

# Guava® CellToxicity™ Assay: A Novel Fluorescent Assay for Measuring NK Effector Function

Katie Gillis and Dianne Fishwild

## ABSTRACT

Cell cytotoxicity as a measure of Natural Killer (NK) or other immune cell effector function has traditionally been examined using <sup>51</sup>-Chromium release assays.<sup>1</sup> Recently, to avoid costs associated with handling and disposal of radioactive waste, a number of fluorescence based flow cytometric assays have been developed as alternatives.<sup>2-5</sup> Here we describe a novel assay in which the target cells, K562, are first labeled or “painted” with CFSE (a fluorescent indicator dye), incubated with NK effector cells and then monitored for the percentage killed using a second fluorescent indicator dye, 7-ADD. Both live and dead targets retain the signal from the CFSE dye, as it is covalently linked to intracellular amine groups upon internalization and

does not leach from cells into the media nor is transferred to adjacent cells. Dead target cells in addition take up the membrane impermeant 7-ADD dye. Thus, on the benchtop microcytometry instruments—the Guava PCA™ and PCA-96 systems—the live cells which fluoresce orange can be readily distinguished from the dead cells which fluoresce orange and red. Using this assay, increasing amounts of dead target cells were detected and quantified as increasing amounts of NK effectors purified from whole blood were added. Results were comparable to those obtained with other NK assays. Increasing amounts of NK activity were also detected when NK cells were pretreated with IL-2. The Guava CellToxicity assay was also used to demonstrate potent T-cell mediated cytotoxicity (CMC) against allogeneic target cells. Similar experiments can be run on the same system for antibody-dependent cell-mediated cytotoxicity (ADCC). Thus, the Guava CellToxicity assay and the Guava platforms provide an intuitive two-color system for assessing cytotoxicity in a number of formats in which live and dead cells are readily distinguished and analyzed based on their differential fluorescence, and the data are displayed in a convenient format for simple interpretation of percent of killed target cells by the user.

## INTRODUCTION

One of the functions of the immune system is to recognize and destroy targets, such as tumor cells or cells infected with microbes (bacteria or viruses). This is accomplished by effector cells, such as T cells, NK cells, macrophages or granulocytes, which employ cell mediated cytotoxicity (CMC), natural killer activity (NK) or antibody-dependent cellular cytotoxicity (ADCC), respectively. CMC and NK activities are commonly monitored in order to assess the potency of immune cell stimulation by small molecules, antibodies or other regulatory molecules, whereas ADCC activity is used to assess the potency of antibodies for therapy.

The Guava CellToxicity assay uses two dyes, a cell painting and a cell impermeant DNA binding dye, to distinguish effector or cytotoxic cells from target cells, and to distinguish dead target cells from live target cells. The cell painting dye, CFSE (also known as CFDA SE), diffuses freely into cells where intracellular esterases cleave off the acetate groups, converting it to a fluorescent, membrane impermeant dye. The dye is retained in the target cell's cytoplasm even after the cell is killed due to covalent crosslinking to proteins through its succinimidyl groups. Painted target cells are subsequently added to unpainted effector cells at various ratios and incubated to allow effector cells to function. A dead cell dye, 7-AAD, is then added to determine which of the painted target cells have been killed.

## MATERIALS AND METHODS

### NK Assays

**Cultured Cells.** A non-adherent human bone marrow cell line, K562 (ATCC Cat. No. CCL-243), and a non-adherent human B cell line, Daudi (ATCC Cat. No. CCL-213), were kept in log phase growth in complete medium designed to stimulate optimal growth. Iscove's modified Dulbecco's medium (Cellgro Cat No. MT 10-016-CV) supplemented with 10% FBS (ATCC Cat. No. 30-2020) and 2 mM L-glutamine (Cellgro Cat. No. MT 25-005-CV) was used for culturing the K562 cells. RPMI 1640 (Cellgro Cat. No. MT-10-04-CV) medium supplemented with 10% FBS, 2 mM L-glutamine, 4.5 g/L glucose (Sigma Cat. No. G8769), and 1 mM sodium pyruvate (Cellgro Cat. No. MT 25-000-CL) was used for culturing the Daudi cells.

**Purification of NK cells.** Peripheral blood mononuclear cells (PBMCs) from anonymous healthy adult blood donors were obtained by gradient centrifugation over Histopaque Plus (Amersham BioSciences Cat. No. 17-1440-02)<sup>6</sup> of day old blood. NK cells were purified from PMBCs using the NK Cell Isolation Kit II (Miltenyi BioTech Cat. No. 130-091-152) following the manufacturer's protocol and resuspended in the target cell medium. NK cells were typically >90% pure.

**Guava CellToxicity Assay.** For the NK cell experiments, the Guava CellToxicity assay (Guava Cat No. 4500-0200) was conducted according to the manufacturer's instructions. Briefly, cells were seeded two days prior to each experiment as follows: the K562 cells were split to a final cell concentration of 300,000 cells/mL and the Daudi cells were split to a final cell concentration of 400,000 cells/mL. The K562 and Daudi cells were incubated with 5  $\mu$ M CFSE and washed to remove excess dye. For those samples to be acquired on the flow cytometer, target cells were painted with a 10-fold lower concentration of CFSE due to the increased excitation of CFSE with 488 nm versus 532 nm laser light. The painted target cells were then mixed with the purified NK cells at various effector to target ratios as described below (including target cells only and effector cells only) in 96-well round bottom plates (unless otherwise indicated) and incubated for 4 hours in a 37 °C humidified 5% CO<sub>2</sub> incubator unless otherwise indicated. In some experiments, Interleukin 2 (IL-2; CalBiochem Cat No. 407623)<sup>7</sup> was added to each well along with the effectors and targets prior to incubation. After incubation, 7-ADD was added to all wells. Samples were then transferred to microcentrifuge tubes for acquisition on the Guava PCA, transferred to 12 x 75 mm polystyrene tubes for acquisition on the flow cytometer, or left in the 96-well microplates for acquisition on the Guava PCA-96 system. In some cases, the Molecular Probes Live/Dead Cell Mediated Cytotoxicity Kit (Cat No. L-7010) was also used according to manufacturer's instructions.

### Cell Mediated Cytotoxicity Assay

**Allogenic Stimulation.** PBMCs from anonymous unrelated healthy adult blood donors were obtained by gradient centrifugation over Histopaque Plus of day old blood. Half the PBMCs from donor A were frozen down in RPMI supplemented with 20% FBS, 2 mM

**Application Note:** A Novel Fluorescent Assay for Measuring NK Effector Function

L-glutamine and 7.5% DMSO (Sigma Cat. No. D2650).<sup>8</sup> To prepare stimulator cells, the second half of the PBMCs from donor A were incubated with 25 µg/mL of mitomycin C (Calbiochem Cat. No.107-409)<sup>9</sup> in RPMI supplemented with 10% FBS and 2 mM L-glutamine, in a 37 °C humidified 5% CO<sub>2</sub> incubator for 30 minutes. The stimulator cells were washed three times with the supplemented RPMI and were then mixed at a 1:1 ratio with responder cells (PBMCs from donor B). The cell concentration of the mixture was adjusted to 1.0 x 10<sup>6</sup> cells/mL and incubated in a 37 °C humidified 5% CO<sub>2</sub> incubator for six days. On day 5, the frozen PBMCs from donor one were thawed<sup>10</sup> and resuspended in supplemented RPMI and incubated overnight in a 37 °C humidified 5% CO<sub>2</sub> incubator.

**Guava CellToxicity Assay.** For the CMC experiments, the Guava CellToxicity Assay was conducted according to the manufacturer's instructions. Briefly, the thawed PBMCs from donor A (targets) were incubated with 5 µM CFSE and washed to remove excess dye. The effector cells (responders from donor B plus mitomycin C-treated stimulators from donor A cultured for a week) were washed once with supplemented RPMI and resuspended in supplemented RPMI. The painted target cells were then mixed with the effector cells at various effector to target ratios as described below in 96-well round bottom plates and incubated for 4 hours in a 37 °C humidified 5% CO<sub>2</sub> incubator. After incubation, 7-ADD was added to all wells. Samples were then acquired on the Guava PCA-96 system.

## RESULTS

Experiments were conducted to demonstrate the utility of the Guava CellToxicity assay to quantify NK activity against K562 and Daudi cells, and CMC activity against allogeneic PBMC targets.

Figure 1 shows typical results obtained using the Guava CellToxicity kit. The dot plot shows a clear distinction between the live and dead target cells. In addition, there is a clear separation between the effector cells and the target cells so that any bystander killing of effector cells does not contribute to the measured percent of target cells killed, as can happen in assays that monitor population effects rather than individual cells. Each cell population is displayed using a unique color code. In this example, purified NK effectors

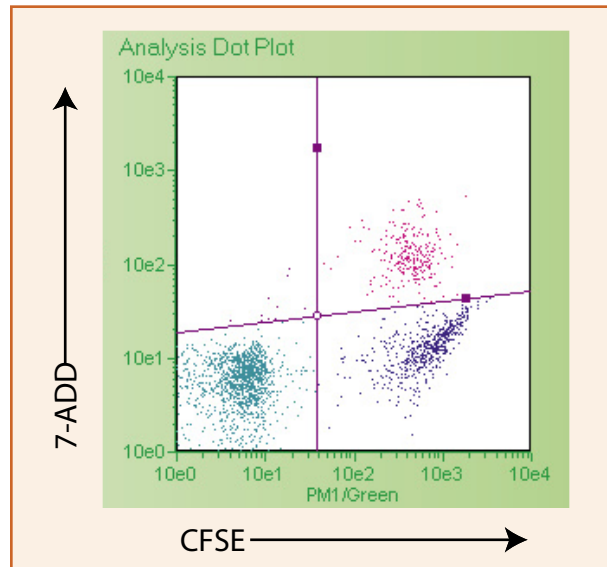


FIGURE 1: Detection of Live and Dead Target Cells. Unpainted, 7-AAD negative, live effector cells appear in the lower left quadrant (teal green), painted, 7-AAD negative, live target cells appear in the lower right quadrant (blue), unpainted, 7-AAD positive, dead effector cells appear in the upper left quadrant (purple), and painted, 7-AAD positive, dead target cells appear in the upper right quadrant (pink).

mixed with painted K562 cells at a 1:1 ratio resulted in approximately 50% killing of the target cells after a 4 hour incubation.

### Guava CellToxicity Accuracy

To assess the ability of the Guava CellToxicity protocol to accurately determine the percent of target cells killed, purified NK cells and CFSE-painted K562 cells were mixed at various effector to target ratios, incubated, and then stained with 7-ADD. These samples were then compared to similar samples prepared as follows:

- 1) following the Molecular Probes Live/Dead Cell-Mediated Cytotoxicity Kit; or
- 2) with CFSE/7-ADD staining optimized to run on a traditional flow cytometer (0.5 µM CFSE).

Samples were run in triplicate on either the Guava PCA-96 (Guava CellToxicity Kit) or a traditional flow cytometer (Live/Dead Cell-Mediated CytoToxicity kit and 0.5 µM CFSE). As shown in Figure 2, the results obtained from the three assays at two time points were similar with very low variation, as indicated by the small error bars.

### Guava CellToxicity Specificity

Daudi cells are much more resistant to NK activity than are K562 cells. To determine whether the Guava CellToxicity assay could demonstrate differential degrees of activity against NK sensitive and NK resistant cells, K562 cells and Daudi cells were separately painted and mixed with purified NK cells obtained from the same donor at various effector to target ratios following the protocol described above. Each sample was then acquired 16 times on two Guava PCA-96 systems. Figure 3 shows that, as expected, much more NK mediated killing of K562 cells was detected than of Daudi cells at all effector to target ratios. Moreover, data in this figure demonstrate that results obtained on different instruments with independently set up assay plates were highly reproducible. Also note that there was little variation between the samples as shown by the small standard deviation bars.

### Guava CellToxicity Precision

Precision of the percentages of target cells killed as well as of the mean fluorescence intensities (MFIs) for each population are important because they measure how reliable any one particular result might be. To assess the precision of the Guava CellToxicity assay for percent of target cells killed, six different mixtures of effector to target ratios were prepared using NK and K562 cells and placed in every sixth well of a 96-well round bottom plate until all the wells of the plate were filled. Figure 4 shows the individual data obtained for percent of target cells killed at five of the six ratios over the entire plate and demonstrates that there is no bias of the results over time or well location. Notice that the percentages of target cells killed for all samples were consistent over the time it took (approximately 1.5 hours) to acquire the entire plate.

FIGURE 4: Precision of Percent of Target Cells Killed. Data are derived from the same experiment using NK cells and K562 cells shown in Figure 3, except here the data for each individual well is shown. The data for the 0.2:1 effector to target ratio are omitted for the sake of clarity.

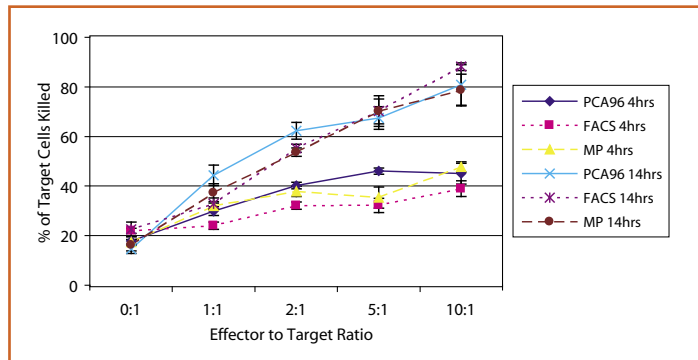


FIGURE 2: Accuracy of Percent of Target Cells Killed. K562 target cell killing was assessed using the Guava CellToxicity Kit (PCA96), CFSE/7-ADD staining optimized for the BD FACSCalibur™ (FACS), and the Molecular Probes Live/Dead Cell-Mediated CytoToxicity Kit (MP). Each point is shown as the mean plus or minus the standard deviation of three samples. K562 cells were painted with CFSE or DiOC18 (Molecular Probes kit), mixed with unpainted NK cells at the indicated ratios and stained with 7-AAD following 4 and 14 hour incubations in a 96-well flat bottom plates.

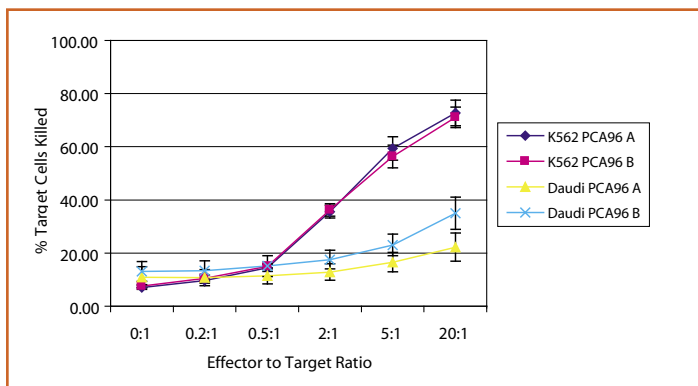
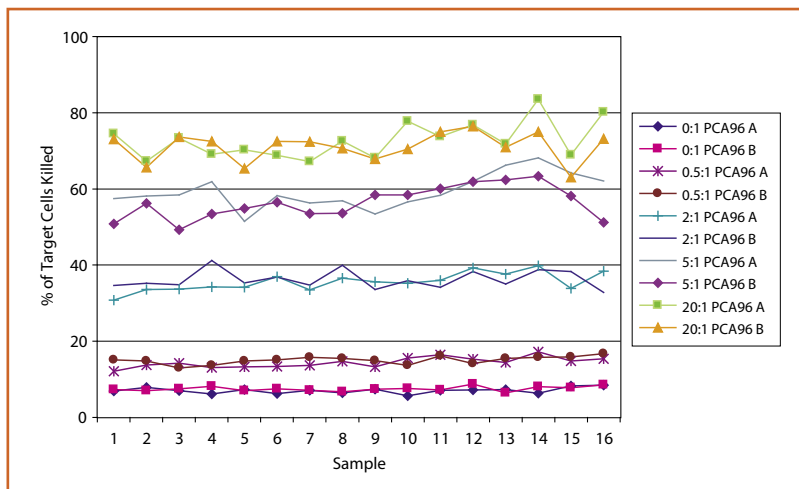


FIGURE 3: Specificity of Target Cells Killed. The percent of target cells killed was determined for wells containing six different effector to target ratios, evenly distributed across a 96-well plate. Purified NK cells were mixed with either painted K562 or painted Daudi cells. Replicate plates were set up and acquired on two different Guava PCA-96 systems. Each point is shown as the mean plus and minus the standard deviation for sixteen replicate samples.



As shown in Table 1, the %CVs for percent of target cells killed were under 10% at effector to target ratios that resulted in killing above background.

While the percent of targets killed data were very uniform over the course of a 96-well plate, it is also important to show that the MFI values are stable. Clear and consistent delineation of the populations of interest—target versus effector and live versus dead—as reflected by the MFI values is critical. Table 2 shows the averages and %CVs for the CFSE (PM1) and 7-ADD (PM2) MFIs for the painted (target) cells. The %CVs for the MFIs were well under 10% in 40 of the 48 test cases, showing that the populations stay consistent over the entire plate. The differences in average MFI values between the two instruments are due to slight differences in how the individual instruments were set up prior to acquiring the data and do not affect the interpretation of the results.

### Post Staining Stability

To determine the stability of the sample post staining, painted K562s were mixed with purified NK cells at the indicated ratios and acquired at various times from immediately after completion of the sample

preparation protocol to 7 hours later on two Guava PCA-96 systems. As shown in Figure 5, the percentage of target cells killed was stable for at least 7 hours for all the effector to target ratios. The MFI values for all populations of cells also remained stable, typically only varying 10% over the 7 hour period (data not shown).

### Optimal Conditions for Cytotoxicity Assays

To determine the optimal conditions for effective killing, various studies to examine the effect of the culture vessel were done. Painted K562 cells were mixed with purified NK cells at various effector to target ratios and incubated in the following vessels: 1.5 mL microcentrifuge tubes, round bottom 96-well plates and flat

bottom 96-well plates. As shown in Figure 6A, there is less NK activity detected in 1.5 mL microcentrifuge tubes than in the round bottom 96-well plates. Similarly, as shown in Figure 6B, flat bottom 96-well plates were also less effective at promoting NK cytotoxicity when compared to the round bottom plates. However, Figure 7 shows that additional cytotoxic activity can be seen if cells in flat bottom plates are incubated for longer times. In this particular

**Table 1: Precision of Percent of Target Cells Killed**

%CV for Target Cells Killed		
Effector: Target Ratio	PCA96 A	PCA96 B
0:1	10.9%	8.7%
0.2:1	10.5%	11.7%
0.5:1	9.3%	6.6%
2:1	6.8%	6.6%
5:1	7.5%	7.6%
20:1	6.6%	5.4%

**Table 2: Precision of Fluorescent Intensity of Live and Dead Target Cells**

Effector: Target Ratio		CFSE ( PM1) MFI				7-ADD (PM2) MFI			
		Dead Targets		Live Targets		Dead Targets		Live Targets	
		PCA96 A	PCA96 B	PCA96 A	PCA96 B	PCA96 A	PCA96 B	PCA96 A	PCA96 B
0:1	Average	395	228	703	405	124	173	12	17
	CV	7.3%	13.2%	4.0%	5.4%	7.9%	8.6%	3.1%	4.6%
0.2:1	Average	399	231	719	401	122	167	12	17
	CV	6.9%	10.8%	3.2%	6.9%	8.3%	8.6%	2.3%	5.4%
0.5:1	Average	418	241	707	404	123	168	12	17
	CV	5.3%	6.6%	2.4%	4.5%	7.3%	6.9%	2.0%	3.1%
2:1	Average	430	246	687	393	127	171	12	18
	CV	3.0%	5.8%	1.9%	4.7%	11.2%	6.5%	2.5%	5.1%
5:1	Average	425	242	672	367	130	177	13	18
	CV	3.7%	6.1%	4.6%	4.7%	14.8%	9.3%	5.0%	4.7%
20:1	Average	433	230	642	347	148	180	13	18
	CV	5.2%	8.5%	9.8%	14.1%	17.8%	11.0%	10.8%	5.6%

**Application Note: A Novel Fluorescent Assay for Measuring NK Effector Function**

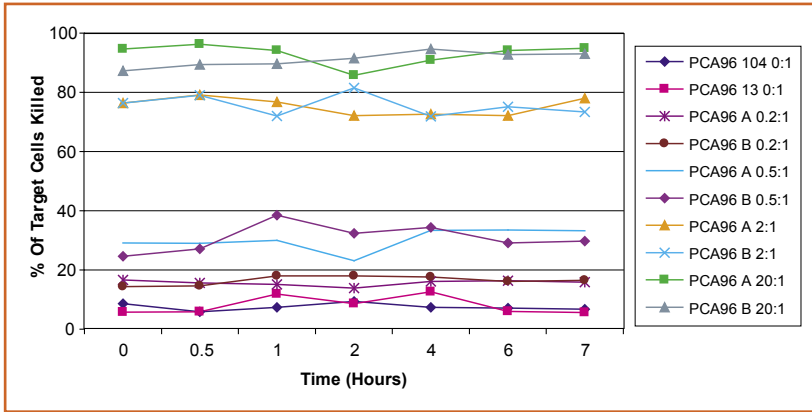


FIGURE 5: Assessing the Stability Over Time of the NK Assay. Replicate samples were prepared at six different effector-to-target ratios, incubated for 4 hours at 37 °C, and then kept in the dark at RT until acquired at the indicated times. Each point on the graph was derived from 3 samples. Similar results were obtained for the 10:1 effector to target ratios, but were omitted from the graph for clarity.

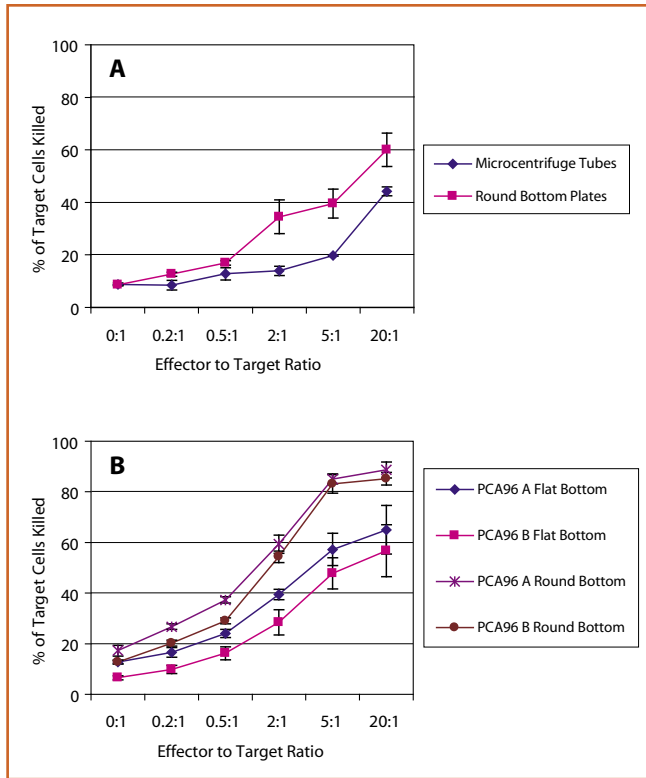


FIGURE 6: Effect of Tissue Culture Vessel on Percent of Target Cells Killed. Incubation of effectors and targets in A) 1.5mL microcentrifuge tubes versus round bottom 96-well plates, or B) flat bottom 96-well plates versus round bottom 96-well plates was compared using K562 cells mixed with purified NK cells. Each point is shown as the average plus and minus the standard deviation for three replicate samples.

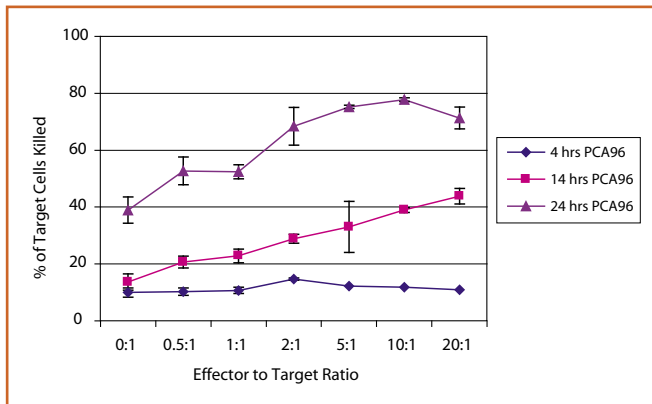


FIGURE 7: Comparison of Incubation Times in Flat Bottom Plates for K562 Cells. Each point is shown as the average plus and minus the standard deviation for three replicate samples.

case, no NK activity was detected at 4 hours but a low level could be detected at both 14 and 24 hours. Note that while the percent of dead targets detected did increase after a 24 hour incubation, addition of effector cells still caused an effector dependent increase in the percent of target cells killed. These results suggest that the shape of the tissue culture vessel and perhaps other variables (different plastic, different pH, etc.) determines how well effector cells can locate and subsequently kill target cells. Thus for optimal NK activity, all incubations should be done in round bottom 96-well plates and if needed samples transferred to other vessels for acquisition.

Generally, for cytotoxic assays, the same number of target cells is placed in every well and different concentrations of effector cells are used. This can greatly affect the total cell concentration per well. To determine if a constant cell concentration could be used with varying amounts of target cells, K562 cells were painted and mixed with purified NK cells at both a constant cell concentration (500 cells/ $\mu\text{L}$  and 125 cells/ $\mu\text{L}$ ) and constant target concentrations ( $1.0 \times 10^4$  and  $2.0 \times 10^4$  target cells per well), stained following the above protocol, and run on the PCA-96. Identical results were obtained, regardless of whether a constant total cell concentration or a constant target cell concentration was used (Figure 8). To minimize the acquisition time on the Guava PCA and PCA-96 systems, it is recommended to use a constant total cell concentration when setting up the effector and target cells.

### IL-2 Titration

IL-2 is known to induce a dose dependent increase in NK activity. To demonstrate this with the Guava CellToxicity Assay, painted K562 cells were mixed with purified NK cells and 0, 2.5, 25 and 100 ng/mL of IL-2 was added to each well prior to the start of the 4 hr incubation. As shown in Figure 9, even at 2.5 ng/mL of IL-2 a large increase in the amount of killing that occurs can be seen at the 1:1 effector to target ratio.

### Cell Mediated CytoToxicity

To ensure that the Guava CellToxicity kit could be used for cytotoxic assays other than NK assays, cell mediated cytotoxicity (CMC) experiments were done. Allogeneically stimulated effector PBMCs were mixed with target cells from the same donor used to stimulate the effector PBMCs as described above at various ratios and run on the Guava PCA-96. As shown in Figure 10, the Guava CellToxicity kit detected CMC. As expected, higher effector to target ratios were necessary to see the same percent of target cells killed as in the NK assays. Also note that there is small variation between samples as shown by the small standard deviation bars.

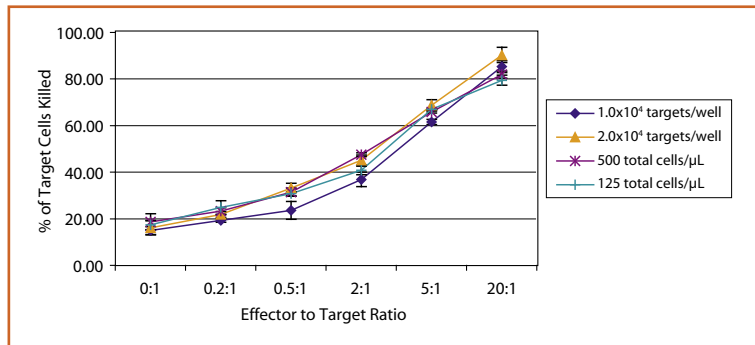


FIGURE 8: Comparison of Target Cell Concentration for K562 Cells. Each point is shown as the average plus and minus the standard deviation for three replicate samples.

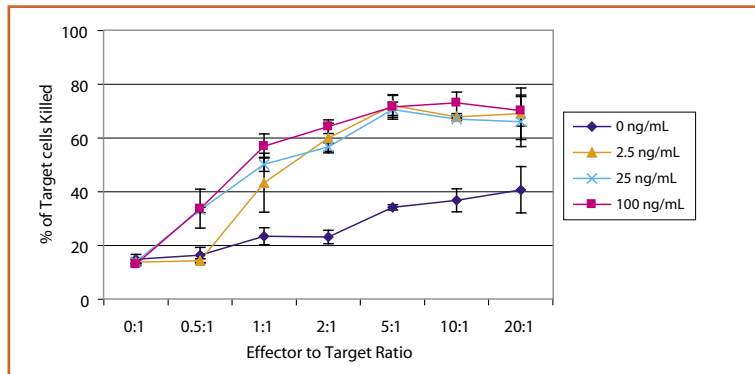


FIGURE 9: Effect of IL-2 on NK Activity. IL-2 was included at the indicated concentrations during the incubation of painted K562 cells mixed with purified NK cells. Each point on the graph is shown as the average plus and minus the standard deviation for three replicate samples.

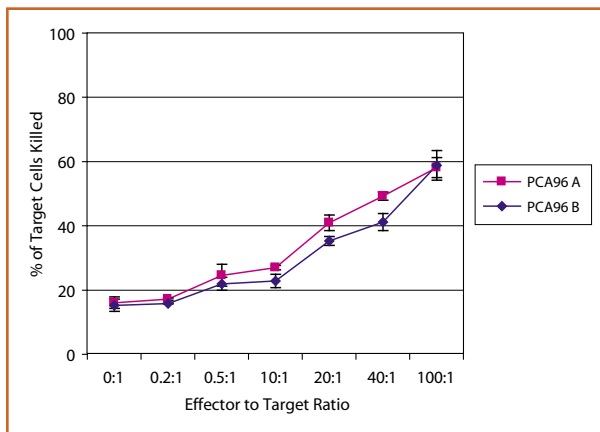


FIGURE 10: Detection of Cytotoxicity Against Allogeneic Target cells. Cell mediated cytotoxicity (CMC) was determined using allogeneically stimulated PBMCs mixed with painted allogeneic PBMCs. Each point on the graph is shown as the average plus and minus the standard deviation for three samples.

## SUMMARY AND CONCLUSIONS

The Guava CellToxicity assay was shown to:

- Enable easy and straightforward sample preparation, permitting completion of the assay in one day;
- Allow the user to easily distinguish between the live and dead target cells in a single sample;
- Differentiate dead effector cells from dead target cells, thereby decreasing the nonspecific background;
- Automatically calculate and report the percent of target cells killed for each sample;
- Report cell concentration and percent of total or gated for each of four cell populations (live and dead effectors and targets)
- Accurately determine the percent of killing present at a variety of effector to target ratios;
- Precisely determine the percent of killing of target cells and the MFI of the target cells, with CVs of typically less than 10% across a 96-well plate;
- Cytotoxicity results stable for 7 hours after end of incubation and prior to acquisition;
- Work for both NK and CMC assays; and
- Detect enhanced NK activity when co-cultured with IL-2.

## REFERENCES

1. Measurement of polyclonal and antigen-specific cytotoxic T-cell function. *Current Protocols in Immunology* 1996, 17.7.1–4.
2. Godoy-Ramirez, K., Franck, K., and Gains, H. A novel method for the simultaneous assessment of natural killer cell conjugate formation and cytotoxicity at the single-cell level by multi-parameter flow cytometry. *J Immunol Methods* 2000; 239:35–44.
3. Lee-MacAry, A.E., Ross, E.L., Davies, D., Laylor, R., Honeychurch, J., Glennie, M.J., Snary, D., and Wilkinson, R.W.. Development of a novel flow cytometric cell-mediated cytotoxicity assay using the fluorophores PKH-26 and TO-PRO-3 iodide. *J Immunol Methods* 2001; 252:83–92.
4. Lecoeur, H., Fevrier, M., Garcia, S., Riviere, Y., and Gougeon, M.L.. A novel flow cytometric assay for quantitation and multiparametric characterization of cell-mediated cytotoxicity. *J Immunol Methods* 2001; 253: 177–87.
5. Jedema, I., van der Werff, N.M., Barge, R.M., Willemze, R., and Falkenburg, J.H. New CFSE-based assay to determine susceptibility to lysis by cytotoxic T-cells of leukemic precursor cells within a heterogeneous target cell population *Blood* 2004; 103:2677–82.
6. Boyum, A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 168; (suppl 97)21:51–76
7. Domzig, W., Standler, B., Herberman R. Interleukin 2 Dependence of Human Natural Killer (NK) Cell Activity. *J Immunol* 1983; 130:1970–1973
8. *Current Protocols in Cytometry*, J.P. Robinson, Ed., 1997; A.3B.5–A.3B.6
9. *Current Protocols in Cytometry*, J.P. Robinson, Ed., 1997; 7.10.3
10. *Current Protocols in Cytometry*, J.P. Robinson, Ed., 1997; A.3B.7



Guava Technologies®

### USA & World

**Guava Technologies, Inc.**  
25801 Industrial Blvd.  
Hayward, CA 94545 USA  
Tel: 866.448.2827  
www.guavatechnologies.com  
info@guavatechnologies.com

### European Office

**Guava Technologies**  
Guava House  
Drope Rd.  
St. Georges Super Ely  
Cardiff CF5 6EP UK  
Tel: +44 1446 760112  
Fax: +44 1446 761015  
info@guavatechnologies.com

© 2004 Guava Technologies Inc. All rights reserved. Guava is a registered trademark and PCA and CellToxicity are trademarks of Guava Technologies, Inc. FACSCalibur is a trademark of Becton-Dickinson Corporation.