Basic Cytotoxicity Assay

Catalog #969, #970

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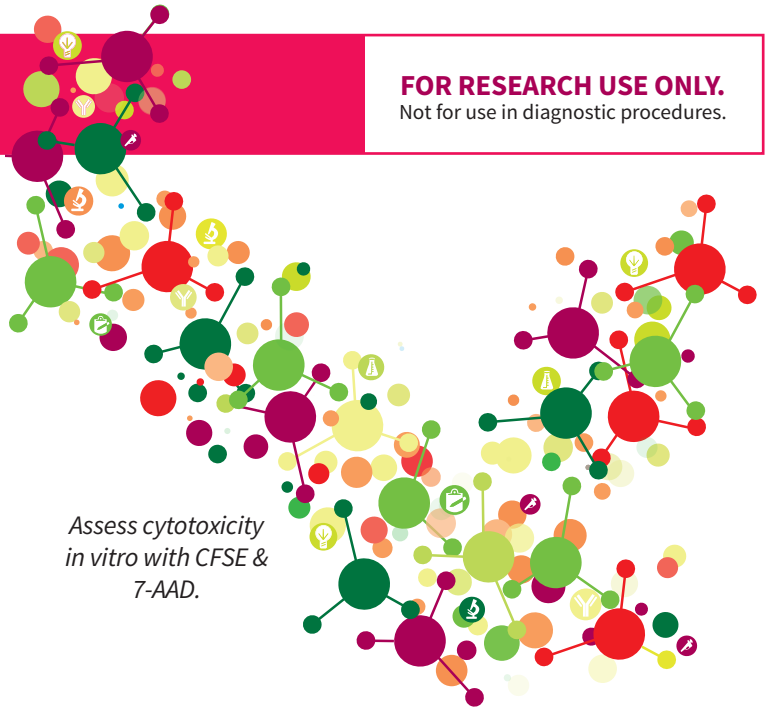


Assess cytotoxicity in vitro with CFSE & 7-AAD.

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].

Flow cytometric assays have been developed to overcome some of the difficulties associated with older assays like lactate dehydrogenase and ^{51}Cr release assays. One 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 Basic Cytotoxicity Assay, a flow cytometry assay combining the green fluorescing cellular stain, (CFSE), with a red fluorescing live/dead stain, 7-aminoactinomycin D (7-AAD). The assay can be used to quantify cytolytic killer activity and distinguish target and effector cells, and live and dead cells within a single sample tube.

In the Basic Cytotoxicity 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 of the target cells are initially labeled with green fluorescing CFSE, and the effector cells are not, these two populations can easily be differentiated (Figure 7). Upon completion of the E:T incubation, the second reagent, 7-AAD, is added to stain all necrotic cells red by binding to the DNA of membrane-compromised cells (Figure 6). With proper compensation and gating of the flow cytometer using the designated instrument controls (Figures 3 and 7-10), researchers can distinguish between target and effector cells, and living and necrotic cells, and assess the level of cytotoxicity in their samples (Figure 10).

2. KIT CONTENTS

Catalog #969, Small Size, 125 Tests, contains:

- 1 vial CFSE, green cellular stain, 250 Tests, #6162
- 1 vial 7-AAD, red live/dead stain, 125 Tests, #6163
- 1 bottle 10X Assay Buffer, 30 mL, #6161

Catalog #970, Large Size, 250 Tests, contains:

- 1 vial CFSE, green cellular stain, 250 Tests, #6162
- 2 vials 7-AAD, red live/dead stain, 125 Tests each, #6163
- 1 bottle 10X Assay Buffer, 60 mL, #685

3. STORAGE

The entire unopened kit should 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.
- 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.
- 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.

ImmunoChemistry TECHNOLOGIES LLC

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

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
- Cultured cells or tissues treated with the experimental conditions ready to be labeled
- Reagents to induce cytotoxicity and create controls
- 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 13)
- 7-AAD red fluorescing live/dead stain: excitation at 546 nm; emission at 647 nm in FL-3 (Section 15)

7. OVERVIEW

Quantifying cell death with ICT's Basic Cytotoxicity Assay can be completed within a few hours. However, the experiment is performed on living cells, which require periodic maintenance and 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 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 Basic Cytotoxicity Assay includes 10X Assay Buffer, and two lyophilized fluorescent reagents: CFSE 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):

1. Dilute 10X Assay Buffer 1:10 with diH_20, forming 1X Assay Buffer and sterile filter (Section 11).
2. Reconstitute CFSE with 200 µL DMSO, forming a 2500X stock concentrate (Section 13).
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 12).
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 1 x 10^5 cells/mL and make 200 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 200 µL aliquots.
12. Add 200 µL unstained effector cells to the CFSE stained target cells. This forms the 'E:T' mixture at 400 µL.
13. Incubate 'E:T' mixture 4-6 hours at 37°C.
14. Create a positive control of killed cells to generate Control E (Section 9).
15. Reconstitute 7-AAD with 260 µL DMSO, forming a 210X stock concentrate (Section 15).
16. Dilute 210X 7-AAD stock 1:10 in sterile 1X Assay Buffer, forming a 21X working solution (40 µL into 360 µL).
17. Add 20 µL diluted 21X 7-AAD to Controls D, E, & F (at 400 µL).
18. Incubate controls 10 minutes on ice.
19. Run the instrument controls to set up gating and compensation on the flow cytometer (Section 8, Figures 7-10).
20. Add 20 µL diluted 21X 7-AAD to samples (at 400 µL).
21. Incubate samples 10 minutes on ice.
22. Read samples and analyze (Section 18, Figures 11-12).

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 16). Follow Figures 7-10 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.

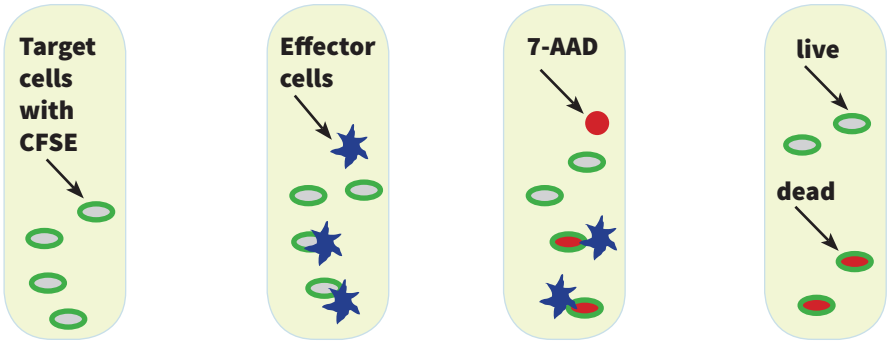
A: Control A contains target cells stained with CFSE and effector cells. It is used to separate the green target cells from the unstained effector cells. Target and effector cells can also be separated by size using forward and side scatter plots (Figure 7).

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 8), the green axis.

D&E: Control D contains live target cells stained green with CFSE and red with 7-AAD. Control E contains killed target cells stained green with CFSE and red with 7-AAD (Section 9). They will determine the shift of 7-AAD from bottom to top along FL-3 (Figure 9), the red axis.

Control D will also act as an experimental control to measure the

FIGURE 1: QUANTITATE CELL DEATH



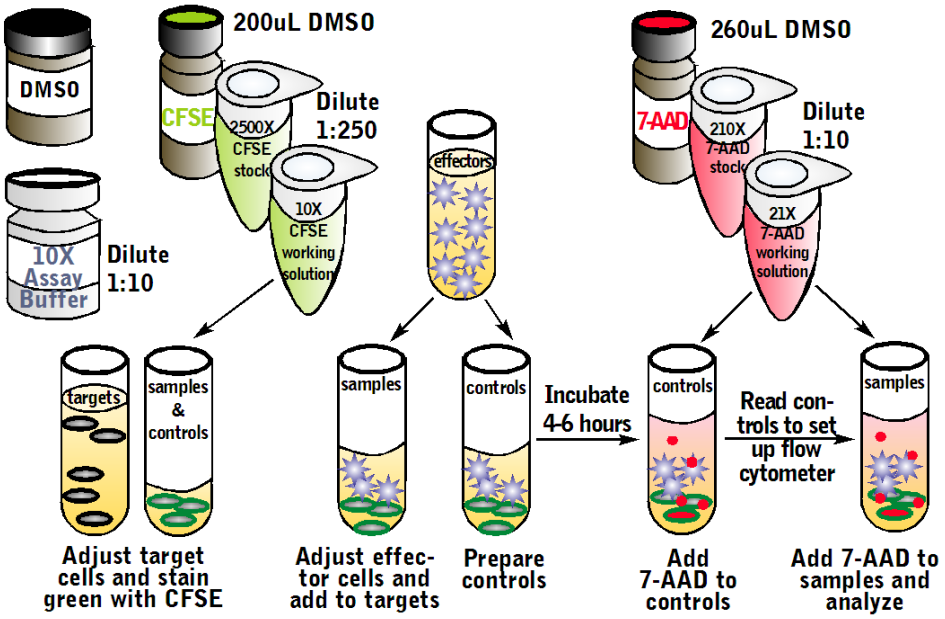
Purify target cells and stain green with CFSE to distinguish them from unstained effector cells during flow cytometry or FACS analysis. If studying the effects of a toxic agent, CFSE is optional.

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 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 2 populations of target cells in each sample:
1) live target cells;
2) necrotic cells.

FIGURE 2: ASSAY PROCEDURE



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basal level of necrosis not caused by the experimental treatment. It will determine the level of spontaneous cell death which normally occurs within the cell line without the influence of the effector cells. Subtract the level of spontaneous cell death from the experimental samples to determine the true level of cytotoxicity.

F: Control F contains non-infected target cells stained with CFSE mixed with effector cells and stained with 7-AAD to determine spontaneous cell death. Prepare Control F if the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc. (Figure 10).

9. PREPARE KILLED CELLS

In Section 8, Control E 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 15).

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 9).

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 15).

10. 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 or cytotoxic process. Do not use target cells that are capable of proliferating more then 4 hours prior to the 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**.

11. 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

FIGURE 3: INSTRUMENT CONTROLS

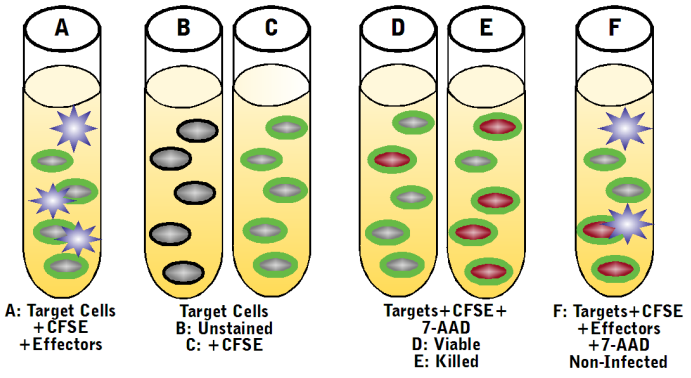


FIGURE 4: STAIN TARGET CELLS WITH CFSE

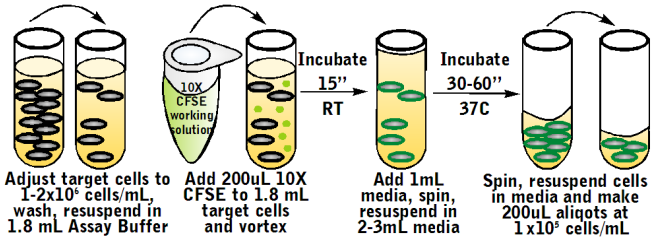


FIGURE 5: ADD EFFECTOR CELLS

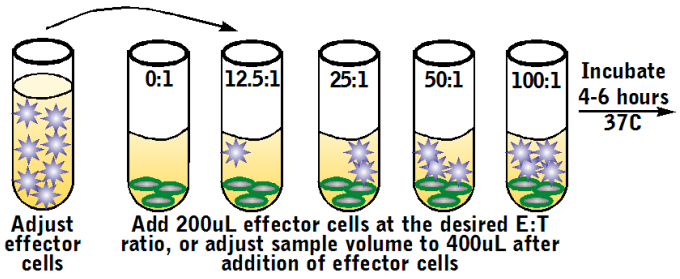
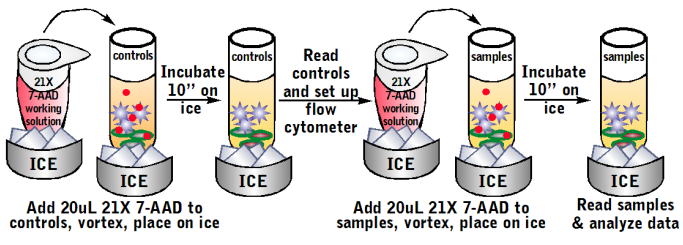


FIGURE 6: LABEL NECROTIC CELLS WITH 7-AAD



(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).
   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.

12. ADJUST TARGET CELLS IN 1X ASSAY BUFFER

1. Adjust target cells to 1-2 x 10^6 cells/mL 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) in 1.8 mL 1X Assay Buffer (5.56 x 10^5 cells/mL to 1.11 x 10^6 cells/mL).

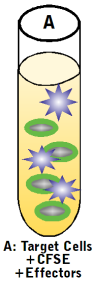
13. STAIN TARGET CELLS WITH CFSE

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.

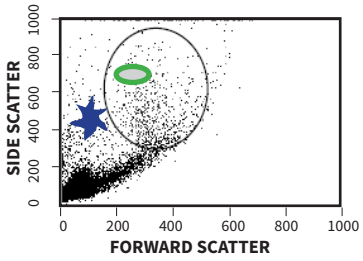
CFSE must first be reconstituted in DMSO, forming a 2500X stock concentrate, and then diluted 1:250 in sterile 1X Assay Buffer to form the 10X working solution that will be used to stain the target cells. Do NOT dilute CFSE in media, as the reactive properties of the CFSE stain will be neutralized. Store 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. At room temperature, this should take just a few minutes. 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.

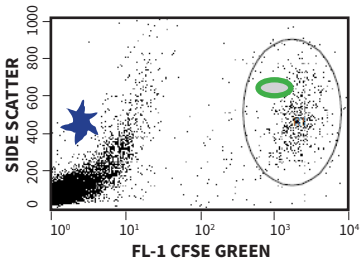
FIGURE 7: CONTROL A DISTINGUISHS GREEN CFSE LABELED TARGET CELLS FROM UNSTAINED EFFECTOR CELLS: FSC VS. SSC AND CFSE (FL-1) VS. SSC PLOTS



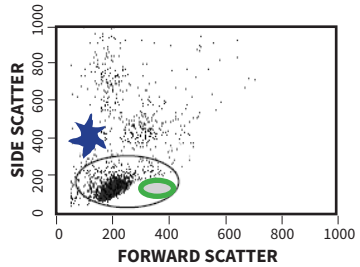
Control A contains CFSE stained target cells and effector cells (E:T). It is used to distinguish the green CFSE stained target cells from the unstained effector cells. Run Control A and create a forward scatter (FSC) vs. side scatter (SSC) plot (Figures 7A and 7C). Large cells, like K562 cells were easy to distinguish from lymphocyte effector cells (upper panel). Then create a plot of CFSE (FL-1) vs. SSC (Figures 7B and 7D) 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). {data 121504}.



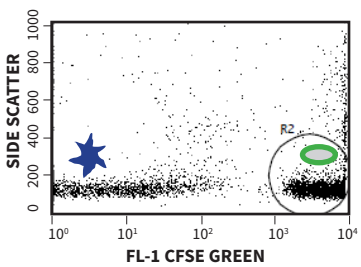
7A: K562 FSC vs SSC



7B: K562 FL-1 vs SSC



7C: *Mycobacterium* FSC vs SSC



7D: *Mycobacterium* FL-1 vs SSC

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 per tube 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 per tube.
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 cell culture media. Adjust the concentration of the target cells to 1 x 10^5 cells/mL, therefore a 200 µL volume will contain 2 x 10^4 target cells. The 200 µL aliquots of target cells will be combined with 200 µL of effector cells, yielding the desired Effector:Target cell ratio (E:T), such as 50:1 (Section 14).

14. ADD EFFECTOR CELLS

1. Adjust the concentration of the effector cells so that approximately 200 µL can be added to the target cells yielding the desired E:T ratio, such as 50:1.

   For example, if the 200 µL target cell suspension contains 2 x 10^4 cells (Section 13), 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 200 µL, the concentration of effector cells needed would be 5 x 10^6 cells/mL (1 x 10^6 cells ÷ 0.2 mL = 5 x 10^6 cells/mL). An optimal E:T cell ratio is required to effectively determine cytolytic activity (Figures 5 and 12).

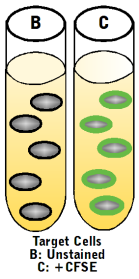
2. Add effector cells to samples and Controls A and F and adjust the total volume to 400 µL.
3. Incubate 4-6 hours at 37°C in a CO_2 incubator. The incubation time may vary depending on the experiment.

15. LABEL CONTROLS WITH 7-AAD

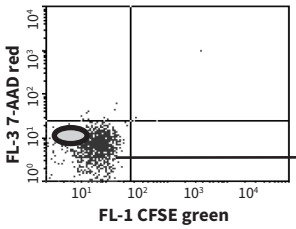
Dead target cells can be identified with 7-aminoactinomycin D (7-AAD, catalog #6163). 7-AAD is a red vital stain that can be used to identify and quantitate dead and dying target cells resulting from the cytolytic activity of the effector cells or toxic agent. This dye will penetrate the structurally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

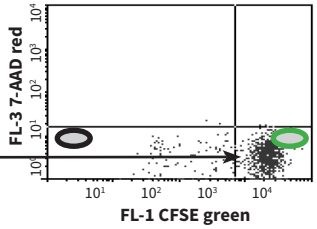
FIGURE 8: CONTROLS B&C DISTINGUISH UNLABELED TARGET CELLS FROM GREEN CFSE LABELED TARGET CELLS: CFSE (FL-1) VS. 7-AAD (FL-3)



Control B contains unstained target cells. Control C contains CFSE stained target cells. Run Control B (Figure 8A) and then run Control C (Figure 8B) 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}.

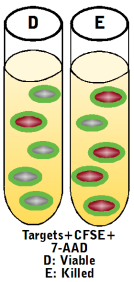


8A: Control B Unstained Target Cells

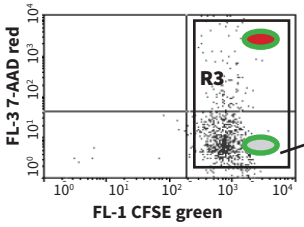


8B: Control C CFSE-Labeled Green Target Cells

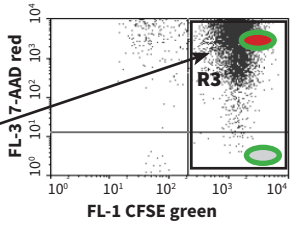
FIGURE 9: CONTROLS D&E DISTINGUISH LIVING FROM DEAD TARGET CELLS AND DETERMINE A BASE LEVEL OF SPONTANEOUS CELL DEATH: CFSE (FL-1) VS. 7-AAD (FL-3)



Control D contains live target cells stained with CFSE and 7-AAD. Control E contains killed target cells stained with CFSE and 7-AAD (see Section 9 for 2 methods to kill cells). Run Controls D (Figure 9A) and E (Figure 9B) to compensate CFSE (FL-1) vs. 7-AAD (FL-3). Control D 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 E and 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 E) migrate up the plot compared to live cells (Control D). Create a gate (R3) on the green target cell population and analyze. {data 072204}.



9A Control D, live target cells



9B Control E, dead target cells

- **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. At room temperature, it should be dissolved within a few minutes.
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 D, E, and F (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.

16. RUN CONTROLS TO SET UP FLOW CYTOMETER

Set up the proper instrument gating and compensation adjustments based on the controls (Section 8) following Figures 7-10.

1. Run Control A (Figure 7) to separate live, green fluorescing target cells from unstained effector cells.
2. Run Controls B&C (Figure 8) to ensure live, green fluorescing target cells fall in the proper region.
3. Run Controls D&E (Figure 9) to ensure dead, red fluorescing target cells fall in the proper region. Control D will also determine the level of spontaneous cell death that normally occurs within the cell line without the influence of effector cells.
4. Run Control F (Figure 10) if the experiment is designed to determine innate or adaptive cytotoxicity using isolated macrophages or monocytes, etc. It contains non-infected target cells stained with CFSE mixed with effector cells and stained with 7-AAD to determine spontaneous cell death.

17. 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 18).

18. ANALYZE SAMPLES

1. Distinguish green target cells from unstained effector cells using FSC vs. SSC or CFSE (FL-1) vs. SSC (Figure 7).
2. Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) to distinguish live green target cells from dead red and green target cells (Figures 10 and 12).
3. Create a gate on the green target cell population (R3 in Figures 9, 10, and 12).
4. Determine the number of red and green necrotic target cells (R2 in Figures 10 and 12).
5. Calculate the percentage of cytotoxicity by dividing the number of red and green dead cells in the R2 region of R3 by the total number of green target cells in both the R1+R2 regions of R3 and multiplying by 100 (Figure 11).
6. Calculate the percentage of cytotoxicity caused by the experimental treatment by subtracting the percentage of spontaneous cell death without the influence of effector cells from the samples (Control D).

FIGURE 10: CONTROL F IDENTIFIES GREEN TARGET CELLS

Control F contains CFSE stained non-infected target cells mixed with effector cells (E:T) and stained with 7-AAD. Once the other controls have been run, import the control settings and run Control F to determine the level of spontaneous cell death, then run the samples. Derive a plot of FSC vs. SSC or CFSE (FL-1) vs. SSC to identify the green target cells from the unstained effector cells (Figure 7). Create a plot of CFSE (FL-1) vs. 7-AAD (FL-3) to further distinguish live green target cells (R1) from necrotic red and green target cells (R2). Gate on all green target cells as R3 (R1+R2=R3) and set acquisition to collect events within R3. Calculate the percentage of cytolytic activity by dividing the number of dead red target cells in R2 by the total number of target cells in R3 and multiplying by 100 (Figures 11 and 12). Traditional enzyme-release assays are often skewed by the large number of necrotic effector cells; data from ICT's Basic Cytotoxicity Assay is not affected by those cells {data 121504}.

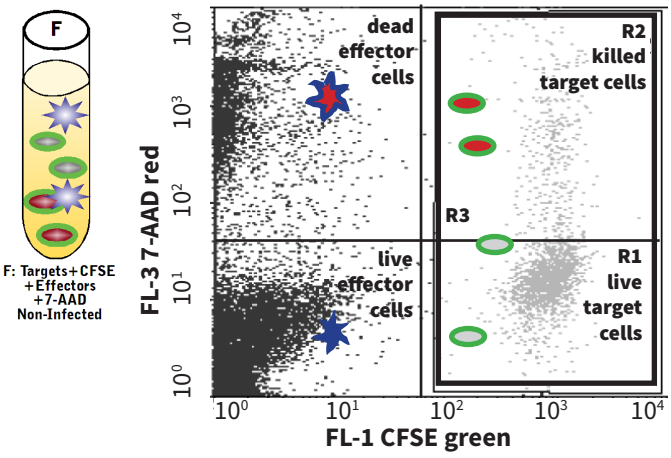


FIGURE 11: CALCULATION OF CYTOTOXICITY

Based on the number of green target cells in R3 (Figures 10 and 12) cytotoxicity can be calculated as the percentage of the green target cells which are also stained red with 7-AAD in R2 (Figure 10). This analysis reveals the population of necrotic target cells without interference from necrotic effector cells. ICT's Basic Cytotoxicity Assay is the easiest test to quantify cytotoxicity.

$$\%\ \text{Cytotoxicity} = \frac{\#\ \text{Necrotic R2}}{\text{R3} = \#\ \text{Live R1} + \#\ \text{Necrotic R2}} * 100$$

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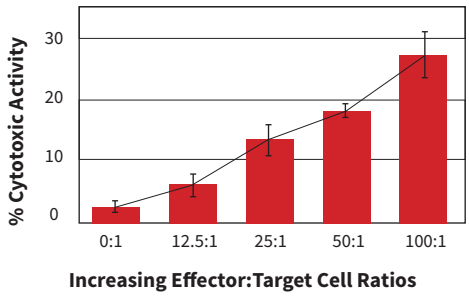
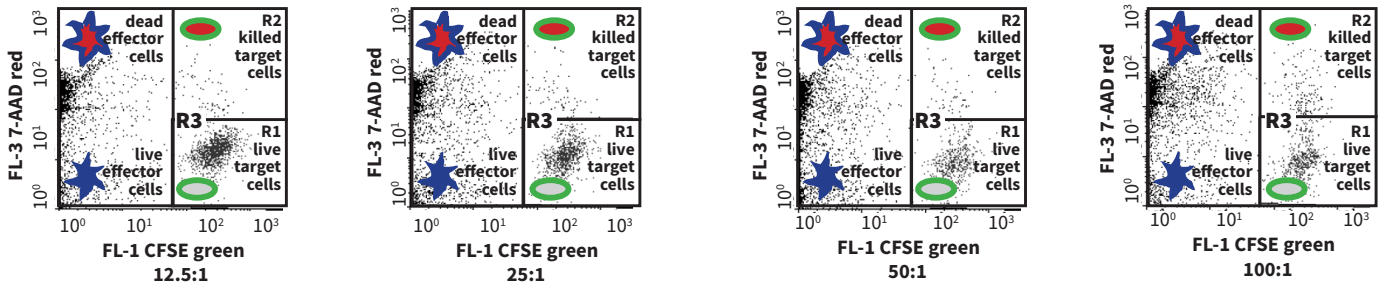
FIGURE 12: EFFECTOR: TARGET CELL RATIOS

An optimal E:T cell ratio is required to effectively determine cytolytic activity (Section 14). Determine this by running an experiment at several different ratios. For example: 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 0:1, 12.5:1, 25:1, 50:1, or 100:1 (Figure 5) and incubated for 4 hours to allow the cytolytic activity to progress. 7-AAD was then added to label necrotic cells red. Samples were analyzed and plotted versus the Effector:Target (E:T) cell ratio. As more effector cells were added, cytolytic activity increased, reaching more than 25% at an E:T ratio of 100:1 {data 072204}.





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