



4600-0590
rev A

Guava® PCA™ -96 MultiCaspase Kit

Cat. No. 4500-0102
100 tests

For Discriminating Apoptotic Cells from Non-Apoptotic Cells on the Guava PCA-96 System

Research Use Only

1. PRODUCT DESCRIPTION AND INTENDED USE

Caspase enzymes play a central role in the apoptotic cell death process.^{1,2} Caspases (cysteine-directed aspartate-specific proteases) form a family of enzymes³ 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 caspases commits a cell to death by the apoptotic pathway.

The Guava PCA-96 MultiCaspase Kit uses a fluorochrome-conjugated inhibitor of caspases called sulforhodamine-valyl-alanyl-aspartyl-fluoromethyl-ketone (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.⁴ Because the SR-VAD-FMK reagent is covalently coupled to the enzyme, it is retained in the cell, while any unbound SR-VAD-FMK reagent can diffuse out of the cell and be washed away. The resulting fluorescent signal in the cell is proportional to the number of active caspase enzymes that were present in the cell when the reagent was added. Cells showing significant positive SR-VAD-FMK staining correspond to cells in the population committed to the apoptotic pathway. 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.

Four populations of cells can be distinguished in this assay:

- Live and early apoptotic (non-committed) cells: SR-VAD-FMK (-) and 7-AAD (-)
- Mid stage (committed) apoptotic cells: SR-VAD-FMK (+) and 7-AAD (-)
- Late stage apoptotic and dying cells: SR-VAD-FMK (+) and 7-AAD (+)
- Dead cells: SR-VAD-FMK (-) or dim and 7-AAD (+)

This assay is intended as an accurate method for determining the number of cells in various stages of the apoptotic pathway.

2. MATERIALS PROVIDED

Guava PCA-96 MultiCaspase Kit (Catalog No. 4500-0102, 100 tests/kit) includes the following reagents:

- SR-VAD-FMK Reagent (Part No. 4100-0212, 50 tests x 2)
- Apoptosis Wash Buffer (10x), (Part No. 4200-0162, 15 mL)
- Caspase Fixative, (Part No. 4200-0170, 6 mL)
- Anhydrous DMSO, (Part No. 4300-0160, 1.0 mL)

- Caspase 7-AAD (Part No. 4000-0064, 250 µL)

3. HANDLING AND STORAGE

1. Store the unopened kit (and each unopened component) at 2 to 8°C.
2. Protect the SR-VAD-FMK Reagent from light exposure.

4. WARNINGS AND PRECAUTIONS

1. The Guava PCA-96 MultiCaspase Kit is intended for research only.
2. All biological specimens and materials should be handled as if capable of transmitting infection and disposed of with proper precautions in accordance with federal, state, and local regulations. Avoid specimen contact with skin and mucous membranes.
3. Exercise care to avoid cross contamination of samples during all steps of this procedure, as this may lead to erroneous results.
4. Use gloves and other appropriate laboratory attire while handling the SR-VAD-FMK and 7-AAD reagents, and the DMSO. Avoid contact with skin and mucous membranes.
5. Dispose of all materials using proper precautions in accordance with federal, state, and local regulations.
6. Material Safety Data Sheets (MSDS) for kit reagents are available from our Web site (www.guavatechnologies.com) or by contacting Guava Customer Care (support@guavatechnologies.com).
7. Guava does not warrant using the reagents after the expiration date.
8. The Apoptosis Wash Buffer contains sodium azide as a preservative. Sodium azide is toxic. Contact with acids liberates toxic gas. Flush plumbing with copious amounts of water when disposing of azide compounds to avoid potentially explosive conditions arising from azide deposits in pipes.
9. Avoid microbial contamination of the solutions, which may cause erroneous results.

5. EQUIPMENT AND MATERIALS REQUIRED

- Guava PCA-96 system with CytoSoft™ software
- Cell suspensions, untreated and treated to induce apoptosis
- Cell culture medium appropriate for cell lines used
- 1X Phosphate buffered saline (PBS) or equivalent, pH 7.2 to 7.4
- Multipipettors, 8 or 12 channel, 2 to 200 µL capacity
- Disposable micropipettor tips
- 8-channel microplate aspirator (VWR Cat. No.29443-120 or equivalent)
- Centrifuge with 96-well microplate holders
- 96-well microplates, round bottom (Falcon Cat. Nos. 353910 or 353918 or equivalent). Refer to the *Guava PCA-96 System User's Guide* for other compatible microplates.
- 96-well microplates, flat bottom (Falcon Cat. Nos. 353075 or 353915), or hydrogel-coated flat bottom (Costar Cat. No. 3474, Myriad Industries Cat. No. 0400-3474) or equivalent. Refer to the *Guava PCA-96 System User's Guide* for other compatible microplates.
- V-bottom reagent reservoirs, 55 mL (VWR Cat. No. 210070-970 or equivalent)
- 1.5 mL microcentrifuge tubes (VWR Cat. No. 20170-215 or equivalent), for cleaning
- 0.5 mL microcentrifuge tubes (VWR Cat. No. 20170-210) for sample acquisition
- Vortex mixer

- Disposable gloves
- Polypropylene or polystyrene centrifuge tubes with cap, sterile
- Serological pipets, sterile
- 37°C CO₂ incubator
- Guava PCA-96 ViaCount Flex Reagent (Cat. No. 4700-0060), optional
- Deionized water
- Guava ICF™ Instrument Cleaning Fluid (Cat. No. 4200-0140), optional
- 20% bleach solution
- ViaCount CDR (Cell Dispersal Reagent) for adherent cells (Cat. No. 4700-0050), optional

6. REAGENT AND SAMPLE PREPARATION

Recommended assay conditions. A typical assay test uses 20 µL of 5X SR-VAD-FMK solution and 20 µL of 0.25X Caspase 7-AAD reagent to stain 5 x 10⁴ cells in a reaction volume of 100 µL. As few as 5 x 10³ cells per well can be used without affecting the quality of the assay results. However, the fewer cells plated per well, the longer each well will require to analyze and the fewer wells will be analyzed within the 30-minute time window for unfixed cells (see the following section, *Time Considerations*). We recommend staining negative control cells (not treated to induced apoptosis) and positive control cells (treated by a proven method to induce apoptosis), in addition to your test samples.

Time Considerations. Staining apoptotic cells with the Guava PCA-96 MultiCaspase Kit and data acquisition on the Guava PCA-96 system can usually be completed within 3 to 4 hours. However, the assay uses living cells, which require periodic maintenance and cultivation several days in advance. Once the proper number of cells for your experiment has been cultivated, additional time is required for the apoptosis induction process (this typically requires 2 to 4 or more hours, depending on your cell type and method). Prepare the kit reagents near the end of the induction period, so they can be used when the cells are ready for staining.

IMPORTANT: Once cells have been stained, they should be analyzed within 30 minutes,* unless the cells have been fixed. After 30 minutes, unfixed cells can show a decrease in apoptotic cell numbers and an increase in the number of dead cells. Fixing the cells, however, may not be a viable alternative, as some cell lines that Guava fixed and tested showed changes in staining patterns and erroneous results compared to unfixed cells. Individual laboratories should test their specific cells for comparable results between fixed and unfixed cells.

* **NOTE:** We suggest using no more than 48 wells for an experiment. This allows time to complete the analysis within 30 minutes.

Reagent Preparation

- **1X Apoptosis Wash Buffer.** The wash buffer is supplied as a 10X concentrate, which must be diluted to 1X with deionized water prior to use (mix 1 part of Apoptosis Wash Buffer (10X) with 9 parts deionized water). If necessary, gently warm (no warmer than 37°C) the Apoptosis Wash Buffer (10X) to completely dissolve any salt crystals that may have precipitated out of solution during storage. Store 1X Apoptosis Wash Buffer at 2 to 8°C for up to 14 days. Approximately 1.0 mL of 1X Apoptosis Wash Buffer is required per sample to be stained.

- **100X SR-VAD-FMK stock solution.** The SR-VAD-FMK reagent is supplied as a highly concentrated lyophilized powder. First, it must be reconstituted with DMSO to make a 100X stock concentrate.

1. Reconstitute each 50 test vial of lyophilized SR-VAD-FMK with 50 μ L DMSO. This yields a 100X concentrate.
2. Mix by swirling and tilting the vial. At room temperature, this reagent should dissolve within a few minutes.
3. If using this solution immediately, dilute it to 5X (see below).
4. If using later, prepare small aliquots of the 100X SR-VAD-FMK Reagent in microcentrifuge tubes and store at (minus) -20°C , protected from light.

NOTE: 50 μ L of 100X SR-VAD-FMK is enough for 50 tests.

- **5X SR-VAD-FMK solution.** Using the freshly reconstituted or thawed 100X SR-VAD-FMK stock, prepare a 5X working solution of SR-VAD-FMK by diluting the stock 1:20 with PBS (for example, 50 μ L of 100 X SR-VAD-FMK and 950 μ L of PBS buffer yields 1 mL of 5X SR-VAD-FMK solution). Using this suggested protocol, each sample to be tested requires 20 μ L of 5X SR-VAD-FMK solution.

NOTE: Use the 5X SR-VAD-FMK solution the same day that it is prepared.

- **Caspase 7-AAD.** Prepare a 0.25X working solution of Caspase 7-AAD by diluting the stock 1:4 with PBS (for example 250 μ L of Caspase 7-AAD and 750 μ L PBS buffer yields 1 mL of 0.25X Caspase 7-AAD). Using this suggested protocol, each sample to be tested requires 20 μ L of 0.25X 7-AAD.
- **Caspase Fixative.** This reagent is ready to use without further preparation.

Cell Sample Preparation for Cells Cultured in Flasks

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol, but not to exceed 1×10^6 cells/mL.
2. Prepare test samples by inducing apoptosis in each cell population using the desired protocol.
3. Prepare positive control samples by treating your cell culture with a proven apoptosis induction method. Contact Guava Technical Support for information regarding proven methods for inducing apoptosis in cell cultures.
4. Prepare negative control samples, which are untreated, uninduced cells from these cultures.

For Non-adherent Cell Cultures When Induction is Complete

1. Transfer the cells from the culture vessels into a tube. Use 1 tube for each culture condition (induced, positive control, negative control).
2. Count the cells using the Guava PCA-96 ViaCount Flex reagent (see the following note).
3. Wash all the cell samples with 1X Apoptosis Wash Buffer to remove the induction agent and medium.
4. Aspirate the supernatant off and adjust the cell concentration to 5×10^5 cells/mL for each culture condition using a 1:1 mixture of 1X Apoptosis Wash Buffer and cell culture medium.
5. Mix the cell suspensions by gently vortexing or repeatedly pipetting.
6. Transfer each cell suspension to a separate V-bottom reservoir.
7. Using a multichannel pipettor, pipet 100 μ L of the induced cell suspension into appropriate wells of a 96-well microplate (5×10^4 cells/well if the concentration of the cell suspension is 5×10^5 cells/mL). Using fresh pipet tips, add the negative and positive control samples to the appropriate wells.

NOTE: You can count the cells using the Guava ViaCount assay. ViaCount may also provide a good estimate of the number of apoptotic cells in your sample. If the level of apoptosis is too high or too low, you can adjust the culture conditions or you can incubate the cells in the inducing agent longer, respectively. From the ViaCount assay results, you can determine whether the cells are suitable for staining, or whether apoptosis has progressed too far and you need to use a fresh culture. Refer to the Guava PCA-96 ViaCount Flex Reagent package insert and *Guava PCA-96 System User's Guide* for detailed information about this assay.

8. Proceed to *Cell Staining Procedure*.

For Adherent Cell Cultures When Induction is Complete

1. Harvest apoptotic cells that may have detached into the culture medium by transferring the medium to a centrifuge tube. Use one tube for each culture condition (induced, positive control, negative control).
2. Rinse each flask once with 5 to 10 mL of PBS and add the rinse PBS to the appropriate centrifuge tube.
3. Dilute the Guava ViaCount CDR (Cell Dispersal Reagent) at 1:3 with PBS (for example, 3 mL of ViaCount CDR in a T75 flask).
4. To remove adherent cells from the culture substrate, add 3 mL of the diluted Guava ViaCount CDR to each flask and incubate for 3 to 5 minutes (or until cells start to detach) in a 37°C CO_2 incubator.
5. Add 6 mL of media (containing at least 5% fetal bovine serum or BSA) to each flask and pipet repeatedly to remove cells from the flask bottom.
6. Add the cell suspensions to the appropriate tube and centrifuge for 5 minutes at room temperature at 300 to 400 \times g.
7. Adjust the cell suspensions to a concentration of 5×10^5 cells/mL using a 1:1 mixture of 1X Apoptosis Wash Buffer and cell culture medium.

NOTE: You can count the cells using the Guava PCA-96 ViaCount Flex reagent. Refer to the Guava ViaCount Flex PCA-96 Reagent package insert and *Guava PCA-96 System User's Guide* for detailed information about this assay.

8. Mix the cell suspensions by gently vortexing or repeatedly pipetting.
9. Transfer each cell suspension to a separate V-bottom reservoir.
10. Using a multichannel pipettor, pipet 100 μ L of the induced cell suspension into appropriate wells of a 96-well microplate (5×10^4 cells/well if the concentration of the cell suspension is 5×10^5 cells/mL). Using fresh pipet tips, add the negative and positive control samples to the appropriate wells.
11. Proceed to *Cell Staining Procedure*.

Cell Sample Preparation for Cells Cultured in Microtiter Plates

1. Using your specific procedure, culture the cells in 96-well microplates to the appropriate density for inducing apoptosis. Do not exceed 1×10^6 cells/mL in a final volume of 100 μ L.
2. Induce apoptosis in each test cell population using your specific protocol.
3. For positive control samples, treat some of the cells in the 96-well microplates with a proven apoptosis induction method. Contact Guava Technical Support for information regarding proven methods for inducing apoptosis in cell cultures.
4. For negative control samples, leave some wells for untreated and uninduced cells.
5. Culture cells in microplates to induce the required amount of apoptosis.
6. *Optional wash step.* Using a multichannel pipettor, add 175 μ L of 1X Apoptosis Wash Buffer to each well. Centrifuge the microplate at 300 \times g for 5 minutes at room temperature. Carefully remove the supernatant

from each well using a multichannel vacuum aspirator fitted with micropipette tips or a multichannel pipettor. To prevent cell loss, do not remove all the supernatant. Resuspend cells with 100 μ L of a 1:1 mixture of 1X Apoptosis Wash Buffer and cell culture medium (5×10^4 cells/well if the concentration of the cell suspension after induction was 5×10^5 cells/mL).

IMPORTANT: If you do not wash the cells at this point, you must add 20 μ L of 10X SR-VAD-FMK to each well instead of 20 μ L of 5X SR-VAD-FMK. Omitting the wash step allows you to prepare only 25 samples instead of 50 samples per vial of SR-VAD-FMK.

7. Proceed to *Cell Staining Procedure*.

7. CELL STAINING PROCEDURE

NOTE: The following procedure for cell staining is a guideline. Different cell types may respond differently to various treatments for apoptotic induction. The level of activated caspases present in a cell can vary with apoptotic progression. You may need to adjust the amount of SR-VAD-FMK Reagent and Caspase 7-AAD for optimal staining of your cell samples.

1. Add 20 μ L of 5X SR-VAD-FMK reagent to each well. If you did not wash the culture medium off the induced cells, add 20 μ L of 10X SR-VAD-FMK. To mix, immediately pipet the cell samples up and down several times, changing tips between wells to avoid cross-contamination.
2. Incubate for 1 hour at 37°C in a 5% CO_2 incubator. Protect samples from light exposure. Gently resuspend the cells once or twice during this incubation period to ensure an even distribution of the SR-VAD-FMK reagent among all cells.
3. Using a multichannel pipettor, carefully add 150 μ L of 1X Apoptosis Wash Buffer to each well.
4. Centrifuge the microtiter plate at 300 \times g for 5 minutes.
5. Carefully remove the supernatant from each well using a multichannel vacuum aspirator fitted with micropipette tips (see section 5, *Equipment and Material Required*). Alternatively, remove supernatant with a multichannel pipettor. To minimize cell loss, do not remove all the supernatant.
6. Repeat steps 4 through 6 one more time (two washes total).
7. Add 20 μ L of 0.25X 7-AAD reagent. Resuspend the cells by pipetting up and down several times, changing tips between wells to avoid cross-contamination. Incubate the samples for 10 minutes at room temperature.
8. Adjust the sample volume to between 150 μ L to 200 μ L with 1X Apoptosis Wash Buffer. You do not need to perform additional wash steps.
9. Cell samples are ready for data acquisition on the Guava PCA-96. If cell samples need to be fixed before data acquisition, proceed to *Cell Fixation Procedure*.

IMPORTANT: Once cells have been stained, they should be analyzed within 30 minutes, unless the cells have been fixed. After 30 minutes, unfixed cells can show a decrease in apoptotic cell numbers and an increase in the number of dead cells. Fixing the cells, however, may not be a viable alternative, since some fixed cell lines can still display altered staining patterns after 30 minutes.

8. CELL FIXATION PROCEDURE

NOTE: Stained samples from certain cell lines may be fixed and stored for data acquisition the next day with little or no loss of fluorescent signal. However, not all cell types can be fixed and stored for extended periods of time (for example, stained apoptotic Jurkat cells show significant loss of

SR-VAD-FMK signal when fixed and stored overnight at 2 to 8°C). We recommend performing data acquisition as soon as possible after cell staining, until proven fixation conditions have been established for your samples. Contact Guava Technical Support for additional information.

1. To fix the samples, add the Caspase Fixative reagent at one tenth (0.1X) of the total volume to each sample well. For example, add 20 µL of Caspase Fixative to a stained cell sample in a 200 µL volume.
2. Store samples at 2 to 8°C.
3. Acquire data from fixed samples within 18 hours, unless previous experiments have indicated the sample and signal remain stable for longer periods of time.

9. EXPECTED RESULTS

The Guava MultiCaspase application displays acquired data in two dot plots: FSC vs. SR-VAD-FMK (PM1) and SR-VAD-FMK (PM1) vs. 7-AAD (PM2) fluorescence intensity. The results are displayed on the computer screen after each sample is acquired. Events to be analyzed can be gated to remove undesired debris events using the Gating Dot Plot (FSC vs. SR-VAD-FMK (PM1)) by checking the **Enable Gating** box. Events above the FSC threshold falling within the rectangular gated region are displayed and analyzed on the Apoptosis dot plot. If **Enable Gating** is turned off on the Gating Dot Plot (box is left unchecked), all of the events collected above the FSC threshold are displayed and analyzed on the Apoptosis dot plot.

The Guava PCA-96 system calculates and presents results automatically in the Apoptosis Analysis Results table. Results include the Count (number of events falling in the indicated Apoptosis Dot Plot region), cells/mL, % of All (% of events falling within each region), and mean fluorescence intensities of each population for PM1 and PM2. SR-VAD-FMK(+)/7-AAD(-) results reflect data analyzed from apoptotic events (positive PM1 signal). 7-AAD+ results reflect data analyzed from late apoptotic/dying and dead cell events (positive PM2 signal).

All data and instrument settings are automatically saved in FCS 3.0 file format. You can select the option to export data to a FCS 2.0 file. This allows you to analyze Guava files with other FCS 2.0- and 3.0-compatible programs.

Guava MultiCaspase assay results can vary with the cell type, induction method, and conditions used. Figures 1 through 3 illustrate examples of results we obtained using the Guava PCA-96 MultiCaspase Kit with cell samples under various conditions.

Figure 1 shows the results of staining an untreated Jurkat sample with the MultiCaspase Kit components. The vast majority of the cells appear live, as expected.

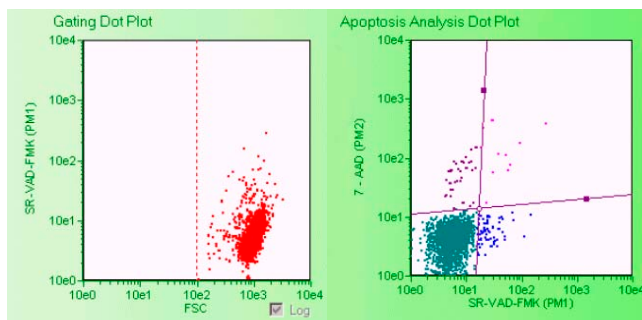


Figure 1 Untreated Jurkat cell sample showing background level of apoptosis. The gating distinguishes the non-apoptotic live cells in teal green (SR-VAD-FMK (-),

Guava® PCA™-96 MultiCaspase Kit

7-AAD (-)) from the mid apoptotic cells in blue (SR-VAD-FMK(+), 7-AAD (-)) and late apoptotic/dying cell populations in pink (SR-VAD-FMK(+), 7-AAD (+)). Dead cell events in purple (SR-VAD-FMK dim or (-), 7-AAD (+)) appear in the upper left area of the dot plot.

Figure 2 shows Jurkat cells rapidly undergoing apoptosis when treated with staurosporine.

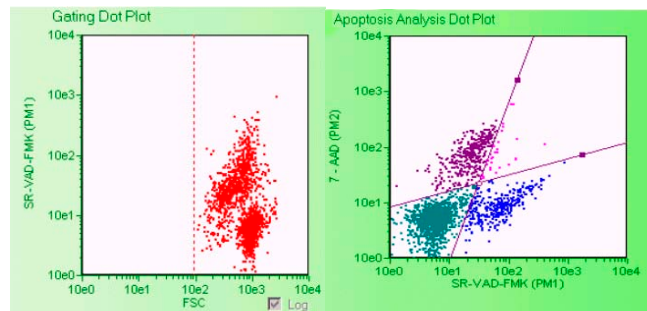


Figure 2 Jurkat cells treated with 1µM staurosporine for 2 hours at 37°C.

Within two hours, 16% of the cells are in early to mid-apoptosis. The equivalent sized population (shown in purple) contains cells that were dead before induction (SR-VAD-FMK (-), 7-AAD(+)) and cells that died via the apoptotic pathway. Very few late apoptotic/dying cells appear in this population. In contrast, CHO-K1 cells take much longer to induce by camptothecin treatment (24+ hours) and show a more gradual apoptotic progression than the Jurkat cells treated with staurosporine (Figure 3).

Figure 3 shows that 13% of the population appears early- to mid-apoptotic (shown in blue), 9% late apoptotic (shown in pink), and 6% dead (shown in purple).

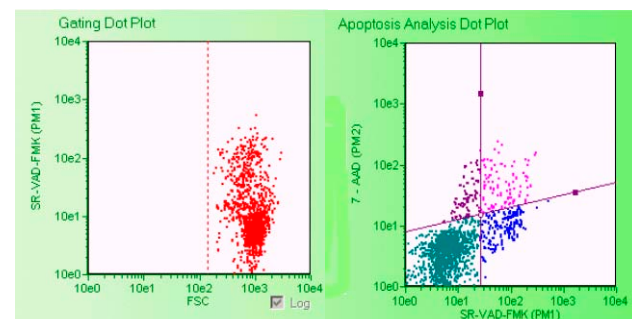


Figure 3 CHO-K1 cells induced for apoptosis (20 µM camptothecin for 24 hours).

Assay Accuracy

We induced thirty one different cell samples, including CHO-K1 adherent cells and Jurkat non-adherent cells, from eight independent experiments to varying levels of apoptosis. We prepared the cell samples according to the PCA and PCA-96 MultiCaspase protocols and analyzed them on both the Guava PCA and Guava PCA-96 systems. Figure 4 presents the results for percent positive apoptotic cells. We obtained comparable results and assay accuracy from both the Guava PCA and Guava PCA-96 systems for the percent of

apoptosis, regardless of the cell type or the extent of apoptotic induction (ranges from 13 to 93%).

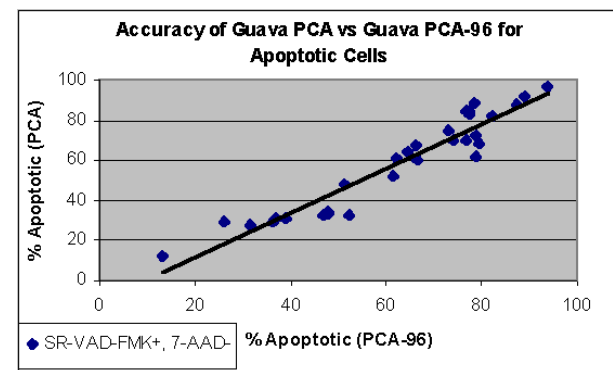


Figure 4 Comparison of % Apoptotic (SR-VAD-FMK+, 7AAD-) results from Guava PCA-96 and Guava PCA. The slope of the trendline is 1.109 and the R squared value is 0.913.

Assay Precision

We induced and stained Jurkat and CHO-K1 cells following the Guava PCA-96 MultiCaspase protocol on two different days. We acquired the replicate samples on two Guava PCA-96 systems over a 30-minute time period (n=48). Tables 1 and 2 present the calculated average values for percent of population and %CV for live cells in uninduced cultures and for apoptotic and dead cells in induced cultures.

Table 1. Precision of MultiCaspase Assay with Jurkat Cells*

Jurkat Cells		Uninduced Cells		Induced Cells			
		Live		Apoptotic		Dead	
Day	PCA-96	Average % of Population	%CV	Average % of Population	%CV	Average % of Population	%CV
1	A	88.7	1.57	79.2	1.87	3.30	14.0
	B	80.3	8.91	79.0	1.82	4.47	11.0
2	A	94.5	0.92	57.9	6.89	3.40	17.6
	B	91.0	2.76	65.6	5.89	3.11	19.7

Table 2. Precision of MultiCaspase Assay with CHO-K1 Cells*

CHO-K1 Cells		Uninduced Cells		Induced Cells			
		Live		Apoptotic		Dead	
Day	PCA-96	Average % of Population	%CV	Average % of Population	%CV	Average % of Population	%CV
1	A	78.9	4.14	33.1	6.87	16.6	12.7
	B	85.8	4.21	29.5	11.3	19.1	11.1
2	A	72.3	5.78	30.3	8.17	11.0	19.6
	B	79.1	4.78	35.8	7.16	9.44	13.0

* All samples (n=48) were acquired within 30 minutes of completion of the staining protocol. On each day, one batch of cells was induced, plated in 48 wells in each of two 96-well microplates, stained using the above protocol and acquired on two Guava PCA-96 systems.

The %CV of live cells in uninduced cultures was less than 9% over the 30-minute time course for both Jurkat and CHO-K1 cell lines. The %CV for the apoptotic population was less than 12%, and the %CV for the dead population was less than 20%. The %CVs for the dead population were somewhat higher than that of live and apoptotic cells because there were so few dead cells. The average % of population values were comparable for the same samples acquired on two Guava PCA-96 systems each day, indicating precision and reproducibility from different instruments.

Cell Concentration

We recommend using at least 5×10^4 cells per well to allow for acquisition within the 30-minute time period; however, you may have less cells in some cases. We compared MultiCaspase results using varying counts of 1×10^4 , 2.5×10^4 , 5×10^4 , and 1×10^5 cells per well. Figure 5 shows the consistency of the varying cell concentrations with apoptotic and dead populations acquired on two Guava PCA-96 systems. We found a difference of less than 10% for the percent Apoptotic population among the various cell concentrations;

likewise, the percentage of the Dead population changed very little over the 10-fold difference in cell concentration.

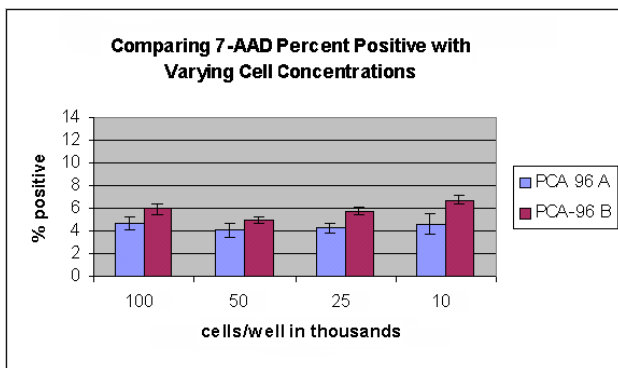
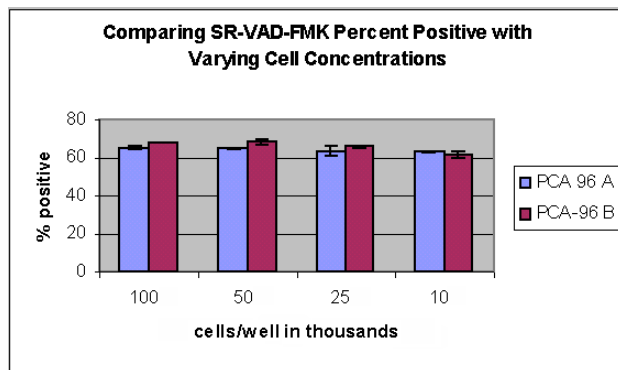


Figure 5 A comparison of the effects on Apoptotic and Dead cell populations using varying cell concentrations for the staining protocol. The percent of apoptotic (SR-VAD-FMK+, 7AAD-) and of late apoptotic/dead (7AAD+) cells were determined. The results shown are the mean +/- SD for 3 replicates at each concentration.

Stability of Stained Samples Over Time

To assess the stability of prepared samples over time, we tested both non-adherent (Jurkat) and adherent (CHO-K1) cells. Our results should be used only as a guideline as different cell lines can exhibit different stabilities over time.

Non-adherent Cells

Non-adherent cells in the Guava MultiCaspase assay are typically stable up to 30 minutes after staining. We observed that after 30 minutes, the percentage of Apoptotic cells in non-adherent Jurkat cells decreased 10 to 25% over the next 30 minutes (see Figure 6).

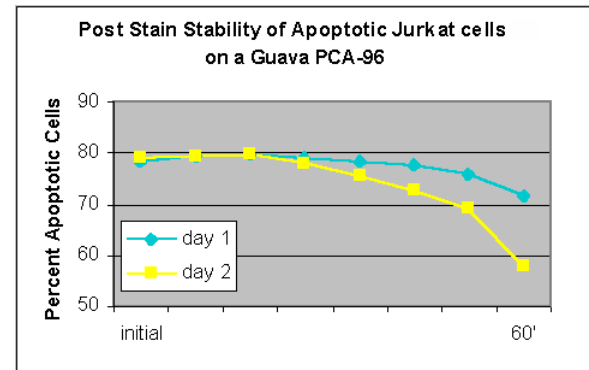


Figure 6 Immediately after staining, Jurkat cell samples were acquired over the course of an hour. Each time point shown corresponds to a row of samples on a 96 well plate (n=12 per time point). The graph shows the % of SR-VAD-FMK+, 7AAD- (apoptotic) cells versus time by row.

Adherent Cells

In the adherent CHO-K1 cells, we observed that the percentage of Apoptotic cells were stable for slightly over 30 minutes. The percentage of Apoptotic cells then decreased approximately 30% between 35 and 60 minutes (see Figure 7).

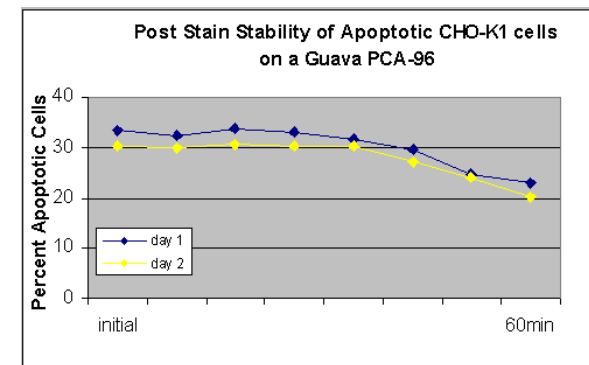


Figure 7 Immediately after staining, the CHO-K1 cell samples were acquired over the course of an hour. Each time point shown corresponds to a row of samples on the 96 well plate (n=12 per time point). The graph shows the % of SR-VAD-FMK+, 7AAD- (apoptotic) cells versus time by row.

10. TROUBLESHOOTING TIPS

1. Dim or false negative staining with the SR-VAD-FMK reagent can indicate reagent degradation. Verify proper storage and handling of the SR-VAD-FMK Reagent and 100X stock solutions. Prepare fresh 5X SR-VAD-FMK working solution just before you are ready to stain cells.
2. If the 7-AAD signal is too strong and causes difficult resolution of the dead vs. late apoptotic/dying population, wash the stained cell sample. Centrifuge the cells and carefully aspirate the supernatant. Resuspend the cell pellet in fresh 1X Apoptosis Wash Buffer or PBS buffer and reacquire the data.

3. If the SR-VAD-FMK or 7-AAD signals are dim or too bright, you may need to optimize the reagent concentration for your cell type by performing a titration.
4. Acquire data from samples as soon as possible after staining. Guava MultiCaspase is a live cell assay; therefore, the staining profiles can change as the cell sample ages. Stability of the stain signals can vary by cell line. Storing stained samples for long periods can adversely affect the results. Guava recommends acquiring data within 30 minutes after completing staining.
5. Avoid excessive exposure of the stained samples to light to prevent fading of the fluorochromes.
6. If the concentration of the stained cell sample for data acquisition is high (greater than 5×10^5 cells/mL), the Guava PCA-96 system may not yield accurate results. Dilute the sample further with additional 1X Apoptosis Wash Buffer or PBS to bring the cell concentration to the recommended range (between 1×10^4 to 5×10^5 cells/mL).
7. We recommend acquiring 2000 events per sample, after gating out debris, to ensure an adequate number of cellular events. You can acquire more or less events, but your statistical error increases as the number of events for acquisition decreases.
8. Check that the mixing option has been selected to ensure accurate readings.
9. Run Guava Check™ (Catalog No. 4500-0020) to verify proper instrument function and accuracy.
10. To prevent a buildup from cell debris in the flow system, run Quick Clean using a deionized water tube after every 12 to 24 sample acquisitions. If your samples contain significant cellular debris, run Quick Clean with Guava ICF followed by water.
11. If you are acquiring data from a sample but the Cell Count number is not increasing and the Events to Acquire bar is not moving, there is probably either insufficient volume to continue to acquire sample, or a blockage of the flow system. Check first for the lack of sufficient sample volume by pausing the CytoSoft application and ejecting the tray. If the sample volume is below 50 μ L, there is not enough sample for the instrument to acquire. Either add additional buffer to bring the sample volume up to greater than 50 μ L, or proceed to the next sample. If the sample volume is more than 50 μ L, then the lack of events acquired is probably due to a clog. A clog or blockage of the flow system can be caused by cell aggregates, cell debris, bleach crystals, or other particulates. Click **Backflush** to flush out the clog into a tube containing 20% bleach. Then 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.
12. For more troubleshooting tips, refer to the *Guava PCA-96 System User's Guide*, or contact Guava Technical Support.

11. LIMITATIONS

1. The Guava PCA-96 yields optimal results when the stained cell sample for data acquisition is between 1×10^4 to 5×10^5 cells/mL. To obtain the most accurate assay results, adjust the concentration of the cell samples to within the recommended range.
2. Guava MultiCaspase assay reagents are formulated to meet most assay requirements. You may need to modify the staining protocol and reagent concentration(s) to ensure optimal performance for your assays.

12. REFERENCES

1. Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. *Cell*, 1997; 91:443-446.
2. Slee EA, et al. Serial killers: ordering caspase activation Events in apoptosis. *Cell Death and Differ.*, 1999; 6:1067-1074.
3. Alnemri ES, et al. Human ICE/CED-3 protease nomenclature. *Cell*, 1996;87:171.
4. Ekert PG, et al. Caspase inhibitors. *Cell Death and Differ.*, 1999;6:1081-1086.

13. DISCLAIMER OF WARRANTY

The product sold hereunder is warranted only to conform to the quantity and contents stated on the label at the time of delivery to the customer. There are no warranties, expressed or implied, which extend beyond the description on the label of the product. The sole liability for Guava Technologies, Inc. is limited to either replacement of the products or refund of the purchase price. Guava Technologies, Inc. is not liable for property damage, personal injury, or economic loss caused by the use of this product.

14. RETURNED GOODS POLICY

Please inspect package(s) upon receipt and inform us immediately of any shortage or shipping errors. Claims must be made within 10 business days. Call our Customer Service department so they can authorize a return and provide shipping instructions.

15. TRADEMARKS AND PATENTS

Microsoft is a registered trademark of Microsoft Corporation.

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

Guava Check, ICF, PCA, and CytoSoft are trademarks of Guava Technologies, Inc.

For ordering information or technical support, call toll-free:

(866) 448-2827 or visit:
www.guavatechnologies.com.

For more information, email:
www.info@guavatechnologies.com

25801 Industrial Blvd.
 Hayward, CA 94545-2991
 Tel 510.576.1400
 Fax 510.576.1500

Revision Date: October 24, 2003
 4600-0590 rev A

© 2003 Guava Technologies, Inc.
 All Rights Reserved
 Printed in U.S.A.

