells:** Annexin V-PE (-), 7-AAD (-)
- **Early apoptotic cells:** Annexin V-PE (+), 7-AAD (-)
- **Late apoptotic, dead cells:** Annexin V-PE (+), 7-AAD (+)
- **Nuclear debris (apoptotic bodies):** Annexin V-PE (- or dim), 7-AAD (+)

#### Guava ViaCount

- **Non-apoptotic, live cells:** PM1 (-), PM2 (+), high FSC
- **Apoptotic cells:** PM1 (dim), PM2 (+), high FSC
- **Dead cells:** PM1 (+), PM2(+), high FSC

	Induced Cells		Uninduced Cells	
	Non-Apoptotic (Live Cells)	Apoptotic and Dead Cells	Non-Apoptotic (Live Cells)	Apoptotic and Dead Cells
<b>Guava MultiCaspase</b>	73.0%	27.0%	94.5%	5.5%
<b>Guava ViaCount</b>	71.0%	29.0%	96.6%	3.4%
<b>Guava Nexin</b>	67.7%	32.3%	91.8%	8.8%
<b>Guava TUNEL</b>	65.9%	34.1%	95.5%	4.5%

**TABLE 1:** Comparison of % Apoptotic and Dead and % Non-Apoptotic cells determined by the four assays in CHO-K1 cells induced to undergo apoptosis and uninduced negative control.

	Live (Non-Apoptotic)	Early Apoptosis	Mid-Apoptosis	Late Apoptosis	Dead
<b>Guava MultiCaspase</b>	73.0%	15.0%		12.0%	
<b>Guava ViaCount</b>	71.0%	16.1%			12.9%
<b>Guava Nexin</b>	67.7%	9.4%	21.8% (1.1% Nuclear Debris)		
<b>Guava TUNEL</b>	65.9%	34.1%			

**TABLE 2:** Distribution of the induced CHO-K1 cell populations in different stages of apoptosis, as determined by the four assays.

## Results and Discussion

We performed four different assays on the Guava PCA, measuring different markers of apoptosis that appear during the stages of apoptotic progression. We assayed CHO-K1 cells prepared from a single induction treatment to look at the differences in quantifying apoptotic cells by the different methods. The results are summarized in Tables 1 and 2.

In Table 1, the distribution of live vs. apoptotic and dead cells is compared in the induced and untreated CHO-K1 cells, as determined by the four different assays. The apoptotic and dead cell percentages were calculated as the sum of the populations staining positively for the apoptotic and dead cell markers. The value shown for each assay result is the average of the two replicate samples.

The results show fairly good agreement across the four assays, with the % of live cells ranging from 65.9% to 73.0% for the induced samples and % of apoptotic and dead cells ranging from 27.0% to 34.1%. For the uninduced samples, the % of live cells ranged from 91.8% to 96.6% and the % of apoptotic and dead cells ranged from 3.4% to 8.8%. As expected, the untreated cells showed higher viability than the camptothecin-induced cells. Results varied by less than 10% across all of the samples in each category for the four assays (n = 8 per type).

In Table 2, data from the camptothecin-treated CHO-K1 culture is presented, reflecting the different distributions through apoptotic progression as defined by each assay. Apoptosis is a process that can exhibit many physiological changes in a variety of ways in an induced cell system. The distinctions between the live to early, mid, late apoptotic, and dead stages are fuzzy because the process is a continuum. However, we have tried to correlate apoptotic stages with the expression of the different assayed markers based on findings in the literature. Populations were identified in the four assays as described in the previous panel.

The categorization of the induced CHO-K1 populations seemed to be fairly consistent across the four assays. There were some factors that contributed to variability in this experiment. Three of the assays used live cells: MultiCaspase, Nexin and ViaCount. The Guava TUNEL assay required fixed cells. Live cells may continue to progress through apoptosis during the experiment, whereas fixed cells will not. Sample preparation (reagent incubation times, wash steps) varied from assay to assay, affecting processing time and sample condition. We prepared and acquired data from samples in a timely manner to try to minimize these variables. Resolution of the different cell populations vary from assay to assay; gating differences can impact results, somewhat.

## Conclusion

The Guava PCA system currently offers four different ways to categorize cells as they progress through apoptosis. We attempted to capture a “snap shot” of the apoptotic status of an induced cell culture by performing the four assays on samples from the same culture. Our model system using CHO-K1 cells induced with camptothecin yielded consistent results across the assays, measuring membrane changes, caspase activity and chromatin/DNA degradation. However, cell types can respond differently to treatments to induce apoptosis and show varying levels of the physiological features of apoptosis. Thus it is important to perform different assays when characterizing apoptotic cells. The apoptosis suite on the Guava PCA allows you to characterize your culture on a cell-by-cell basis using small numbers of cells with optimized reagent kits and custom, easy to use software applications.

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