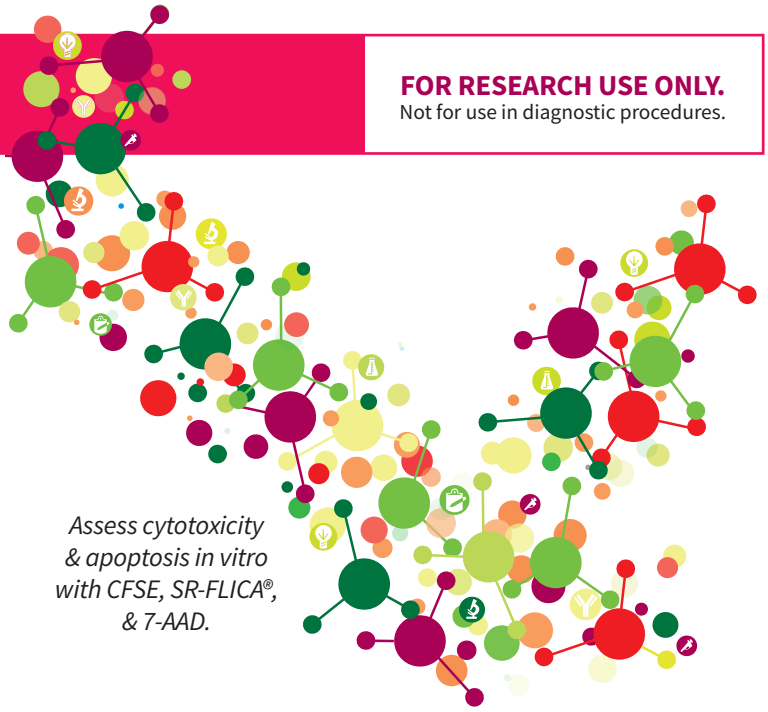
Total Cytotoxicity & Apoptosis Assay

Catalog #971, #972

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.



Assess cytotoxicity & apoptosis in vitro with CFSE, SR-FLICA®, & 7-AAD.

1. INTRODUCTION

Cytolytic activity is an important process for eliminating intracellular pathogens and cancer cells. This process is accomplished through various immune effector mechanisms including natural killer (NK) leukocytes. NK activity is facilitated by non-specifically lysing infected targets through the use of NK receptors, or the FcγII (CD16) receptor, recognizing IgG bound to specific antigens on the target cell surface[1]. NK cells may also induce apoptosis in target cells. The activity of natural killer cells, and their effect on target cells, is frequently studied in immunomodulation experiments.

Older methods to assess NK cytolytic activity include measuring the release of lactate dehydrogenase, and more commonly, the release of radioactive ^{51}Cr from lysed target cells[1]. Unfortunately, these techniques have several drawbacks. Traditional enzyme-release assays are often skewed by the large number of necrotic effector cells. Problems associated with ^{51}Cr release methods include high spontaneous leakage resulting in high backgrounds, high cost, short half-life, and the health risks due to exposure to radioactive material[2]. Beyond these limitations, these assays frequently underestimate the true level of cytotoxicity, as they are unable to detect early-stage apoptotic cells.

Flow cytometric assays have been developed to overcome some of the difficulties associated with older assays like lactate dehydrogenase and ^{51}Cr release assays. Once such early version involved the detection of NK cytotoxicity activity by staining target cells with the green fluorescent dye, F-18, in combination with the DNA intercalating dye, propidium iodide[3]. Since then, a red fluorescent membrane dye, PKH-26, has been used in preference to F-18, and in combination with the viability probe, TO-PRO-3 iodide[4-7]. However, despite correlations of greater than 95% when compared with the ^{51}Cr release assay[1], the PKH-26 method is problematic. It is difficult to use at a constant concentration, leading to unreliable staining, and the staining procedure requires multiple steps, often decreasing the viability of the target cells.

More recently, the problems with older flow cytometric assays were overcome with the use of 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE), a green fluorogenic reagent that diffuses into target cells and covalently binds to primary amino groups on intracellular molecules[8]. Intracellular esterases quickly cleave the acetate groups from the dye, thus converting it to its green fluorescent form. Any unbound reagent diffuses back out of the cell. Building upon these techniques, ImmunoChemistry Technologies has developed the Total Cytotoxicity & Apoptosis Assay, a flow cytometric assay combining the green fluorescing cellular stain, (CFSE), with a red fluorescing live/dead stain, 7-aminoactinomycin D (7-AAD), and ICT's SR-FLICA® apoptosis detection reagent to concurrently quantify caspase-positive cells. The assay can be used to determine total cytotoxicity in the form of apoptosis and necrosis. It will quantify 4 populations of cells: live; early apoptotic; late apoptotic; and necrotic cells within a single sample tube.

While other methods often underestimate the true level of cytotoxicity, ICT's Total Cytotoxicity & Apoptosis Assay is the best method to accurately quantify cell death because it can detect cells in early apoptosis. This often reveals a significant percentage of cells that are 7-AAD negative (indicating that they are alive and do not have compromised membranes), but are SR-FLICA positive (meaning that they are becoming apoptotic and dying and have active caspase enzymes).

In the assay, CFSE is first used to label the target cell population green (Figure 4). The unstained effector cells are then added and incubated with the target cells (referred to as the Effector:Target, or 'E:T' mixture, Figure 5). As all the target cells are initially labeled with green fluorescing CFSE, and the effector cells are not, these two populations can be easily distinguished (Figure 8). Apoptotic target cells can then be identified by labeling with the second reagent, SR-FLICA (Figure 6). SR-FLICA is an orange/red fluorescent poly caspase inhibitor, SR-VAD-FMK, which binds to active caspase enzymes up-regulated for apoptosis[9]. Upon completion of the E:T incubation (which includes exposure to the apoptosis detection reagent), the last reagent, 7-AAD, is added to stain all dead cells red by binding to the DNA of membrane-compromised cells (Figure 7). With proper compensation and gating of the flow cytometer using the designated instrument controls (Figures 3 and 8-13) researchers can distinguish between target and effector cells, and living, necrotic, and apoptotic cells, and assess the level of cytotoxicity in their samples (Figures 14-15).

2. KIT CONTENTS

Catalog #971, Small Size, 125 Tests, contains:

- 1 vial CFSE, green cellular stain, 250 Tests, #6162
- 1 vial SR-VAD-FMK, orange/red poly caspase inhibitor, 125 Tests, #6221
- 1 vial 7-AAD, red live/dead stain, 125 Tests, #6163
- 1 bottle 10X Assay Buffer, 30 mL, #6161

Catalog #972, Large Size, 250 Tests, contains:

- 1 vial CFSE, green cellular stain, 250 Tests, #6162
- 2 vials SR-VAD-FMK, orange/red poly caspase inhibitor, 125 Tests each, #6221
- 2 vials 7-AAD, red live/dead stain, 125 Tests each, #6163
- 1 bottle 10X Assay Buffer, 60 mL, #685

3. STORAGE

The entire kit may be stored frozen until the expiration date; however, some of the components may be stored refrigerated.

- Store CFSE at ≤-20°C. Once reconstituted with DMSO, use immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

ImmunoChemistry TECHNOLOGIES LLC

ImmunoChemistry Technologies, LLC
9401 James Ave. S. #155, Bloomington, MN 55431 USA 800-829-3194
immunochemistry.com

- 7-AAD may be stored frozen or refrigerated. Once reconstituted with DMSO, use immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.
- SR-VAD-FMK may be stored frozen or refrigerated. Once reconstituted with DMSO, use immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.
- 10X Assay Buffer may be stored frozen or refrigerated. 1X Assay Buffer may be stored at 2-8°C for 1 week, or frozen and used within 6 months.

4. SAFETY DATA SHEETS (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- DMSO, up to 1 mL to reconstitute reagents
- DiH_20, 270-540 mL to dilute 10X Assay Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute SR-FLICA and handle cells
- Cultured cells or tissues treated with the experimental conditions ready to be labeled
- Reagents to induce apoptosis and create controls for SR-FLICA staining, such as staurosporine (cat. #6212) or camptothecin (cat. #6210)
- 15% ETOH (in PBS or 1X Assay Buffer) to create live/dead controls for 7-AAD staining
- Hot water bath, to create live/dead controls for 7-AAD staining
- Hemocytometer
- Centrifuge at 200 x g
- 37°C incubator
- FACS tubes
- 15 mL polypropylene centrifuge tubes
- Ice bath

6. DETECTION EQUIPMENT

Flow Cytometer, 488 nm argon excitation laser (a BD FACS Caliber was used for the examples in this manual). Use filter pairings that best approximate these settings:

- CFSE green fluorescing cellular stain: excitation at 492 nm; emission at 520-540 nm in FL-1 (Section 14)
- SR-FLICA orange/red fluorescing caspase inhibitor reagent: excitation at 550-580 nm; emission at 590-600 nm in FL-2 (Section 16)
- 7-AAD red fluorescing live/dead stain: excitation at 546 nm; emission at 647 nm in FL-3 (Section 17)

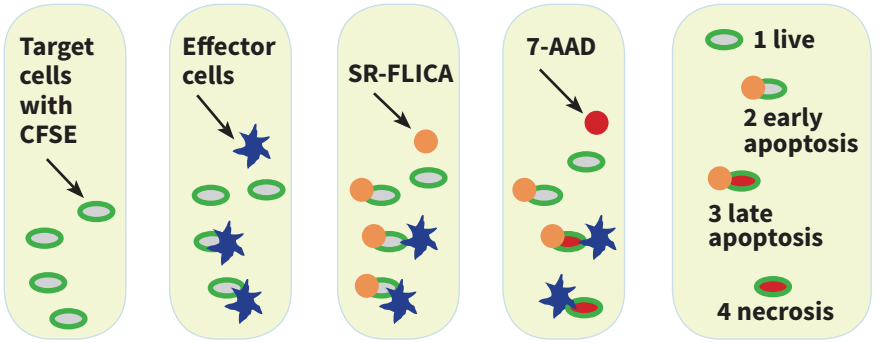
7. OVERVIEW

Quantifying cell death with ICT's Total Cytotoxicity & Apoptosis Assay can be completed within a few hours. However, the experiment is performed on living cells, which require cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental treatment or cytotoxic process, or to induce apoptosis and necrosis, and create controls. Each investigator should adjust the amount of the reagents and incubation times to accommodate their particular cell line(s) and research conditions.

Control populations must be made for the experimental conditions. If the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc., prepare a control of non-infected target cells combined with effector cells to determine cell death which normally occurs within the healthy target cells. In addition, several control tubes must be prepared for compensation and gating of the flow cytometer (Section 8).

ICT's Total Cytotoxicity & Apoptosis Assay includes 10X Assay Buffer, and three lyophilized fluorescent reagents: CFSE, SR-FLICA, and 7-AAD, which must be reconstituted and diluted prior to use. First dilute and filter the 10X Assay Buffer, as it is used to dilute the other reagents. Then reconstitute the lyophilized reagents with DMSO to create the stock concentrates and store on ice. Once it is time to use the reagent, prepare the working solution by diluting the stock. Here is a quick overview of the procedure (Figure 2):

FIGURE 1: QUANTITATE 4 POPULATIONS IN EACH SAMPLE



Purify target cells and stain green with CFSE to distinguish them from unstained effector cells during flow cytometry or FACS analysis. If studying the effects of a toxic agent, CFSE is optional.

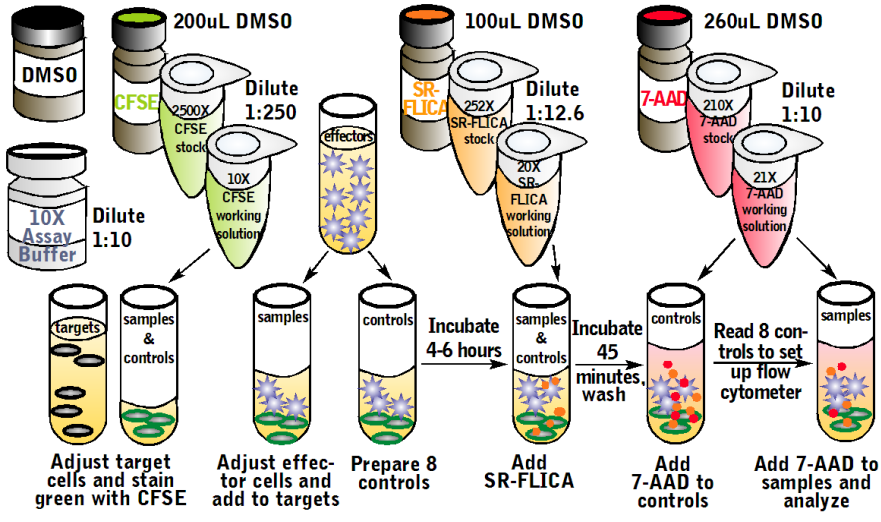
Add effector cells (PBMC, CD8, NK, etc.) or toxic agent to the target cells. Effector cells or toxic agent will act on target cells.

Add the orange/red SR-FLICA apoptosis reagent. It will enter the cells and bind to active caspases. If the cells are not apoptotic, it will wash out of the cells.

Add the red live/dead stain, 7-AAD. It will enter cells with compromised membranes in necrosis and late apoptosis and bind to DNA. It will not enter cells with intact membranes.

Run controls and analyze using flow cytometry to quantify 4 populations of target cells in each sample, including cells in early apoptosis which are not detectable by any other method: 1) live target cells; 2) early apoptotic cells; 3) late apoptotic cells; and 4) necrotic cells.

FIGURE 2: ASSAY PROCEDURE



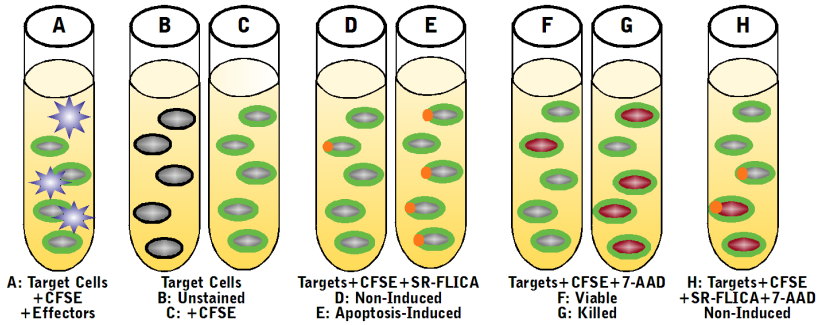
1. Dilute 10X Assay Buffer 1:10 with di$H_2$0, forming 1X Assay Buffer and sterile filter (Section 12).
2. Reconstitute CFSE with 200 µL DMSO, forming a 2500X stock concentrate (Section 14).
3. Dilute 2500X CFSE stock 1:250 in sterile 1X Assay Buffer, forming a 10X working solution (4 µL into 996 µL).
4. For each sample and control, adjust target cells to 1-2 x 10^6 cells/mL, wash, and resuspend each in 1.8 mL 1X Assay Buffer (Section 13).
5. Prepare control tubes (Section 8).
6. Add 200 µL diluted 10X CFSE to target cells, and all controls except B (at 1.8 mL).
7. Incubate 15 minutes at room temperature.
8. Wash cells: add 1 mL media, centrifuge, remove supernatant, and add 2-3 mL media.
9. Incubate 30 minutes at 37°C.
10. Adjust stained target cells to 2 x 10^5 cells/mL and make 100 µL aliquots (2 x 10^4 cells/aliquot).
11. Adjust unstained effector cells to the desired concentration, such as 50 times the concentration of the target cells, in 100 µL aliquots.
12. Add 100 µL unstained effector cells to the CFSE stained target cells and Control A. This forms the 'E:T' mixture at 200 µL.
13. Incubate 'E:T' mixture 4-6 hours at 37°C.
14. Create a positive control of apoptotic cells to generate Control E (Section 9).
15. Reconstitute SR-FLICA with 100 µL DMSO, forming a 252X stock concentrate (Section 16).
16. Dilute 252X SR-FLICA stock 1:12.6 in media, forming a 20X working solution (10 µL into 116 µL).
17. Add 10 µL diluted 20X SR-FLICA to 'E:T' experimental samples, and Controls D, E, & H (at 200 µL).
18. Incubate 45 minutes at 37°C. Wash cells and resuspend in 400 µL media; place cells on ice.
19. Create a positive control of killed cells to generate Control G (Section 10).
20. Reconstitute 7-AAD with 260 µL DMSO, forming a 210X stock concentrate (Section 17).
21. Dilute 210X 7-AAD stock 1:10 in sterile 1X Assay Buffer, forming a 21X working solution (40 µL into 360 µL).
22. Add 20 µL diluted 21X 7-AAD to Controls F, G, & H (at 400 µL).
23. Incubate controls 10 minutes on ice.
24. Run the instrument controls to set up gating and compensation on the flow cytometer (Section 8, Figures 8-13).
25. Add 20 µL diluted 21X 7-AAD to samples (at 400 µL).
26. Incubate samples 10 minutes on ice.
27. Read and analyze samples (Section 20, Figures 14-16).

8. FLOW CYTOMETRY CONTROLS

Several control populations (Figure 3) are needed to properly gate the flow cytometer and set up compensation on the instrument (Section 18). Follow Figures 8-13 to create the control tubes and set up the flow cytometer. Examples shown here were generated on a BD FACS Caliber; compensation requirements may differ among instruments.

A: Control A contains target cells stained with CFSE and effector cells. It is used to separate the green target cells from the unstained effector cells (Figure 8). It is also used as a negative control to ensure proper gating of SR-FLICA and 7-AAD reagents (Figure 12).

FIGURE 3: INSTRUMENT CONTROLS



B&C: Control B contains unstained target cells. Control C contains target cells stained green with CFSE. They are used to determine the shift of target cells from left to right along FL-1 (Figure 9).

D&E: Control D contains healthy target cells stained green with CFSE and orange/red with SR-FLICA (non-induced negative control). Control E contains apoptotic target cells stained green with CFSE and orange/red with SR-FLICA (induced to undergo apoptosis) (Section 9). They determine the shift of SR-FLICA from left to right along FL-2 (Figure 10).

F&G: Control F contains live target cells stained green with CFSE and red with 7-AAD. Control G contains killed target cells stained green with CFSE and red with 7-AAD (Section 10). They will determine the shift of 7-AAD from bottom to top along FL-3 (Figure 11).

H: Control H contains target cells stained with CFSE, SR-FLICA, and 7-AAD that are not induced to undergo apoptosis. It will determine background levels of apoptosis and necrosis without the influence of effector cells (Figure 13).

9. APOPTOSIS INDUCTION

In Section 8, Control E is created as an apoptosis-positive control to verify staining with SR-FLICA. Prior to commencing the experiment, determine a reproducible method for obtaining a positive control by triggering caspase activity. This process varies significantly with each cell line. For example, apoptosis may be induced with 2-4 µg/ml camptothecin for >4 hours (catalog #6210); or 1-2 µM staurosporine for >4 hours (catalog #6212).

10. PREPARE KILLED CELLS

In Section 8, Control G contains target cells labeled green with CFSE that have been killed and then labeled red with 7-AAD. For compensation and gating, it is preferable to use a control that contains a mixture of live and dead cells. Below are 2 methods for preparing such a control.

Method 1: Hot water bath.

1. Immerse the tube of cells in a 56°C water bath for 10-20 minutes. The optimal heat exposure period may vary with cell type. For best results, determine a reproducible method for killing 30-60% of the cell population prior to commencing the experiment.
2. Place on ice.
3. Add 7-AAD to stain necrotic cells (Section 17).

Method 2: Ethanol is an effective killer, however, ethanol may decrease the fluorescence output of the CFSE cellular stain, therefore the population may not shift as far to the right along FL-1, the green axis (Figure 11).

1. Centrifuge cells at 200 x g for 5-10 minutes at room temperature (RT).
2. Carefully remove the supernatant.
3. Resuspend cells in 15% ethanol. For example, add 150 µL ethanol plus

850 µL PBS or 1X Assay Buffer to resuspend cells.

4. Incubate 10 minutes at RT.
5. Add 2-3 mL 1X Assay Buffer.
6. Centrifuge at 200 x g for 5-10 minutes at RT.
7. Carefully remove the supernatant.
8. Add 400 µL media to resuspend cells.
9. Add 7-AAD to stain necrotic cells (Section 17).

11. PREPARE SAMPLES AND CONTROLS

All target cells must be stained green with CFSE to distinguish them from non-stained effector cells (Figure 4). If studying the effects of a toxic agent rather than cell mediated cytolytic activity, CFSE staining is optional as the only cells present will be the target cells.

Cultivate the proper number of target and effector cells for the sample and control populations. Allow time for the experimental treatment, cytotoxic process, or induction of apoptosis. Do not use target cells that are capable of proliferating more then 4 hours prior to assay; when a CFSE labeled cell divides, its progeny each inherit half the number of fluorescent tagged molecules as the parent cell. Therefore, proliferation will decrease the average fluorescence intensity of the target cell population. **As cell media will quench CFSE fluorescence, the media must be replaced with 1X Assay Buffer before staining with CFSE.**

FIGURE 4: STAIN TARGET CELLS WITH CFSE

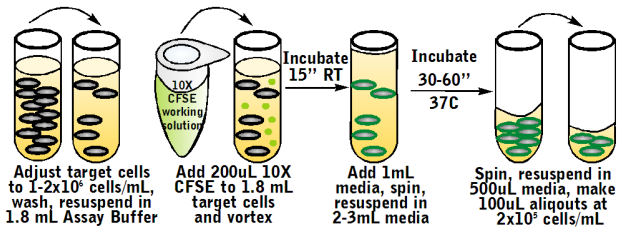


FIGURE 5: ADD EFFECTOR CELLS

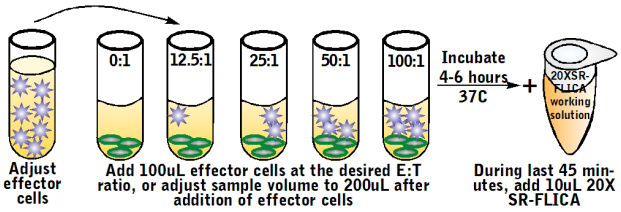


FIGURE 6: LABEL APOPTOTIC CELLS WITH SR-FLICA

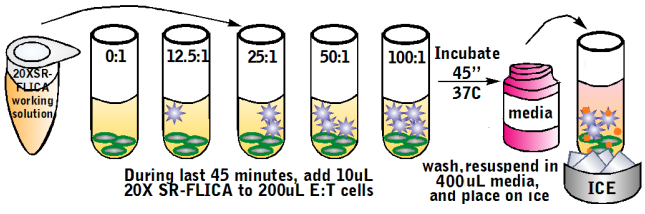
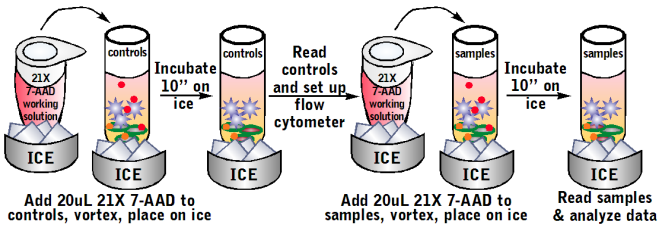


FIGURE 7: LABEL NECROTIC CELLS WITH 7-AAD



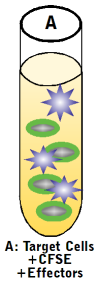
12. PREPARE 1X ASSAY BUFFER

ICT's 10X Assay Buffer (catalog #6161 and #685) is used to replace cell culture media, dilute reagents, and wash cells. It is a PBS-based buffer that does not contain any preservatives and should be stored at ≤2-8°C (precipitates may form in the 10X buffer during cold storage). It is supplied as a 10X concentrate which must be diluted to 1X with sterile/endotoxin-free diH_20 prior to use and sterile filtered. 1X Assay Buffer may be stored at 2-8°C for 1 week or frozen and used within 6 months.

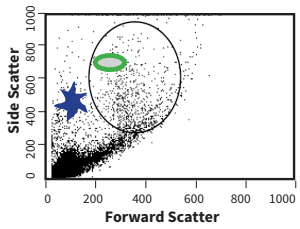
Instead of using 1X Assay Buffer to dilute the reagents, sterile PBS can be used. In some steps, fresh cell culture media can be used in place of 1X Assay Buffer (but not while staining with CFSE).

1. 10X Assay Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Assay Buffer 1:10 in diH_20. For example:
   a. Add 30 mL 10X Assay Buffer to 270 mL diH_20 (forming a total volume of 300 mL).

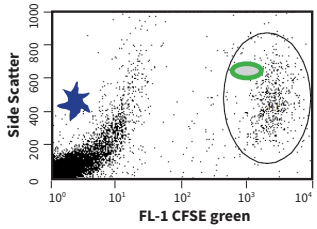
FIGURE 8: CONTROL A DISTINGUISHES GREEN CFSE-LABELED TARGET CELLS FROM UNSTAINED EFFECTOR CELLS: FSC VS. SSC AND CFSE (FL-1) VS. SSC PLOTS



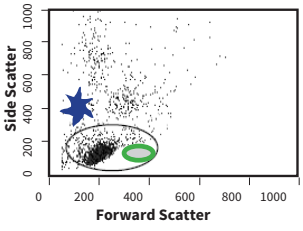
Control A contains CFSE stained target cells and effector cells. It is used to distinguish the green CFSE stained target cells from the unstained effector cells. Run Control A and create a forward scatter (FSC) vs. side scatter (SSC) plot (Figures 8A and 8C). Large cells, like K562 cells were easy to distinguish from lymphocyte effector cells (upper panel). Then create a plot of CFSE (FL-1) vs. SSC (Figures 8B and 8D) which becomes important when target cells are the same size as effector cells. When *Mycobacterium* infected monocytes were used as the target cells, they were easily distinguished from effector lymphocytes by creating these dot plots (lower panel). Set Control A aside, as it will be used again as a negative control to ensure proper gating of SR-FLICA and 7-AAD reagents (Figure 12) {data 121504}.



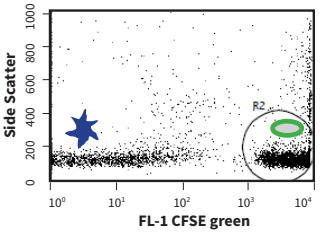
8A: K562 FSC vs SSC



8B: K562 FL-1 vs SSC



8C: *Mycobacterium* FSC vs SSC



8D: *Mycobacterium* FL-1 vs SSC

b. Add 60 mL 10X Assay Buffer to 540 mL diH_20 (600 mL total).

3. Mix for 5 minutes or until all crystals have dissolved.
4. Sterilize by filtration.

13. ADJUST TARGET CELLS IN 1X ASSAY BUFFER

1. Adjust target cells to 1-2 x 10^6 in 1 mL 1X Assay Buffer (Figure 4). Do not use media as it will quench CFSE fluorescence.
2. Wash target cells twice with 1X Assay Buffer to remove any media. Centrifuge at 200 x g for 5-10 minutes at RT and discard supernatant.
3. Resuspend target cells with 2-3 mL 1X Assay Buffer.
4. Centrifuge at 200 x g for 5-10 minutes at RT; discard supernatant.
5. Resuspend target cells (1-2 x 10^6) with 1.8 mL 1X Assay Buffer (5.56 x 10^5 cells/mL to 1.11 x 10^6 cells/mL).

14. STAIN TARGET CELLS WITH CFSE

5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, catalog #6162), is used to label cells with a green fluorescence potential stain. In this assay, it is used to label all the target cells green prior to exposure to the effector cells. CFSE is supplied as a highly concentrated lyophilized powder; the amber vial may appear empty as the reagent is lyophilized onto the insides of the vial. Once reconstituted in DMSO, it should have a slight hint of color.

CFSE must first be reconstituted in DMSO, forming a 2500X stock concentrate, and then diluted 1:250 in sterile 1X Assay Buffer to form the 10X working solution that will be used to stain the target cells. Do NOT dilute CFSE in media, as the cell reactive properties of the CFSE stain will be neutralized. Store the lyophilized CFSE and 2500X stock at ≤-20°C protected from light.

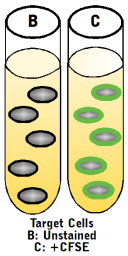
1. Reconstitute CFSE with 200 µL DMSO. This yields a 2500X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the vial until completely dissolved. Protect from light.
2. If not using all of the 2500X stock concentrate at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.
3. When ready to use in the assay, dilute the 2500X stock 1:250 with sterile 1X Assay Buffer. For example, add 4 µL of 2500X CFSE stock to 996 µL sterile 1X Assay Buffer and mix. This yields 1 mL of 10X CFSE working solution. For best results, the 10X working solution should be used within 2 hours, stored on ice and protected from light. Do NOT dilute in media, as the fluorescence will be quenched.
4. Add 200 µL 10X CFSE working solution to each 1.8 mL suspension of target cells, and to all control tubes except B. Gently vortex.

   The optimal concentration of CFSE may vary among cell types. Adjust the concentration of CFSE and the incubation time to adequately stain the target cells for the experiment. Excessive staining may cause problems when compensating the flow cytometer.
5. Incubate 15 minutes at room temperature.
6. Add 1 mL cell culture media to stop the CFSE binding reaction.
7. Centrifuge at 200 x g for 5-10 minutes at RT; discard supernatant.
8. Resuspend in 2-3 mL cell culture media.
9. Incubate 30-60 minutes at 37°C in a CO_2 incubator (or other conditions appropriate for the experiment). Plan the experiment so the cells incubate no more than 1 hour while setting up the assay.
10. Centrifuge at 200 x g for 5-10 minutes at RT; discard supernatant.
11. Resuspend with 500 µL cell culture media. Adjust the concentration of the target cells to 2 x 10^5 cells/mL, therefore a 100 µL volume will contain 2 x 10^4 target cells. The 100 µL aliquots of target cells will be combined with 100 µL of effector cells, yielding the desired Effector:Target cell ratio (E:T), such as 50:1 (Section 15).

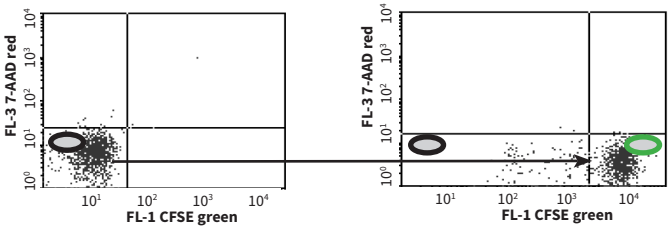
15. ADD EFFECTOR CELLS

1. Adjust the concentration of the effector cells so that approximately 100 µL can be added to the target cells yielding the desired E:T ratio, such as 50:1.

FIGURE 9: CONTROLS B&C DISTINGUISH UNLABELED TARGET CELLS FROM GREEN CFSE LABELED TARGET CELLS: CFSE (FL-1) VS. 7-AAD (FL-3)



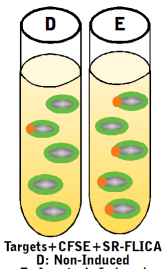
Control B contains unstained target cells. Control C contains CFSE stained target cells. Run Control B (Figure 9A) and then run Control C (Figure 9B) to compensate for CFSE (FL-1) vs. 7-AAD (FL-3). When stained green with CFSE (Control C), target cells shift to the right compared to unstained cells (Control B). Adjust the PMT voltage so that the green fluorescing target cells fall within the 3rd or 4th decade and save the data to ensure the target cells are properly gated {data 072204}.



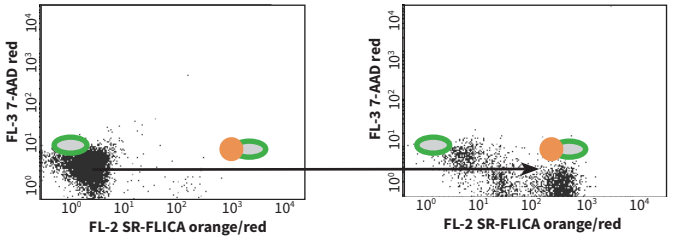
9A: Control B Unstained Target Cells

9B: Control C CFSE-Labeled Green Target Cells

FIGURE 10: CONTROLS D&E DISTINGUISH NON-APOPTOTIC FROM APOPTOTIC TARGET CELLS: SR-FLICA (FL-2) VS. 7-AAD (FL-3)



Control D contains healthy target cells labeled with CFSE and SR-FLICA that were not induced to undergo apoptosis. Control E contains target cells labeled with CFSE and SR-FLICA that were induced to undergo apoptosis. Run Controls D (Figure 10A) and E (Figure 10B) to compensate SR-FLICA (FL-2) vs. 7-AAD (FL-3). In Control D, depending upon the cell line, a small percentage of the non-induced control population will naturally undergo apoptosis and appear to the right. Run Control E and compensate the flow cytometer to ensure apoptotic cells shift to the right along the X-axis. Apoptotic target cells stained green with CFSE and orange-red with SR-FLICA (Control E) exhibit high levels of staining with SR-FLICA and migrate to the right compared with non-induced cells (Control D). {data 021705}.



10A: Control D Non-Induced Target Cells

10B: Control E Apoptotic Target Cells

For example, if the 100 µL target cell suspension contains 2 x 10^4 cells (Section 14), in order to add 50 times that number of effector cells (50 x (2 x 10^4 target cells) = 1 x 10^6 effector cells) in 100 µL, the concentration of effector cells needed would be 1 x 10^7 cells/mL (1 x 10^6 cells ÷ 0.1 mL = 1 x 10^7 cells/mL). An optimal E:T cell ratio is required to effectively determine cytolytic activity (Figures 5 and 17).

2. Add effector cells to samples and Control A and adjust the total volume to 200 µL.
3. Incubate 4-6 hours at 37°C in a CO_2 incubator. The incubation time may vary depending on the experiment.
4. Approximately 1 hour prior to the end of the incubation period, prepare SR-FLICA and add it to samples and Controls D, E, and H (Section 16).

16. LABEL APOPTOTIC CELLS WITH SR-FLICA

Cells that are in the early stages of apoptosis can be detected by ICT's orange-red poly caspase SR-FLICA inhibitor reagent, sulforhodamine labeled Valine-Alanine-Aspartic-Acid fluoromethyl ketone (SR-VAD-FMK, catalog #6221). Just add the inhibitor to the mixture of target and effector cells, and caspase-positive cells will fluoresce orange-red. SR-FLICA is cell-membrane-permeant; it will enter the cell and form a covalent bond with active caspase enzymes inside the cell undergoing apoptosis. Because SR-FLICA is always fluorescent, it is necessary to remove any unbound reagent from the cells or media by washing the cells after labeling.

SR-FLICA allows detection of cells in the early stages of apoptosis that would otherwise be missed by 7-AAD alone. This assay will often reveal a significant percentage of early apoptotic cells that were 7-AAD negative (indicating they are alive), yet SR-FLICA positive (indicating they are entering apoptosis). These early apoptotic cells can then be included in the overall percentage of total cell death, leading to more accurate results.

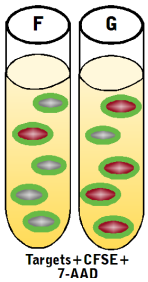
The SR-FLICA inhibitor reagent is supplied as a lyophilized powder that may be slightly visible as an iridescent sheen inside the vial. The amber vial may appear empty; once reconsitituted in DMSO, it may appear red in color. It must first be reconstituted in DMSO, forming a 252X stock concentrate, and then diluted 1:12.6 in sterile 1X Assay Buffer (or media) to form a final 20X working solution that will be used to label the cells. Store the lyophilized SR-FLICA refrigerated or frozen; store 252X stock at ≤-20°C protected from light.

1. Reconstitute SR-FLICA with 100 µL DMSO. This yields a 252X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the amber vial until completely dissolved. Store the 252X stock concentrate at ≤-20°C protected from light.
2. If not using all the 252X stock at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.
3. When ready to use in the assay, dilute the 252X stock 1:12.6 with sterile 1X Assay Buffer or media and mix. For example, add 10 µL of the 252X stock to 116 µL 1X Assay Buffer. This yields 126 µL of the 20X working solution. For best results, the 20X working solution should be used within 4 hours, stored on ice, and protected from light.
4. Add 10 µL 20X SR-FLICA working solution to Controls D, E, and H and sample tubes (at 200 µL) and mix.
5. Incubate for the remaining 45 minutes of E:T incubation at 37°C in a CO_2 incubator protected from light.
6. Wash cells to remove any unbound SR-FLICA from the media: centrifuge at 200 x g for 5-10 minutes at RT, discard supernatant, and resuspend in 400 µL media. Place samples on ice.

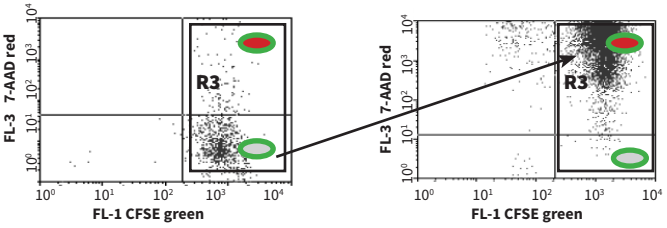
17. LABEL CONTROLS WITH 7-AAD

Dead target cells can be identified with 7-aminoactinomycin D (7-AAD, catalog #6163). 7-AAD is a red vital stain that can be used to identify and quantitate dead and dying target cells resulting from the cytolytic activity of the effector cells or toxic agent. This dye will penetrate the structurally com-

FIGURE 11: CONTROLS F&G DISTINGUISH LIVING FROM DEAD TARGET CELLS: CFSE (FL-1) VS. 7-AAD (FL-3)



Control F contains live target cells stained with CFSE and 7-AAD. Control G contains killed target cells stained with CFSE and 7-AAD. Run Controls F (Figure 11A) and G (Figure 11B) to compensate CFSE (FL-1) vs. 7-AAD (FL-3). Control F will also determine the level of spontaneous cell death that normally occurs within the cell line without the influence of effector cells and may be used as an experimental baseline control. Run Control G to compensate the flow cytometer to ensure that dead cells shift straight up the Y-axis. Killed target cells stained green with CFSE and red with 7-AAD (Control G) migrate up the plot compared to live cells (Control F). Create a gate (R3) on the green target cell population and rerun Control A (Figure 12). {data 072 204}.



11A Control F, live target cells

11B Control G, dead target cells

FIGURE 12: RERUN CONTROL A: SR-FLICA (FL-2) VS. 7-AAD (FL-3)

Control A contains CFSE stained target cells and effector cells (E:T). It is also used in Figure 8. Using the compensation set up for Controls F and G, draw a gate around the CFSE stained target cells (R3 in Figure 11A). Set acquisition to collect events within R3. Create a dot plot of SR-FLICA (FL-2) vs. 7-AAD (FL-3) gated off R3. Rerun Control A under new conditions, SR-FLICA vs. 7-AAD, as a negative control to ensure proper compensation of the instrument. Once gating is set, CFSE stained target cells appear in the lower left quadrant of this plot. Since Control A has not been stained with either SR-FLICA or 7-AAD, there should be very few cells outside this region {data 012706}.

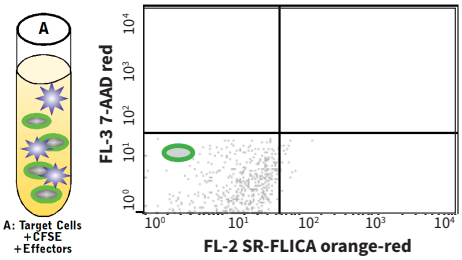
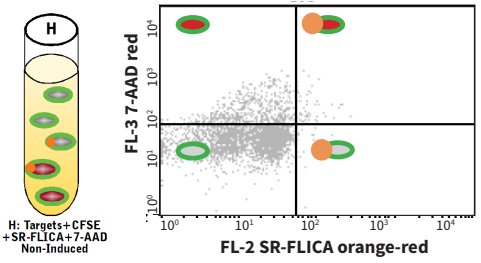


FIGURE 13: CONTROL H, SR-FLICA (FL-2) VS. 7-AAD (FL-3)

Control H contains non-induced CFSE stained target cells labeled with SR-FLICA and 7-AAD. Run Control H in SR-FLICA (FL-2) vs. 7-AAD (FL-3). The quadrants may need to be readjusted: create negative and positive quadrants based on cut-offs. This control reveals background levels of spontaneous death and apoptosis in the non-induced cells without the influence of the effector cells {data 012706}.



promised cell membranes of dead and dying cells and complex with DNA. The intercalated 7-AAD dye exhibits a red fluorescence in the FL-3 region with maximum output at 647 nm. Staining with 7-AAD should be done just prior to analysis; it is the last step due to its toxic effect on most cell types.

7-AAD is supplied as a highly concentrated lyophilized powder; the amber vial may appear empty as the reagent is lyophilized onto the insides of the vial. It must first be reconstituted in DMSO, forming a 210X stock concentrate, and then diluted 1:10 in sterile 1X Assay Buffer to form a final 21X working solution. Store the lyophilized 7-AAD refrigerated or frozen; store 210X stock at ≤-20°C.

- **Danger:** 7-Aminoactinomycin D (7-AAD) is fatal if swallowed, may cause cancer if swallowed, and may damage the unborn child if swallowed. See SDS for further information.

1. Reconstitute lyophilized 7-AAD with 260 µL DMSO. This yields a 210X stock concentrate. Mix by swirling or tilting the vial, or gently vortexing, allowing the DMSO to travel around the base of the amber vial until completely dissolved.
2. If not all of the 210X 7-AAD stock concentrate will be used at the time it is reconstituted, store it at ≤-20°C for 6 months protected from light. To avoid repeated freeze-thaw cycles, make small aliquots in amber vials or polypropylene tubes.
3. When ready to use in the assay, dilute the 210X stock 1:10 with sterile 1X Assay Buffer or media. For example, add 40 µL of the 210X stock to 360 µL 1X Assay Buffer and mix. This yields 400 µL of the 21X working solution. For best results, the 21X working solution should be used within 2 hours, stored on ice and protected from light.
4. Add 20 µL 21X 7-AAD to Controls F, G, and H (at 400 µL) just prior to analysis and mix or gently vortex. **Do not add 7-AAD to the experimental samples until the flow cytometer has been set up.**
5. Incubate 10 minutes on ice protected from light.

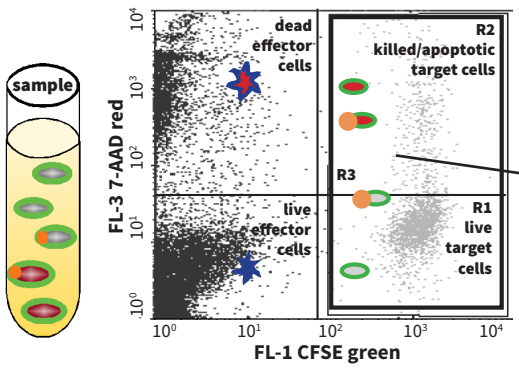
18. RUN CONTROLS AND SET UP FLOW CYTOMETER

Set up the proper instrument gating and compensation adjustments based on the controls (Section 8) following Figures 8-13.

1. Run Control A (Figure 8) to separate live, green fluorescing target cells from unstained effector cells. Control A also serves as a negative control on SR-FLICA (FL-2) vs. 7-AAD (FL-3) dot plots (Figure 12).
2. Run Controls B&C (Figure 9) to ensure live, green fluorescing target cells fall in the proper region.
3. Run Controls D&E (Figure 10) to ensure apoptotic, orange-red fluorescing target cells fall in the proper region. Control D will also determine the basal level of apoptosis that normally occurs within the cell line without the influence of effector cells.
4. Run Controls F&G (Figure 11) to ensure dead, red fluorescing target cells fall in the proper region. Control F will also determine the level of spontaneous cell death that normally occurs within the cell line without the influence of effector cells.
5. Run Control H (Figure 13) to determine the background levels of necrosis and apoptosis without the influence of effector cells.

FIGURE 14: IDENTIFY GREEN TARGET CELLS

Run the samples. Derive a FSC vs. SSC plot or CFSE (FL-1) vs. SSC to identify the target cells (see Figure 8). Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) to further distinguish the green target cells from the unstained effector cells. Identify all green target cells and gate on them as R3 (R1+R2=R3). Set acquisition to collect events within R3. Traditional enzyme-release assays are often skewed by the large number of necrotic effector cells; data from ICT's Total Cytotoxicity & Apoptosis Assay is not affected by those cells {data 121504}.



19. LABEL SAMPLES WITH 7-AAD

1. Add 20 µL 21X 7-AAD to the experimental samples (at 400 µL) and mix or gently vortex.
2. Incubate 10 minutes on ice protected from light and analyze as soon as possible (Section 20).

20. ANALYZE SAMPLES

1. Distinguish green target cells from unstained effector cells using FSC vs. SSC or CFSE (FL-1) vs. SSC (Figure 8)
2. Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) and gate on the green CFSE stained target cells (R3 in Figure 14).
3. From this population of green target cells, prepare a dot plot of SR-FLICA (FL-2) vs. 7-AAD (FL-3) (Figure 15).
4. Calculate total cytotoxicity by adding the percentage of early apoptotic cells in the R5 region, late apoptotic cells in R6, and necrotic cells in R7 (Figures 15 and 16).

FIGURE 15: QUANTITATE 4 POPULATIONS

Once the green target cells have been gated (R3, Figure 14), derive a plot of the poly caspase apoptosis reagent (SR-FLICA) in FL-2 vs. live/dead stain (7-AAD) in FL-3. This plot reveals 4 populations of cells: live cells and 3 populations of cells in the death process, including cells in early apoptosis which are not detectable by any other method. All apoptotic events can now be included in the calculation of total cytotoxicity, leading to more accurate results. Cytotoxicity is accurately quantitated as 32.59% (7.98% R5 early apoptotic + 22.63% R6 late apoptotic + 1.98% R7 necrotic).

Live Cells: R4 Lower left: Viable live cells are SR-FLICA apoptosis negative and 7-AAD live/dead negative (67.41%).

Early Apoptosis: R5 Lower right: Cells in early apoptosis are SR-FLICA apoptosis positive (they have active caspase enzymes and are becoming apoptotic and dying), but are 7-AAD live/dead negative (they are alive but do not have compromised membranes yet); these cells are not detectable by other methods (7.98%).

Late Apoptosis: R6 Upper right: Late apoptotic cells are SR-FLICA apoptosis positive and 7-AAD live/dead positive (22.63%).

Necrosis: R7 Upper left: Necrotic cells are SR-FLICA apoptosis negative and 7-AAD live/dead positive (1.98%) {data 101805}.

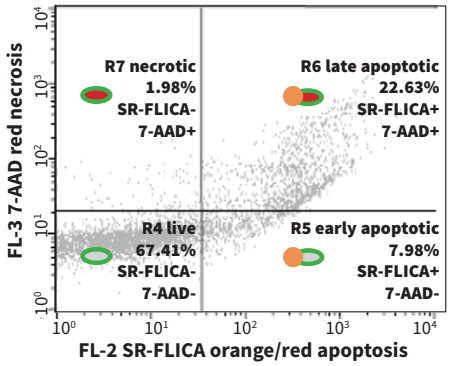


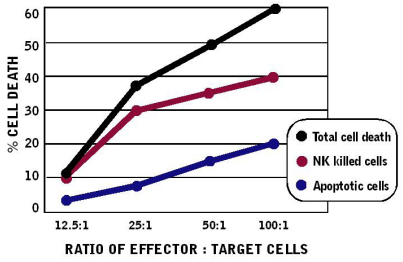
FIGURE 16: CALCULATE TOTAL CYTOTOXICITY

Based on the percentage of cells in each quadrant, total cytotoxicity can be calculated (see Figure 15). This sample analysis reveals a population in the lower right quadrant of early apoptotic cells that is not detectable by 7-AAD staining or any other method (7.98% are SR-FLICA positive, but 7-AAD negative). These early-apoptotic cells can then be included in the overall percentage of total cell death, leading to more accurate results. By including all apoptotic events to analyze cytotoxicity, total cytotoxicity can now be accurately quantitated as 32.59% (7.98%, early apoptotic + 22.63%, late apoptotic + 1.98%, necrotic). Other assays would calculate cytotoxicity at only 24.61% because they miss the 7.98% of cells that are in early apoptosis, thereby underestimating the true level of cytotoxicity. {data 101805}.

32.59%		**7.98%**		**22.63%**		**1.98%**
Total Cytotoxicity	**=**	**Early Apoptosis R5**	**+**	**Late Apoptosis R6**	**+**	**Necrosis R7**

FIGURE 17: EFFECTOR:TARGET CELL RATIO VS CYTOTOXICITY

K562 target cells were stained with CFSE and adjusted to 1.5 x 10^4 cells/tube. Effector cells were added at a ratio of 12.5:1, 25:1, 50:1, or 100:1. Cells were incubated 4 hours to allow the cytolytic activity to progress. Cells were labeled with SR-FLICA and 7-AAD and analyzed via flow cytometry. The maroon line reveals the amount of SR-FLICA + 7-AAD labeled, natural killer (NK) killed target cells detected (necrotic and late stage apoptotic). The blue line reveals the amount of SR-FLICA labeled, early apoptotic cells detected. When combined, the total % of cell death is calculated (black line), and the results were plotted versus the effector:target (E:T) cell ratio. Total cytotoxicity increased when more effector cells were used, reaching 60% at an E:T ratio of 100:1 .



21. REFERENCES

1. Lee-MacAry, A. E. et al. Development of a novel flow cytometric cell-mediated cytotoxicity assay using the fluorophores PKH-26 and TO-PRO-3 iodide. J Immunol Methods 252, 83-92 (2001).
2. Slezak, S. E. & Horan, P. K. Cell-mediated cytotoxicity. A highly sensitive and informative flow cytometric assay. J Immunol Methods 117, 205-214 (1989).
3. Radosevic, K., Garritsen, H. S., Van Graft, M., De Grooth, B. G. & Greve, J. A simple and sensitive flow cytometric assay for the determination of the cytotoxic activity of human natural killer cells. J Immunol Methods 135, 81-89 (1990).
4. Hatam, L., Schuval, S. & Bonagura, V. R. Flow cytometric analysis of natural killer cell function as a clinical assay. Cytometry 16, 59-68, doi:10.1002/cyto.990160109 (1994).
5. Lowdell, M. W. et al. The *in vitro* detection of anti-leukaemia-specific cytotoxicity after autologous bone marrow transplantation for acute leukaemia. Bone Marrow Transplant 19, 891-897, doi:10.1038/sj.bmt.1700756 (1997).
6. O'Brien, M. C. & Bolton, W. E. Comparison of cell viability probes compatible with fixation and permeabilization for combined surface and intracellular staining in flow cytometry. Cytometry 19, 243-255, doi:10.1002/cyto.990190308 (1995).
7. Van Hooijdonk, C. A., Glade, C. P. & Van Erp, P. E. TO-PRO-3 iodide: a novel HeNe laser-excitable DNA stain as an alternative for propidium iodide in multiparameter flow cytometry. Cytometry 17, 185-189, doi:10.1002/cyto.990170212 (1994).
8. Olin, M. R., Hwa Choi, K., Lee, J. & Molitor, T. W. Gammadelta T-lymphocyte cytotoxic activity against *Mycobacterium bovis* analyzed by flow cytometry. J Immunol Methods 297, 1-11, doi:10.1016/j.jim.2004.10.002 (2005).
9. Bedner, E., Smolewski, P., Amstad, P. & Darzynkiewicz, Z. Activation of caspases measured *in situ* by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. Exp Cell Res 259, 308-313, doi:10.1006/excr.2000.4955 (2000).

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

Thank you for using this kit! If you have any questions, or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788, or send an email to help@immunochemistry.com.

ImmunoChemistry TECHNOLOGIES LLC

BRIGHT MINDS, BRIGHT SOLUTIONS.

ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product, including the creation and illustration of its strategy and protocol.

Immunochemistry Technologies, LLC
9401 James Ave. S. #155, Bloomington, MN 55431 USA 800-829-3194
immunochemistry.com